ORAL GENE THERAPY FOR HEMOPHILIA TREATMENT AND TOLERANCE

CHITOSAN-MEDIATED ORAL GENE THERAPY FOR HEMOPHILIA TREATMENT AND PROPHYLACTIC TOLERANCE

By

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Abstract

Hemophilia A and B are X-linked recessive bleeding disorders caused by the deficiency of coagulation factor VIII (FVIII) and Factor IX (FIX), respectively. Current treatment involves life-long protein replacement therapy which is invasive, expensive and inaccessible to the majority of hemophiliacs worldwide. Treatment is further compromised by the development of neutralizing antibodies. Thus, the development of an alternative treatment that is safer, cost effective and non-invasive that circumvents immune response induction is desirable.

To this end, a chitosan-mediated gene therapy strategy delivered orally was developed to provide clinically relevant plasma expression of FVIII or FIX. Hemophilia A mice that ingested chitosan nanoparticles containing FVIII DNA transiently expressed canine FVIII reaching >100 mU one day post treatment, together with partial phenotypic correction. Residual FVIII activity was detected for several days. Repeated administration of nanoparticles restored FVIII expression for 4 weeks and reduced clotting time in treated mice. Interestingly, inhibitors and non-neutralizing antibodies were not detectable throughout the experiment.

The immunomodulatory effects of chitosan-mediated oral gene delivery was investigated in naive hemophilia A mice and mice with pre-existing inhibitors. Administration of nanoparticles containing human FVIII DNA in naive mice suppressed systemic antibody responses and provided long-term tolerance to rhFVIII protein immunizations for at least 8 weeks. This tolerance was transferable to naive mice, suggesting development of regulatory T cells. In contrast, repeated oral nanoparticle

administration was unable to suppress FVIII-specific antibody responses in hemophilia A mice with pre-existing inhibitors.

Treatment of hemophilia B is challenged by a 25-50 fold higher therapeutic threshold. Nevertheless, hemophilia B mice fed chitosan nanoparticles containing CpG-FIXi plasmid transiently expressed therapeutically relevant human FIX >14mU/mL plasma.

Chitosan nanoparticle formulation was optimized *in vitro* for improved transfection efficiency. Nanoparticles formulated at a chitosan:DNA charge ratio of >2:1 (N:P) provided DNA protection against proton and enzymatic degradation that mimic conditions of the stomach and intestine, respectively. The inclusion of 25 mM sodium acetate-acetic acid decreased transfection of HEK 293 cells 4-fold, while 50 mM sodium sulphate increased uptake by ~40%. Optimal transfection was achieved with chitosan chloride (CL 213) formulated at a charge ratio of 3:1 in 50 mM sodium sulphate.

These findings suggest chitosan nanoparticles can provide clinically relevant FVIII and FIX transgene expression, which is amenable to a one-tablet-a-day dosing strategy. Taken together, chitosan-mediate gene therapy delivered orally is proposed as a potential non-invasive alternative strategy for hemophilia treatment and without inducing neutralizing and non-neutralizing antibody production.

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I am proud to acknowledge the endless encouragement from my parents Mr. and Mrs. Kirpal Dhadwar. I also recognize my siblings Neena and Rupy for their boundless support. I have great gratitude to my family and friends for understanding my absence from a number of functions spent conducting research. Finally, I thank my lovely wife Gurjeet for making every day an adventure.

Dedication

This thesis is dedicated to the memory of my grandfather Sardar Singh Dhadwar. I will always remember our family heritage in the small farming village of Shakoh Pur, Punjab, India.

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Chapter 1

1.0 Oral gene therapy strategy for the treatment of hemophilia

Gene therapy represents an attractive therapeutic treatment for genetic disorders by delivering functional gene(s) to correct defective DNA. However, effective therapies must provide a means of delivering the functional gene to the nucleus of cells for subsequent expression of the transgene product(*1*). Hemophilia A, a monogenic bleeding disorder, can be corrected by expression of functional coagulation factor VIII (FVIII) protein whereas hemophilia B is corrected by restoring functional coagulation factor IX (FIX) expression(*2*). These disorders represent an ideal target for gene therapy since restoration of as little as 1% normal coagulation factor levels improves clinical presentation(*3-5*). Current hemophilia treatment, although effective, involves life-long infusions of recombinant or plasma derived coagulation factor FVIII or FIX which is invasive, expensive and FVIII replacement is prone to antibody formation. The development of neutralizing antibodies or inhibitors greatly reduces the effectiveness of treatment and is considered a major obstacle in conventional FVIII replacement. To overcome these challenges, the objective of this research is to develop and optimize a chitosan-mediated gene therapy strategy delivered orally for the treatment of hemophilia (Figure 1.0).

Chitosan, a naturally occurring polysaccharide, has been investigated as a non-viral gene delivery vehicle offering advantages such as biocompatibility, biodegradability

and low toxicity(*6-9*). The high cationic potential of chitosan enables self-assembly with DNA at low pH by complex coacervation(*10*). The formation of these nanoparticles is mediated by electrostatic attraction between the positively charged amine groups of chitosan and the negatively charged phosphate groups of the DNA backbone (*11, 12*).

The transfection efficiency of chitosan/DNA nanoparticles can be modulated by a number of factors(*13*). These include the degree of deacetylation and molecular weight of chitosan, pH, electrolytes and chitosan/DNA charge ratio(*13-15*). Chitosan functional groups also allows coupling of extracellular and intracellular targeting ligands(*13, 16-22*). However, the optimum conditions for transfection efficiency of chitosan/DNA nanoparticles must be elucidated for its practical application as a gene carrier *in vitro* and *in vivo*(*9, 11, 14, 18*).

Chitosan has proven to be amenable to oral delivery of gene therapeutics(*19, 23*). Strong electrostatic interactions of chitosan with DNA protects the latter against acid degradation in the stomach and enzymatic degradation in the intestines(*23, 24*). The neutral pH of the intestines facilitates release of DNA from chitosan. The mucoadhesive properties of chitosan mediates DNA binding to mammalian cells while facilitating its transcellular and/or paracellular transport across mucosal epithelium(*7, 18, 25-28*). Also, chitosan-mediated delivery of therapeutics has been detected in small intestine, liver, spleen and other tissues following oral administration(*7, 29*). Furthermore, the use of chitosan-mediated delivery systems for therapeutics provides encouraging precedence for its application in hemophilia(*9, 10, 16-19, 23, 28-30*). Finally, the oral delivery of gene

therapeutics may provide a tolerogenic presentation of the transgene and suppress antibody formation.

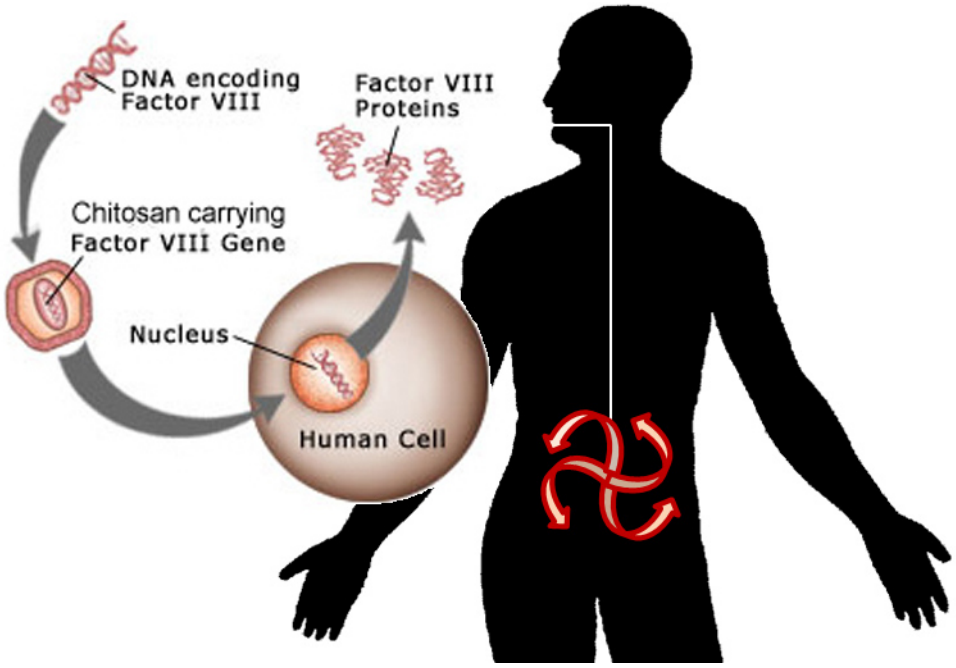

Figure 1.0: Chitosan-mediated oral gene therapy strategy for the treatment of hemophilia A. Plasmid DNA encoding coagulation factor VIII is encapsulated by chitosan to form nanoparticles via electrostatic interaction. Oral administration of nanoparticles protects DNA from degradation and delivers the FVIII gene to cells of the gastrointestinal tract. Transfected cells ectopically express and secrete functional FVIII protein into circulation. This strategy is also amenable to FIX delivery for the treatment of hemophilia B(*31*).

1.1 Hemophilia- an ideal target for gene therapy

Hemophilia is a recessive X-linked bleeding disorder in which an essential clotting factor is either partly or completely missing(*32*). The main forms are hemophilia A and hemophilia B which occur as a result of a deficiency in coagulation factor VIII (FVIII) and factor IX (FIX) proteins, respectively. Hemophilia in females is rare since a wild type allele in female heterozygotes maintains production of FVIII/FIX factors, which

protects against bleeding. Nevertheless, carriers of hemophilia have lower levels of FVIII or FIX than normal and can experience bleeding symptoms through lyonization(*33*). Hemophilia A is most prevalent with 1 in 5000 males inflicted by the disorder while hemophilia B occurs at a frequency of 1 in 25,000 males. This life-long inherited genetic condition also results from spontaneous gene mutation in 30% of incidents where there is no previous family history(*34*).

Moreover, the severity of hemophilia depends on the concentrations of FVIII or FIX available in circulation. Normal human physiological levels of FVIII and FIX are 100-200 ng/ml and 5 µg/ml, respectively(*35*, *36*). Hemophilia is defined as severe when levels of circulating FVIII or FIX are less than 1% of the physiological value, as moderate when levels are between 1 and 5% and as mild when FVIII or FIX levels are between 5 and 50% (Table 1.0)(*34*). Approximately 60% of hemophilia A and 30-40% of haemophilia B patients have the severe form of the disorder(*37*).

The activity of circulating FVIII/FIX protein dictates the phenotype of haemorrhaging into joints/muscles, easy bruising, and prolonged bleeding from wounds (*38*). During blood vessel injury a temporary scab forms, but the missing coagulation factors prevent effective fibrin formation, which is necessary to maintain the integrity of blood clots. Thus, a haemophiliac does not bleed more intensely than a normal person, but they bleed significantly longer(*39*). Cuts and grazes are not a challenge as a little pressure is usually enough to stop bleeding, however, major complications include hemarthrosis, haemorrhage, gastrointestinal bleeding, menorrhagia and particularly

intracranial bleeding(*39*). Treatment of hemophilia is required to prevent acute pain and severe joint damage leading to disability.

Table 1.0: Hemophilia phenotype severity.

Diagnosis (% normal)	Characteristics	FVIII levels* (ng/mL)	FIX levels (ng/mL)
Severe (<1%)	Spontaneous bleeding	<1	<50
Moderate (1-5%)	Minor injury causes prolonged bleeding	1-5	50-250
Mild (5-50%)	Does not interfere with normal living	5-50	250-2500

*Based on physiological FVIII level of 100 ng/ml.

Hemophilia is an ideal target for gene therapy as this monogenic disorder is well characterized and has a broad therapeutic window. A slight increase in plasma clotting factor above 1% of normal levels is sufficient to prevent morbidity and mortality, while supraphysiological levels of FVIII or FIX is associated with an increased risk for arterial and venous thrombosis(*40-43*). FVIII and FIX are endogenously expressed in the liver. FVIII is synthesized in hepatic sinusoidal endothelial cells or Kupfer cells while FIX is synthesized in hepatocytes, although the transgene can also be expressed by many other nucleated cell types(*44*). Furthermore, animal models of hemophilia A and B including murine, canine and rhesus macaque are available for preclinical evaluation. The effects of gene therapy can be monitored by measuring factor levels in peripheral blood.

1.1.1 The royal disease

Hemophilia is referred to as 'the royal disease' as this disorder inflicted several members of Europe's royal families. Recent evidence suggests that the affected members had hemophilia B (*45*). This recessive disorder is caused by a mutation in the X-chromosome resulting in heterozygous females being carriers while males inherit a mutant FIX gene. This life-long inherited genetic condition, also results from spontaneous gene mutation in 30% of incidents where there is no previous family history(*34*). Queen Victoria (1837-1901) was a carrier for hemophilia believed to be acquired by spontaneous mutation. Only her eighth child, Leopold (1853-1884) was affected by the disease and died of brain haemorrhage at age 31. Queen Victoria's daughters passed the mutation to various royal houses across Europe including the royal families of Germany, Spain and Russia into which they married.

Hemophilia has played a critical role in European history. Interestingly, Alexei Nikolaevich (1904-1918), the only heir of Nicholas II of Russia (1868-1918) and great grandson of Queen Victoria also had hemophilia. The 'mad monk' Rasputin (1869-1916) was entrusted to treat Alexei and gained influence with the Russian royal family. Rasputin's publicized influence of the Royals along with poor decisions made by the Tsar may have unintentionally influenced the Russian revolution of 1917.

1.1.2 History of hemophilia treatment

The majority of hemophilia males die before the age of 20 without treatment(*46*). This bleeding phenotype is associated with frequent bleeding, damage to joints and

intracranial haemorrhaging. In the early 1900's hemophilia treatment was limited to transfusions with whole blood or fresh plasma. However, these products did not contain sufficient quantities of FVIII or FIX to prevent internal bleeding.

The discovery of large quantities of FVIII in cryoprecipitate by Dr. Judith Poole advanced hemophilia treatment in the 1960's(*32*). Plasma fractions of cryoprecipitates were used in the treatment of hemophilia A while prothrombin complex concentrates were used in hemophilia B treatment. Prothrombin complex concentrates are enriched prothrombin, FVII, FIX and FX with trace amounts of FVIII, FVIIa, FIXa, protein C and protein S. The separation of clotting factors from plasma was facilitated by the Cohn fractionation process develop during WWII to separate albumin(*47*). Although these replacement products helped control bleeding, life threatening haemorrhaging was still a concern.

The introduction of freeze-dried FVIII- and FIX-enriched products allowed for home treatment. Plasma-derived clotting factors were produced from pools of ~20,000 donors which were administered on demand. The development of prophylactic treatment regimens prevented a majority of bleeding episodes and joint damage and represents the standard level of care endorsed by the World Health Organization (*48-50*).

An epidemic of HIV and Hepatitis C inflicted many hemophilia patients through contaminated blood-pooled products. By 1985, 75% of severe hemophilia A patients in the United States had transfusion-acquired HIV(*46*). Production of safer heat-treated products to inactivate blood-borne pathogens was introduced by major pharmaceutical companies Alpha Therapeutic, Armour Pharmaceutica, Bayer Corporation and Baxter

International for western markets. However, many of these companies continued to produce and supply HIV-contaminated products to Asia and Latin America (*51*).

It was later discovered that over 95% of hemophiliacs in the United States contracted hepatitis C also from contaminated blood products(*52*). The hepatitis C virus was only identified in 1989 while symptoms of infection including cirrhosis or liver cancer can take 10-20 years to develop.

Production of recombinant coagulation factor replacement products was possible by cloning the FVIII and FIX genes in 1984 and 1982, respectively(*53*). These proteins are produced in mammalian cell cultures to maintain the post translational modifications required for full factor activity. These plasma-free products are considered as a safer, although more expensive, treatment for hemophilia. Recombinant factor concentrates were commercially available in the early 1990's and manufacturing protocols have evolved to improve product safety and yield (*54, 55*).

1.2 Current hemophilia treatment

Hemophilia A and B patients are currently treated with protein replacement therapy involving frequent prophylactic infusions of FVIII or FIX, respectively (*32*). Therapeutic management include the use of either plasma-derived or recombinant products(*56*). This protein replacement therapy reconstitutes circulating coagulation proteins to prevent spontaneous bleed and restore normal hemostasis following trauma. Typical hemophilia A treatment involves 3-4 injections a week of 20 IU of FVIII/kg body weight, although various regimens have been explored(*57*). Current treatment cost is

$100,000 per patient annually and can increase to $1,000,000 in case of inhibitor formation. One third of hemophilia A patients develops inhibitors to FVIII which limits the effectiveness of conventional treatment. Patients with inhibitors are treated with Immune Tolerance Induction (ITI) protocols involving very frequent doses of large quantities of FVIII (50-300 IU of FVIII/kg of body weight) or with bypassing agents including recombinant FVIIa and a plasma-derivative said to contain Factor Eight Inhibitor By-passing Activity (FEIBA).

Hemophilia B treatment involves 2-3 injections per week of 30 IU of FIX/kg body weight to restore normal hemostasis(*57*). These patients have a lower incidence of inhibitor formation to treatment of approximately 2-3%. The frequent injection required for treatment is attributed to the short half-life of FVIII and FIX of 8-12 hours and 24 hours, respectively(*34*). Development of new recombinant concentrates focus on providing improved production, pharmacokinetics, and function with reduced immunogenicity by employing strategies that include pegylated liposomes, pegylated protein conjugates, fusion proteins (albumin, Fc fragment of IgG), and porcine/human hybrid sequences(*54, 58-67*).

The global demand for protein replacement products is growing exponentially. The world-wide hemophilia therapeutics market was valued at $6.5 billion dollars in 2009 and projected to increase to $10.4 billion by 2017. The growth of the FVIII market is primarily in industrialized nations while developing markets contribute to growing demand. The global supply for FVIII concentrates have increased from 3.7 billion units in 2000 to 6.2 billion in 2007 (Figure 1.1). Recombinant FVIII usage grew fastest in Europe

and North America, while other regions used almost exclusively plasma-derived FVIII. Indeed, the gap between industrialized and emerging countries remains significant as a majority of hemophiliacs' worldwide do not have access to treatment.

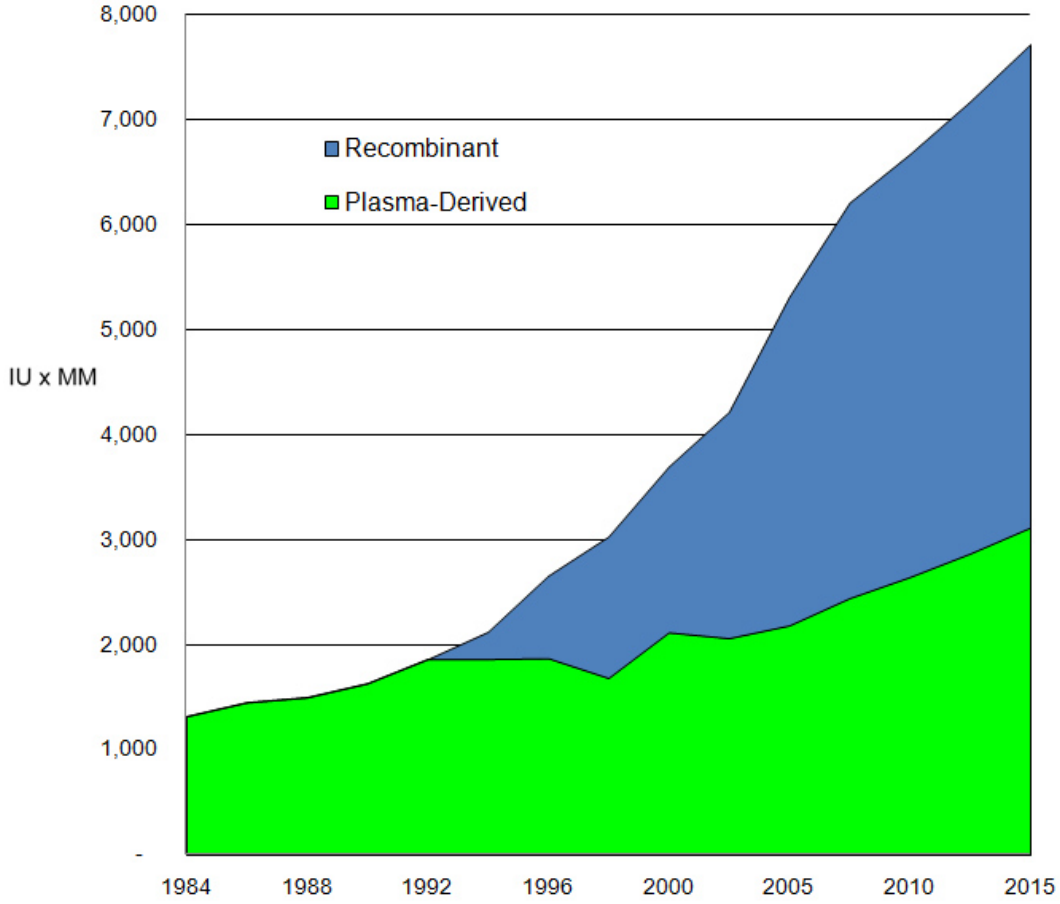

Figure 1.1: Global FVIII market demand. Conservative forecast of plasma-derived and recombinant products from 1984 to 2015 expressed as international units (IU) x MM. (source data: Marketing Research Bureau 2009)

1.2.1 Hemophilia treatment challenges

Current hemophilia treatment, while effective, is invasive and expensive. Patients ~2 years of age can be enrolled in prophylactic protocols involving frequent life-long

injections. Repeated venous access may require a central venous catheter, which present risk of infection and thrombosis. Furthermore, protein replacement therapy is expensive with an annual cost of $100,000 per patient. Hemophiliacs living in low and middle income countries do not have access to the large amount of concentrates needed for regular prophylaxis (Figure 1.2). It is estimated that 75% of hemophiliacs world-wide do not have access to treatment(*68*). In the case of inhibitor formation, cost of treatment increases 10-fold with the application of immune tolerance induction protocols and bypassing agents(*69, 70*).

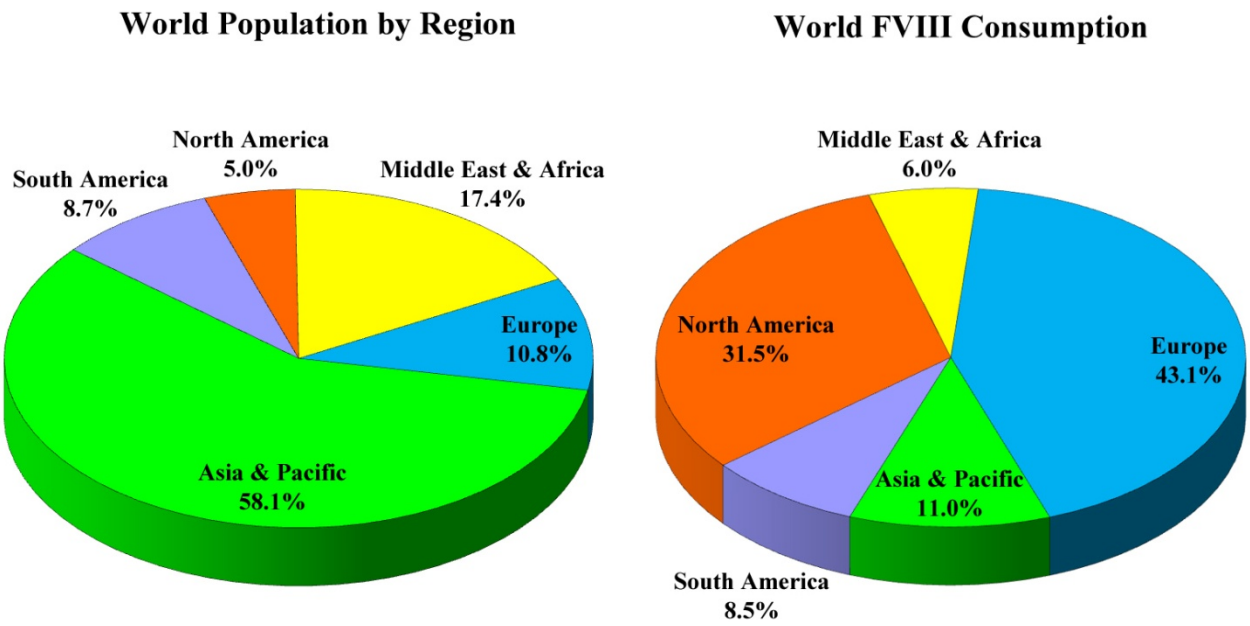

Figure 1.2: Global population and FVIII consumption in 2007. (Source data: Marketing Research Bureau 2009)

An alternative hemophilia treatment should address these challenges by providing a cost effecting, non-invasive strategy devoid of immune response complications. Gene therapy may provide a viable solution for the delivery of functional FVIII and FIX.

1.3 Blood coagulation cascade

A number of proteins in the blood participate in the coagulation pathway to repair an injury by forming a stable fibrin clot (Table 1.1). However, deficiency in either FVIII or FIX delays stable clot formation and results in prolonged bleeding resulting in phenotypes diagnosed as hemophilia A and hemophilia B, respectively.

Table 1.1: Coagulation factors that participate in stable clot formation.

Coagulation factor	Name
Factor I	Fibrinogen
Factor II	Prothrombin
Factor III	Tissue factor
Factor IV	Calcium
Factor V	Labile factor
Factor VI	Recognizes as factor Va
Factor VII	Stable factor
Factor VIII	Antihemophilic factor
Factor IX	Christmas factor
Factor X	Stuart-Prower factor
Factor XI	Plasma thromboplastin
Factor XII	Hageman factor
Factor XIII	Fibrin-stabilizing factor
VWF	Von Willebrand factor

During vascular damage, dilation decreases blood flow to the wound while activated platelets, in processes that depend on the participation of vWF and exposed collagen, plug the site of injury. Simultaneously, proteins of the blood are activated in a cascade that leads to strengthening of the plug by fibrin strands (Figure 1.3). FVIIa is activated by the membrane protein ‘tissue factor’ from exposed cells at the site of injury. This process is short-lived and is deactivated by tissue factor pathway inhibitor. FVIIa is able to activate both FXa and FIXa to produce a ‘thrombin burst’, a process by which

thrombin (Factor IIa) activates positive feedback pathways(*34, 71*). Thrombin activates FVIIIa which forms a complex with FIXa and calcium on the negatively charged phospholipid membranes of activated platelets. The phospholipid membrane surface provides a 2D surface that facilitates protein orientation and interactions. The activated FVIIIa acts as a cofactor that enhances FIXa catalysis by ~200,000-fold to activate FXa(*72*). Once activated, FXa participates in the prothrombinase complex (FXa-FVa), which in the presence of calcium and phospholipids converts prothrombin (FII) to thrombin (FIIa). In turn, thrombin (FIIa) cleaves fibrinogen into soluble fibrin monomers that are cross-linked by FXIIIa to form a haemostatic clot. Thus, a deficiency in either of FVIII (hemophilia A) or FIX (hemophilia B) arising from genetic mutations prevents stable clot formation.

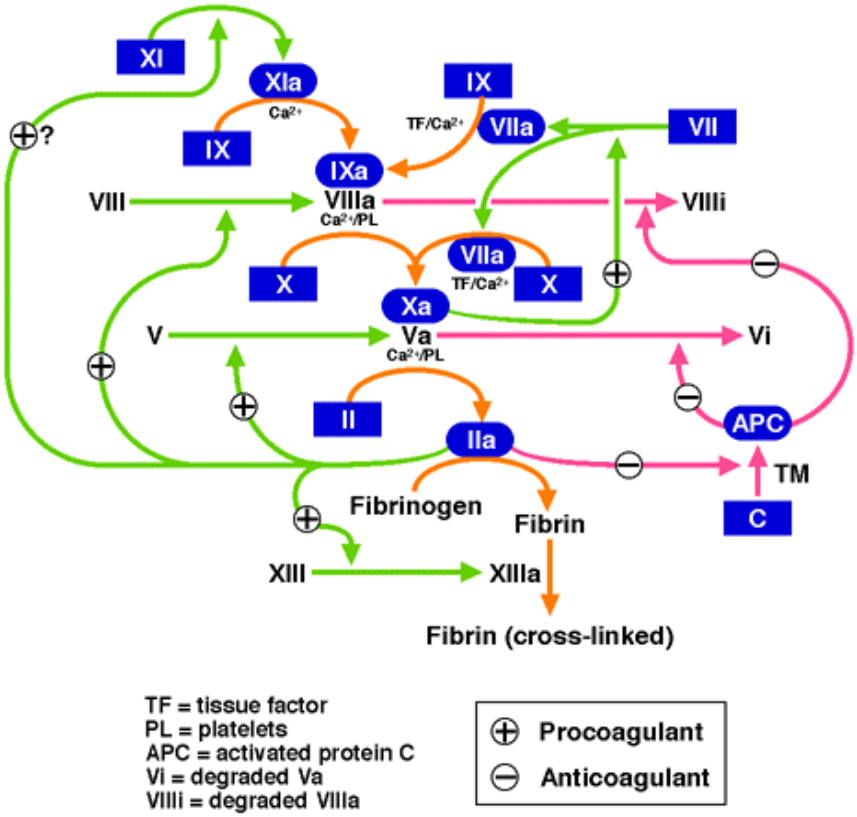

Figure 1.3: Coagulation cascade. FVIII and FIX plays a critical role in coagulation. Following initiation of the cascade by tissue factor, factor X is activated to create an initial 'thrombin burst'. Tissue factor pathway inhibitor quickly inactivates this pathway. However, the thrombin created activates FVIII and FIX via positive feedback to complete the fibrin. Following clot formation negative feedback systems limit the extent of clot formation. Activated FVIII is proteolytically inactivated by Protein C and Factor IXa(*73*).

1.3.1 Coagulation factor VIII

The lack of functional FVIII in circulation results in the prolonged bleeding phenotype identified as hemophilia A. Coagulation factor VIII is an essential clotting factor that enhances the FIX activity required for haemostasis. This FVIII protein is produced in the liver, more specifically in hepatic sinusoidal endothelial cells and Kupfer cells although other tissues have also been implicated including endothelial cells (*74*). In

fact, FVIII can be potentially produced by a number of other nucleated cell types. However, the complex glycosylation and post translational modifications required for FVIII's full cofactor activity which enhances FIXa enzymatic activity demands FVIII production in mammalian cells(*75, 76*).

The protein sequence of FVIII is derived from its gene sequence located on the X chromosome (Xq28). Its 26 exons code for a 2332 amino acid protein that has the structure A1-A2-B-A3-C1-C2(*72*). Processing of the protein structure results in heterodimer composed of a heavy chain (A1-A2-B) and a light chain (A3-C1-C2) bound together by a divalent cation (Figure 1.4).

The secreted FVIII protein is stabilized in circulation by binding to von Willebrand factor (vWF) which extends its half-life in circulation and acts as a transporter to the site of injury (*77*). The FVIII heterodimer is activated by thrombin or FXa by cleavage at R372 in the heavy chain and R1689 in the light chain resulting in the release of vWF. The heterotrimer (A1, A2, and A3-C1-C2) binds to activated platelets through the C2 domain at the site of vascular injury (*78*). Serine protease FIXa is recruited into a complex with FVIIIa that catalyzes the proteolytic activation of FX and subsequent generation of fibrin.

The activated FVIII (FVIIIa) can be inactivated through a number of mechanisms. For example, FVIIIa is subject to dissociation of the A2 subunit (*40*). Also, FVIIIa is inactivated by serine proteases including activated protein C, FXa, FIXa, thrombin and plasmin (*79, 80*).

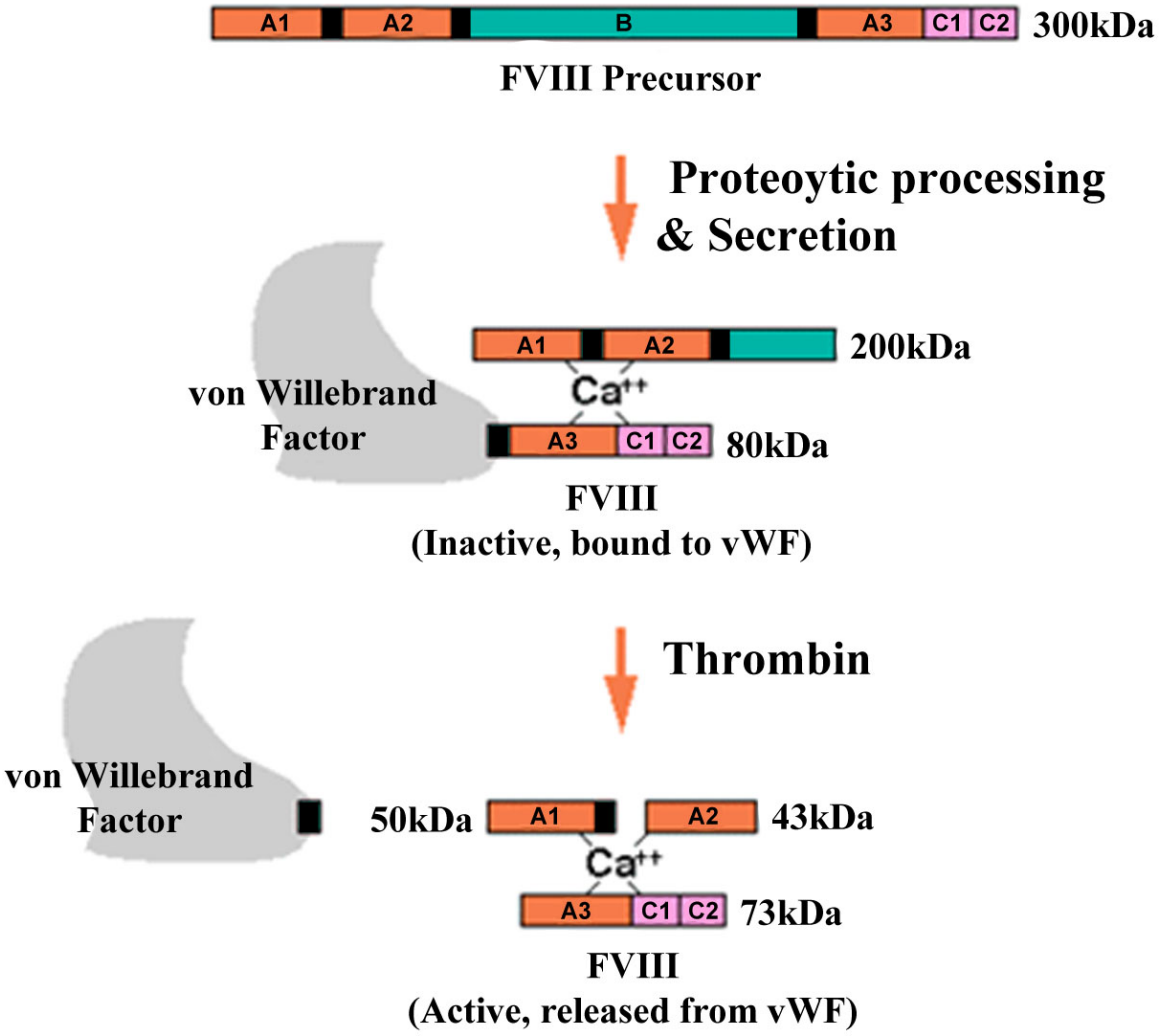

Figure 1.4: Coagulation factor VIII processing. FVIII is synthesized as a 300 kDa precursor protein, which is a 2332-residue single-chain glycoprotein. During processing the B-domain is removed as FVIII forms a heterotrimer. FVIII circulates in plasma in a noncovalent complex with von Willebrand factor (VWF). This binding with VWF stabilizes FVIII in circulation, protects it from degradation and transports FVIII to the bleeding sites where coagulation is required (*72, 81*).

Several mutations of the FVIII gene leading to hemophilia include point mutations, deletions, insertions and inversions. A large number of disease causing mutations has been identified for FVIII have been reported. The most common FVIII

mutation is inversion of intron 22(*82-84*). Intron 22 contains a 9.5 kb domain that share homology with two sequence segments located upstream of the FVIII gene. Inversion is mediated by intrachromosomal recombination between the copies of homologous regions. This inversion is seen in 50% of severe cases of hemophilia A(*79*). A similar inversion occurs within intron 1, albeit at a much lower frequency.

Table 1.2 FVIII Homology:

Species	Protein (% Identity)	DNA (% Identity)
C.lupus *(canine)*	77.6	85.2
M.musculus *(mouse)*	74.0	81.9
R.nervegicus *(rat)*	57.1	65.3
G.gallus *(chicken)*	58.5	62.3
D.rerio *(fish)*	45.9	52.9

Gene therapy for hemophilia A often utilizes modified plasmids coding for coagulation factor VIII. The highly glycosylated B domain, which represents 38% of the entire sequence, is dispensable for procoagulation activity(*85*). Hence, the smaller B-domain deleted (BDD)FVIII variant is often used in gene delivery strategies for hemophilia A. The BDDFVIII provides a 17-fold increase in mRNA translation but only a 30% increase in FVIII secretion(*86*). The FVIII gene contains sequences that inhibit its own expression (*12, 87*). Misfolded FVIII accumulate in the endoplasmic reticulum resulting in oxidative damage and apoptosis(*88*). Conversely, FVIII codon optimization removes these transcriptional silencers to facilitate efficient protein translation and folding resulting in a 44-fold increase in expression (*89, 90*). Moreover, FVIII variants have been engineered for higher stability, secretion or reduced immunogenicity (*12, 85,*

91, *92*). Engineered FVIII variants may also be created to mimic the high FVIII expression and secretion observed in other species (Table 1.2) (*93*-*98*).

1.3.2 Coagulation factor IX

FIX is a 56 kDa (461 aa) vitamin K-dependant glycoprotein required for the activation of FX in the coagulation cascade. FIX is primarily expressed in hepatocytes and undergoes extensive posttranslational modifications to become fully γ-carboxylated zymogen that is secreted into blood. The N-terminal non-catalytic region contains the γ-carboxyglutamic acid domain required for calcium and phospholipids binding. The C-terminal trypsin-like peptidase domain is responsible for catalytic cleavage of FX. These two FIX domains are separated by an 11kDa (35aa) activation peptide. Activation of FIX occurs through cleavage of the activation peptide by plasma serine protease FVIIa or FXIa. The resulting FIXa serine protease is composed of a 145aa light chain and 236aa heavy chain joined together by a disulfide bond (Figure 1.5).

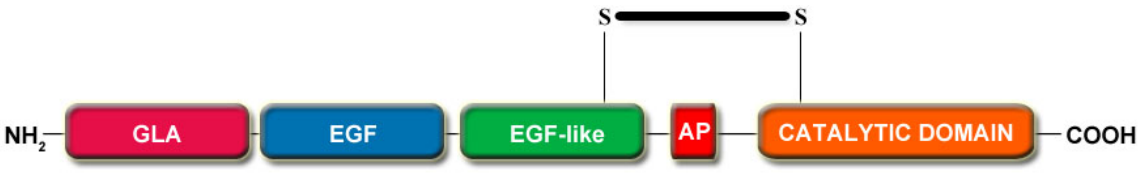

Figure 1.5: Domain structure of FIX protein. FIX consists of an N-terminal non-catalytic γ-carboxyglutamic acid (GLA) domain linked to a catalytic C-terminal catalytic domain through a 35 aa activation peptide (AP). Activation occurs by FXIa or FVIIa cleavage of Arg145 and Arg180(*44*).

The FIX gene, located on the X chromosome (Xq27), contains 8 exons spanning 34 kb, although 92% of the total gene length codes for introns. This gene is susceptible to mutations including point mutations, insertions and deletions. The predominant disease associated FIX alterations are missense mutations that cause 60% of severe hemophilia B cases.

Gene delivery strategies for hemophilia B may utilize high expression FIX variants. For example, Lin *et al.* discovered that a triple substitution in FIX (V86A/E277A/R338) enhanced activity 7-fold compared to wild type(*99*). These engineered variants can be applied to increase the efficacy of gene delivery strategies.

1.4 Antibody formation to protein replacement therapy

The treatment of hemophilia is associated with the formation of neutralizing antibodies to protein replacement therapy. Antibody formation is prevalent in 33% of severe hemophilia A patients and only 2-3% of hemophilia B cases (*40*). These antibodies can neutralize the activity of exogenous clotting factors and greatly reduce their circulation half-life. Interestingly, the highest incidence of inhibitor formation is generally associated with gene mutations that cause truncation or absence of endogenous protein. These include inversions, large deletions and nonsense mutations (*100-102*). Although a single point mutations can also result on inhibitor formation(*101*).

The absence or the truncation of endogenous FVIII or FIX protein can prevent self-tolerance. Central tolerance is mediated by deletion of auto-reactive effector T-cell in the thymus, or through development of regulatory T cells in the periphery. The absence of

self-tolerance can lead to an adaptive immune response to exogenous therapeutic proteins that are perceived as foreign antigens.

Protein replacement therapy with exogenous FVIII or FIX is thus susceptible to inhibitor formation. These circulating proteins are endocytosed by antigen presenting cells such as dendritic cells of the spleen(*103*). Degraded protein fragments are then presented on MHC class II antigen receptors. Activation of T cell proliferation requires interaction with peptide bound MHC II (signal I) and co-stimulatory 'danger' signal molecules that induce stimulation of cytokine secretion (signal II). The activated T cells bind to peptide bound MHC-II epitopes on B cells to induce proliferation and differentiation into antibody-secreting plasma cells or memory B cells(*104*). The T cell dependency of this process is demonstrated by the loss of FVIII inhibitors in patients with HIV(*105*). Interestingly, it is suggested that early prophylactic therapy may reduce inhibitor formation by presenting exogenous clotting factors when inflammatory signals are not present (*106*). However, this strategy may be complicated by the numerous vaccine-adjuvant regimens given to infants.

Antibodies generated are polyclonal and target both functional and non-functional domains as in the example of FVIII. Protein function is neutralized by steric hindrance that prevents FVIII interactions with other molecules of the coagulation pathway. Inhibitors that bind to the A3-domain prevent binding to FIXa while A2-domain directed inhibitors prevent normal function of the FVIIIa/FIXa complex(*107*). Anti-C2 domain inhibitors can prevent FVIII binding to phospholipids and vWF(*108*). Furthermore, non-neutralizing antibodies may increase the clearance rate of FVIII while others have been

found to enhance FVIII activity (*109*). Interestingly, auto antibodies to FVIII can occur naturally and has been found in the plasma of healthy individuals(*110, 111*). The lack of inhibition of endogenous FVIII in healthy individuals may be attributed to the presence of anti-idiotypic antibodies, which is the basis for intravenous immunoglobulin (IVIg) treatment in patients with autoantibodies(*112, 113*).

1.4.1 Immunogenicity of protein replacement products

Several groups have discussed the coagulation product-specific inhibitor development in hemophilia A patients (*114, 115*). It is observed that hemophiliacs treated with recombinant FVIII encounter a higher frequency of inhibitor formation than plasma-derived FVIII recipients (*116*). The lower immunogenicity of plasma-derived products may be attributed to the presence of vWF in the preparations, the natural carrier protein of FVIII, that binds immunodominant epitopes in the A3 and C2 domain(*117*). Alternatively, it has been suggested that vWF may act as a competitive antigen to FVIII (*118*). However, vWF is present at normal levels in hemophilia A patients and may not provide the basis for lower immunogenicity of pdFVIII. Instead, the inability for ~20% rFVIII to bind vWF may contribute to the greater immunogenicity of rFVIII(*75*).

The two-fold higher frequency of inhibitor formation in black haemophilia A patients is also linked to treatment (*100*). This is attributed to unique non-synonymous single-nucleotide polymorphisms that are unique in the black population but not represented in the currently available FVIII treatment products. Current treatment is based

on two haplotypes that are predominant in white, black and Chinese patients. Thus, conventional treatment although effective, is sub-optimal for hemophilia treatment.

1.4.2 Immune tolerance induction in the hemophilia treatment

Patients with inhibitors are treated with Immune Tolerance Induction protocols. Immune tolerance induction (ITI) is based on the concept of gradually eliminating the anti-FVIII inhibitors using frequent infusions of high dose FVIII protein. These ITI protocols may involve administration of 50-300 IU FVIII/kg body weight daily in conjunction with immunosuppressive agents. Eradication of FVIII inhibitors may take months to years to achieve with a success rate of 50-80%. However, low success rates occur in patients with large deletions, inversions and nonsense mutations. Furthermore, a number or retrospective studies have indicated that use of plasma-derived FVIII concentrates results in greater ITI success than rFVIII(*57, 119*). More specifically, high purity FVIII products have lower ITI success rates than vWF-rich products concentrates, although the data is not definitive and remains controversial(*120*).

The exact mechanism involved in immune tolerance induction has not been elucidated (*121*). This may involve removal of effector cells through clonal deletions, anergy or immune ignorance(*104*). Alternatively, ITI may be achieved through development of anti-idiotypic antibodies(*122*).

ITI protocols are less effective in the treatment of hemophilia B patients with inhibitors. Life-threatening anaphylactic reactions to FIX are common in 50% of these

patients. These complications demand safer alternative treatment methods and prophylactic tolerance strategies.

1.5 Gene therapy in hemophilia treatment

Gene therapy presents a potential solution to an expanding range of genetic disorders(*123*). The classical gene therapy strategy is to insert functional genes into an individual's cell or tissues to restore functional protein. This cell-mediated delivery of therapeutic proteins could provide persistent, on-demand expression at the site of activity. Many gene therapy strategies have been applied towards the treatment of hemophilia and reconstitution of functional FVIII or FIX proteins necessary for proper homeostasis(*68*).

The therapeutic gene must reach the nucleus to result in mRNA synthesis and subsequent protein expression. The cell membrane restricts spontaneous internalization of large molecules like DNA. These exogenous genes can be degraded by enzymes and require protection and transport mediated by a gene delivery vehicle. The most extensively studied gene delivery vehicles are viral vectors which have been applied in both *ex vivo* and *in vivo* settings.

Ex vivo gene transfer introduces genes into viable cells that have been removed from the donor and are then transferred to the recipient (*124*). This method eliminates unpredictable ectopic expression of transgenes in other cells and provides a safer application of integrating vectors. These genetically modified cells can be engrafted into the recipient directly or immunoisolated with biomaterials such as enclosed microcapsules to prevent immune response following allogeneic transplant (*125-127*).

Ectopic FVIII or FIX expression has been demonstrated in a number of cell types including platelets(*128-135*), erythrocytes(*136*), dendritic cells(*137*), endothelial cells(*127*), skeletal muscles(*138*) and stem cells(*139-143*). Nevertheless, persistent transgene expression of genetically modified cells requires pre-treatment with conditioning regimens to ensure engraftment. For example, the Doering lab has shown that porcine FVIII expression in hematopoietic stem cells and mesenchymal stem cells induced anti-porcine neutralizing antibodies following transplant (*69, 144*). Successful reconstitution of supraphysiological FVIII levels (~200% normal) was achieved only with prior irradiation-mediated myeloablative conditioning or pre-treatment with myelosuppressive agents and immunosuppressive drugs (*142, 145*). This strategy may be impractical for clinical application as hemophilia patients would be susceptible to opportunistic infections.

In vivo gene therapy strategies have also been examined for haemophilia treatment. Adeno-associated virus (AAV) is a non-integrating viral vector that has been used to target FVIII or FIX expression in skeletal muscles or hepatocytes (*146-150*). The promising results achieved in animal studies have not been recapitulated in the clinical settings (*151-155*). Indeed, many humans have been pre-exposed to wild-type AAV and may possess memory T cells that can eliminate the gene delivery vector and transduced cells (*156, 157*).

Lentiviral vectors can integrate the therapeutic gene into the host genome to potentially provide persistent FVIII or FIX expression as a cure for hemophilia. Transgene expression in hepatocytes is believed to mediate tolerance by induction of

regulatory T cells. However, liver-directed expression has shown to elicit an immune repose to the transgene (*158*). This has been attributed to the off-target transgene expression in immune cells. In contrast, liver-restricted FVIII or FIX expression in hepatocytes achieved persistent transgene expression *in vivo* (*159, 160*). Nevertheless, genomic integration may result in insertional mutagenesis and oncogene activation. These viral vectors need to be reassessed for the safety in human gene therapy(*157*). Thus, a safer alternative gene therapy strategy is desirable.

1.5.1 Chitosan-mediated gene therapy

Chitosan is a polysaccharide derived from the deacetylation of chitin, a structural component in the exoskeleton of crustaceans and the cell wall of fungi(*161*). Each deacetylated subunit of chitosan contains a primary amine group with a pKa value of 6.5. In physiological conditions, chitosan is digested by lysosymes or chitosanase which is produced by flora in intestines or present in the blood. This biocompatible and biodegradable material has been FDA approved for food and pharmaceutical applications (*6-9, 162, 163*). It has been suggested that chitosan chelates fat and reduces cholesterol. The properties of chitosan can be modified through functional groups (*164-171*). Chitosan is versatile material that has been used in applications of tumour targeting, vaccine delivery, insulin delivery, drug delivery and DNA vaccines delivery (*162, 172-177*). This material has also gained interest as a safe, non-viral delivery system for oral gene delivery(*10, 162, 178, 179*).

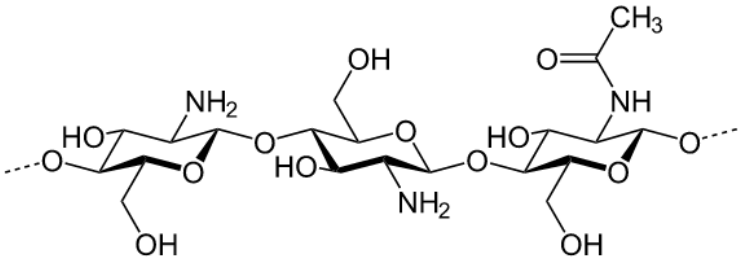

Figure 1.6: Chitosan structure. Chitosan is a polymer composed of β-(1-4)-linked D-glucosamine and N-acetyl-D-glucosamine with a pKa of 6.5.(*29*) Nanoparticles are formed by electrostatic interactions with DNA at low pH while plasmid release is mediated at neutral pH.

The potential of chitosan as a gene delivery agent is based on its cationic properties. In acid conditions below pKa 6.5, primary amines are positively charges. This enables binding to negatively charged DNA by electrostatic interactions and the spontaneous formation of nanoparticles (*10-12*). This formation also involves hydrogen bonding and hydrophobic interactions. The electrostatic interaction is described as the N:P charge ratio between the positive amine groups of chitosan to the negative phosphate backbone of DNA. Nanoparticles can be prepared using a variety of methods and techniques which can influence transfection efficiency (*162, 176, 180, 181*).

Chitosan nanoparticles have favourable characteristics that facilitate oral gene delivery. Nanoparticles can protect DNA from proton degradation in the stomach and enzymatic degradation in the intestine. Mucoadhesive properties increase residence time and induce transient opening of tight junctions (*161, 182, 183*). It has been suggested that chitosan nanoparticles are absorbed by the gastrointestinal tract in a size dependant manner by M cells and enterocytes (*165, 184*). Nanoparticles can enter cells through macropinocytosis and caveolae-mediated endocytosis (*161, 185, 186*). The high charge

density at low pH also facilitates escape from endocytic vesicles via proton sponge effect(*187*). A balance must be reached between DNA protection and DNA release to provide efficient gene transfection (*177, 188, 189*).

1.5.2 Chitosan as oral gene delivery agent

Chitosan-mediated oral gene delivery is an attractive treatment strategy that facilitates transgene expression, mucosal immunity and non-invasive administration that may be applicable to mass vaccination especially in developing nations. Roy *et al.* first demonstrated the delivery of the peanut allergen Arah2 DNA in chitosan nanoparticles in a murine model of peanut hypersensitivity. Treatment induced secretory IgA mucosal immune response that protected against anaphylactic reactions to peanut allergen (*190*). Li *et al.* examined oral delivery of TGF-β DNA to alleviate food allergy symptoms (*191*). TGF-β is involved in the induction and maintenance of oral tolerance by facilitating IgA secretion and development of regulatory T cells. Immune response modulation was also demonstrated by oral gene delivery of dust mite allergen (*192, 193*). Furthermore, Chen *et al*. used chitosan nanoparticles in the delivery of erythropoietin DNA for the stimulation of red blood cells production (*194*). This gene therapy strategy has also been examined in the delivery of FVIII DNA for the treatment of hemophilia. Transient human FVIII expression was detectable on day 21 post treatment that was unsustainable with repeated administration (*195*). Recently we have reported a chitosan-mediated FVIII DNA delivery formulation that provided sustainable therapeutically FVIII expression that is amenable to one-tablet-a-day administration (*196*). Antibody response to transgene

expression was not detectable and may imply formation of mucosal tolerance. Chitosan mediated gene delivery may provide both therapeutic transgene expression and the development of prophylactic tolerance.

Chapter 2

Objectives

Hemophilia is an ideal target for gene therapy as modest transgene expression is sufficient to provide phenotypic correction. Current hemophilia treatment involves protein replacement therapy of coagulation factors, which is invasive, expensive, prone to antibody formation and inaccessible to a majority of patients world-wide. Oral gene therapy has the potential to address these challenges. More specifically, chitosan-mediated gene delivery has demonstrated *in vivo* expression of peanut allergen (*190*), erythropoietin (*194*) and transforming growth factor-beta (*191*). Also, chitosan-mediated expression of human FVIII was reported by Bowman *et al.*(*195*), although, the transgene was only detected three weeks post administration by indirect methods and was not sustainable. Recently, we have demonstrated *in vivo* FVIII expression that was sustainable and amenable to daily administration for the treatment of hemophilia(*196*). Transgene expression in the mucosa may provide tolerogenic protein presentation and immune response modulation.

It is hypothesized that orally administered chitosan/DNA nanoparticles can provide clinically relevant transgene expression of coagulation factor FVIII or FIX in hemophilic mice. To achieve this goal plasmid CMV-BDD-cFVIII coding for canine plasmid was utilized to permit gavage of chitosan nanoparticles and subsequent detection of modest gene delivery and anti-hemophilic activity. FVIII expression kinetics and phenotypic correction was examined *in vivo* using C57BL/6$^{FVIII-/-}$ hemophilia A mice.

Furthermore, this study investigates the potential immune response modulation of oral chitosan-mediated gene delivery containing human FVIII DNA in naive hemophilic mice and mice with pre-existing FVIII inhibitors. Another aim of this study is to demonstrate the chitosan-mediated transgene expression of human FIX in C57BL/6$^{FIX-/-}$ hemophilia B mice. Oral gene delivery of FIX was facilitated by nanoparticles formulated with a CpG-FIXi plasmid. Finally, this study characterizes chitosan nanoparticle formulations and factors contributing to improved therapeutic performance.

Aim I: To evaluate the feasibility of oral administration of chitosan nanoparticles containing FVIII DNA to provide sustainable FVIII activity in hemophilia A mice. More specifically:

a. Dose response to oral administration of nanoparticles in hemophilic mice
b. FVIII kinetics in hemophilic mice following chitosan-FVIII DNA ingestion
c. Phenotypic correction following oral FVIII gene delivery
d. Repeated oral administration of cFVIII DNA in hemophilic mice
e. Characterization of immune response to transgene expression

Aim II: Examine the immune response modulation of orally administered chitosan nanoparticle-mediated hFVIII gene delivery in hemophilia A mice. More specifically:

a. Chitosan/DNA nanoparticle formulation for the delivery of human FVIII
b. Immune response modulation following oral hFVIII administration

c. Oral nanoparticle administration induce long-term tolerance in naive hemophilia A mice
d. Immune response modulation in the presence of pre-existing FVIII inhibitors
e. Adoptive transfer of splenocytes from tolerized mice into naïve recipient mice

Aim III: Demonstrate chitosan nanoparticle-mediated hFIX gene delivery for the treatment of hemophilia B. More specifically:

a. Selection of hFIX plasmid
b. FIX transgene expression in hemophilia B mice
c. Batch variation

Aim IV: Optimize and characterize transfection efficiency of Chitosan/DNA nanoparticle formulations. More specifically:

a. Size & morphology of nanoparticles
b. Surface charge of nanoparticles
c. Stability of DNA against proton and enzymatic degradation
d. Effect of electrolyte concentration & N:P charge ratio
e. Effect of chitosan type
f. Production of concentrated nanoparticles

Chapter 3:

Aim I: To evaluate the feasibility of oral administration of chitosan nanoparticles containing FVIII DNA to provide sustainable FVIII activity in hemophilia A mice

Hemophilia A is an inherited bleeding disorder caused by a deficiency of coagulation factor VIII (FVIII) that affects 1:5,000 males(*34*). Current treatment relies on regular infusions of FVIII protein, either plasma-derived or recombinant(*197*). Albeit effective, this treatment is invasive and expensive (>$100,000 per patient annually), and not immune from clinical complications. A challenge for hemophilia management is the development of neutralizing antibodies (inhibitors) to infused FVIII that occur in 30% of hemophiliacs, which decrease treatment efficacy(*198*). Thus, an alternative treatment is desirable.

Hemophilia is a suitable model for gene therapy, as restoration of modest FVIII activity can provide phenotypic correction. Current gene therapy strategies, effective in expressing high levels of functional FVIII and FIX in murine and canine models of hemophilia (*199, 200*), have shown limited efficacy in clinical trials (*154, 201*). These challenges have sparked interest in novel non-viral gene therapy strategies (*195, 202, 203*).

Oral administration of DNA or gene tablet may provide a cost effective, non-invasive approach with enhanced patient compliance. The gastrointestinal (GI) tract is able to absorb plasmid DNA and subsequently secrete the transgene product into blood

(*190, 191, 193-195*). To increase uptake efficiency, strategies are being devised to protect and chaperone DNA through the GI tract as a site for tolerance induction.

Chitosan, a cationic polymer, forms nanoparticles through electrostatic interactions with anionic DNA that can facilitate delivery in the gut(*204, 205*). These nanoparticles protect entrapped DNA from digestion(*206*), increase transcellular and paracellular transport across mucosal epithelium(*207, 208*), mediate uptake by the Peyer's patches(*209*) and have been used for the delivery of a variety of therapeutic genes(*190-195*).

Oral administration of chitosan hFVIII-DNA nanoparticles has been reported by Bowman *et al*(*210*). The authors observed evidence of hFVIII in plasma by thrombin generation assay, and a peak of 2-4% of FVIII activity(*195*). Here, we examined the effect of repeated oral administration of chitosan/DNA nanoparticles containing canine FVIII cDNA, of higher activity than human FVIII. The delivered FVIII plasmid DNA was detected in intestine and, to a lesser extent, in the liver(*196*). Oral delivery of nanoparticles provided transient FVIII activity that was sustainable upon re-administration. Importantly, no antibodies to FVIII were detected in response to recurrent FVIII delivery. Our data suggest that oral administration of chitosan nanoparticles containing FVIII DNA may provide an alternative treatment for hemophilia.

3.1 Materials & Methods

Plasmid vectors

Plasmid pBK-CMV-cFVIIIcDNABDD, containing the canine FVIII cDNA (cFVIII), was provided by Dr. D. Lillicrap (Queen's University, Kingston, Ontario, Canada). Plasmid DNA was prepared using a GigaPrep Kit (Qiagen, Mississauga, Ontario, Canada).

Chitosan/DNA nanoparticle formulation

Chitosan/DNA nanoparticles were formed by complex coacervation method described previously (*190*). Chitosan (CL 213; degree of deacetylation >84%; Novamatrix, Drammen, Norway), in 25 mM sodium acetate-acetic acid and plasmid DNA dissolved in 50 mM Na_2SO_4 was heated to 55°C. The chitosan and DNA solutions were mixed and vortexed for 20 s. Nanoparticles were formulated at an N:P charge ratio of 4:1.

Animal Procedures

Hemophilia A mice ($C57BL/6^{FVIII-/-}$) were fed chitosan nanoparticles containing either 25, 50 or 100 µg cFVIII DNA via gavage using a 20G needle (Popper & Sons, New Hyde Park, NY). Blood samples were collected via orbital plexus in citrate. All experiments were conducted in adherence to the Animal Ethics Guidelines of McMaster University.

FVIII activated partial thromboplastin time (aPTT)

Plasma clotting time was determined by aPTT in an automated coagulometer (General Diagnostics Coag-A-Mate). Samples and canine reference standards were mixed with equal parts human FVIII deficient plasma, veronal buffer and aPTT reagent

(Organon Tecknika Corp, Durham NC, USA) for 3 min at 37°C. Subsequently, 25 mM $CaCl_2$ was added and the time to clot formation was measured. Normal canine plasma was used as standard. The aPTT detection limit was 2.4 mU canine FVIII activity.

cFVIII antigen and antibody ELISA

Plasma cFVIII antigen levels were detected using a cFVIII ELISA kit (Affinity Biologicals, Hamilton, Ontario, Canada), using normal canine plasma as standard. The ELISA detection limit was 3.6 mU canine FVIII antigen.

A modified ELISA was developed to detect antibodies to canine FVIII expression in mice. Briefly, wells were coated first with goat anti-canine FVIII capture antibody (Affinity Biologicals, Hamilton, Ontario, Canada), followed by normal canine plasma and finally mouse plasma samples. Normal canine plasma provided a source of canine FVIII to capture mouse anti-canine FVIII antibodies in test samples. Bound mouse anti-cFVIII antibodies were detected with anti-mouse IgG antibodies (Promega, USA). Positive control plasma obtained from mice immunized with genetically-modified cells-secreting cFVIII (inhibitor titer of 3.4 BU) was used. The antibody ELISA had a detection limit of OD 0.37.

Bethesda assay

Plasma samples were examined for cFVIII inhibitors using a modified Bethesda assay(*211*). Briefly, samples were mixed with equal volumes of buffered normal plasma pH 7.4 and incubated for 2 h at 37°C. Bethesda Units were determined using aPTT assay by comparing test samples with human FVIII deficient control plasma activities.

Tail clip assay

Phenotypic correction in hemophilia A mice following nanoparticle administration was determined by tail clip assay. Hemophilia A mice were treated with nanoparticles (100 µg) and examined on days 1 (n=10), 4 (n=13) or 7 (n=10) post-treatment. Tails were transected at a 2-mm diameter and immediately immersed in normal saline at 37°C. Bleeding was followed visually for 15 minutes. Blood loss was quantified by hemoglobin content that accumulated in the saline solution. Red blood cells were resuspended in lysis buffer (NH_4Cl 8 g/L; $KHCO_3$ 1g/L; EDTA 0.037 g/L) and absorbance was measured at 575 nm. Controls include naive hemophilia A mice (n=6) and normal BALB/c mice (n=5).

Statistics

Data analysis performed with GraphPad Prism 5 statistical software package. A value of $p<0.05$(*) was considered significant while $p<0.01$(**) and $p<0.001$(***) were considered highly significant.

3.2 Results

a. Dose response to oral administration of nanoparticles in hemophilic mice

Chitosan nanoparticles are formed through electrostatic interactions between the positively charged amine groups on chitosan and negatively charged phosphate backbone of DNA. The nanoparticles in this study were formulated at an N:P charge ratio of 4:1 using 25 mM acetic acid and 50 mM sodium sulphate. A plasmid containing the canine

FVIII (cFVIII) cDNA was used for its greater stability and reportedly 5-7 times higher specific activity than its human homologue (*212*). This higher expression permits detection of modest transgene expression in plasma. Canine FVIII cDNA sequence has a high sequence identity to human FVIII(*213*). In humans, a small nucleotide change in the hFVIII gene can lead to inhibitor formation(*100*). Nevertheless, mucosal FVIII presentation may induce tolerance and permit use of FVIII variants including canine FVIII.

The dose-response to chitosan nanoparticles containing canine FVIII was examined in hemophilia A mice. Hemophilia A mice (n=4) were fed nanoparticles containing 25 µg, 50 µg or 100 µg cFVIII plasmid. One day following treatment the plasma cFVIII antigen level was analyzed by cFVIII ELISA. Plasma cFVIII levels >50mU and >100mU were achieved following administration of nanoparticles containing 25 µg and 100 µg, respectively (Figure 3.0). However, variation in the resulting plasma levels did not provide statistically significant difference between transgene expressions ($p<0.523$).

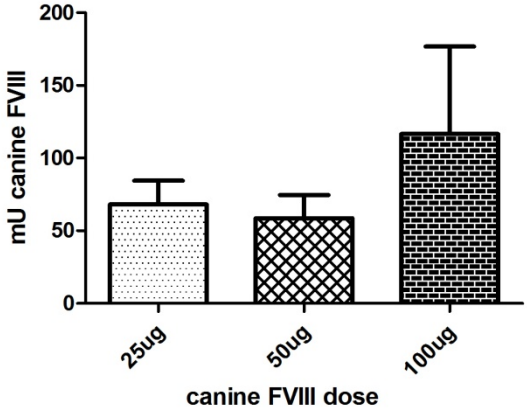

Figure 3.0: Dose dependence transgene expression following chitosan nanoparticles administration containing canine FVIII plasmid. Hemophilia A mice (n=4) were gavaged chitosan nanoparticles formulated with 25 mM acetic acid and 50 mM sodium sulphate containing 25 µg, 50 µg or 100 µg canine FVIII plasmid. Plasma samples were taken one day following treatment and examined using ELISA. Bars represent mean ± SEM.

b. FVIII kinetics in hemophilic mice following chitosan-FVIII DNA ingestion

The kinetics of circulating FVIII was evaluated following a single administration of chitosan nanoparticles. The change in plasma FVIII activity following oral DNA administration was monitored over a four-week period. Hemophilia A mice (n=30) were fed a single dose of chitosan nanoparticles containing 100 µg cFVIII DNA. Blood samples were collected two weeks pre-treatment, and for groups of mice (n=6) on days 0.5, 1, 2, 3, 4, 7, 14, 21, and 28 post-treatment. Untreated hemophilia A mice, and wild type mice were used as controls. Circulating FVIII on day one showed a statistically significant increase ($p<0.001$) over pre-treatment values, which reflects an average FVIII activity of 77 mU in treated mice (Figure 3.1A). However, FVIII activity was temporary,

as it regressed back to baseline by day 2 post-treatment. Nevertheless, trace FVIII activity was observed in the days following peak expression.

c. Phenotypic correction following oral FVIII gene delivery

The oral administration of a single dose of chitosan nanoparticles protected hemophilic mice against trauma, in the form of a standardized tail transection. A group of FVIII-deficient mice (n=33) was administered nanoparticles containing 100 µg cFVIII plasmid DNA. A tail clip assay was performed on days 1, 4 or 7 post-treatment to determine tail bleeding times (Figure 3.1B). All wild type mice (n=5) stopped bleeding within 15 minutes while all naive Hemophilia A mice (n=6) bled beyond 15 minutes. In contrast, on day one post-treatment, 50% of treated hemophilia mice bled for less than 15 minutes. The transient nature of phenotypic correction was demonstrated by excessive bleeding on days 4 and 7 post-treatment. Interestingly, a subset of treated hemophilic mice displayed phenotypic correction on day 4. This trend was further confirmed by measuring the volume of blood loss as reflected by hemoglobin concentration of blood collected following tail transaction (Figure 3.1C). The blood loss in wild type mice was minimal while excessive in naive hemophilia A mice. In mice treated with nanoparticles, blood loss was reduced one day after feeding but regressed to the hemophilic phenotype by day 4 or 7 post treatment (Figure 3.1C).

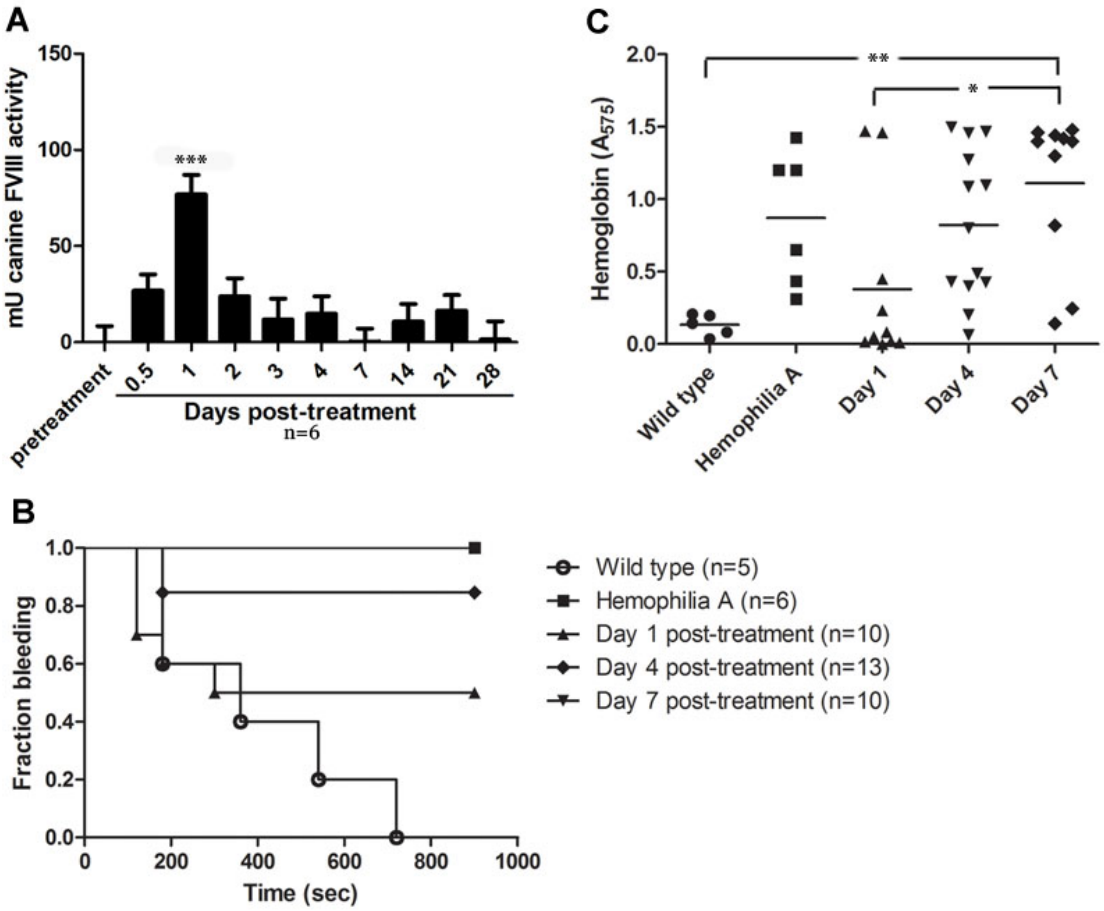

Figure 3.1: *in vivo* cFVIII activity and phenotypic correction of hemophilic mice following oral administration of chitosan nanoparticles containing 100 µg cFVIII DNA. (A) Plasma clotting time examined two weeks pre-treatment then in select groups of mice (n=6) on days 0.5, 1, 2, 3, 4, 7, 14, 21 and 28 post-treatment. Control group include wild type mice (n=4, 281.6±27.9 mU) and pooled human plasma (n=3, 375.8±7.7 mU). (B**)** Phenotypic correction of hemophilic mice following oral nanoparticle administration. Tail clip assay performed on mice on days 1 (n=10), 4 (n=13), and 7 (n=10) post-treatment. Control groups include wild type mice (n=5) and naïve hemophilia A mice (n=6). Tail clip experiments were terminated after 15 minutes. Tail bleeding times presented as a fraction of mice still bleeding as a function of time (C) Blood loss determined by measuring hemoglobin absorbance (575nm) of blood collected following tail transection. Bars represent mean±SEM. *p<0.05, **p<0.01, ***p<0.001, compared with untreated hemophilic mice.

d. Repeated oral administration of cFVIII DNA in hemophilic mice

Following the transient cFVIII expression observed after single administration of nanoparticles in treated mice, we determined whether repeated administrations of nanoparticles could restore cFVIII activity in previously treated mice. A group of hemophilia A mice (n=7) were fed nanoparticles containing 100 µg of cFVIII DNA once a week for a total of four weeks. Untreated hemophilic mice were used as controls. The plasma FVIII activity in treated mice was on average 100 mU on day 1 (Figure 3.2A) and remained elevated at statistically significantly levels for all four weeks of treatment. A week after the last feed, FVIII activity decreased markedly, although some residual activity remained (Figure 3.2A).

Complementing these results, canine FVIII antigen levels increased to statistically significant levels on day 1 ($p<0.001$) to >100 mU, and remained detectable throughout four weeks (Figure 3.2B). Canine FVIII antigen was undetectable on week 5, confirming the transient nature of cFVIII expression. These results indicate the potential of re-administration of nanoparticles in providing sustainable FVIII levels for the treatment of hemophilia.

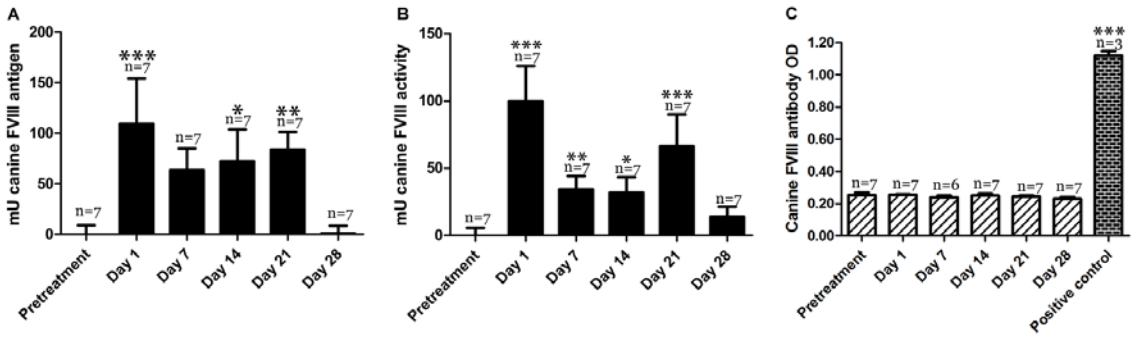

Figure 3.2: Plasma cFVIII activity, antigen and antibody detection following repeated weekly oral administration. Hemophilia A mice were administered chitosan nanoparticles containing 100 µg cFVIII on four consecutive weeks. Plasma samples collected two weeks pre-treatment then 24 h following each administration on days 1, 7, 14, 21 and a week following final administration. (A) Plasma clotting time. (B) Plasma cFVIII antigen levels determined by ELISA. (C) Antibody response to repeated weekly oral administration of chitosan nanoparticles. Antibody production to canine FVIII expression was detected by anti-cFVIII ELISA. Positive control consists of plasma from hemophilia A mice injected with engineered cells secreting cFVIII (3.4 BU). Bars represent mean±SEM. *p<0.05, **p<0.01, ***p<0.001, compared with untreated hemophilic mice.

e. Characterization of immune response to transgene expression

A major challenge for hemophilia management is the presence of inhibitors. However, despite weekly repeated oral administration of chitosan nanoparticles, inhibitors were not detected in any of the treated mice, as determined by a Bethesda Assay (data not shown, zero values). Additionally, a specific ELISA protocol was developed for the detection of anti-cFVIII antibodies. Interestingly, antibodies to cFVIII were undetectable in any of the treated mice during the experiment (Figure 3.2C), in agreement with the lack of inhibitors.

3.3 Discussion

The purpose of this study was to determine whether oral administration of chitosan/DNA nanoparticles could provide sustainable delivery of functional FVIII in hemophilic mice. Similar studies have previously shown that oral administration of DNA provides subsequent expression of transgenes such as mEpo(*194*) or the peanut allergen Arah2(*190*).

Oral administration of chitosan nanoparticles containing cFVIII plasmid provided detectable levels of FVIII expression. The absence of dose-dependent transgene expression may suggest that a limit for gene delivery was reached or that the chitosan nanoparticle batch-variation must be controlled. Alternatively, the use of a larger number of mice may have produced a significant dose response. Nevertheless, oral administration led to a partial and transient increase in FVIII plasma activity in treated hemophilia mice, with an average of >100 mU cFVIII activity. This level of activity, if achieved in humans, would convert severe or moderate hemophilia to a mild phenotype(*34*). The protection against trauma of mice following the tail clip assay also suggests correction of the hemophilic bleeding phenotype. However, the fact that canine FVIII has greater stability and reportedly 5-7 times higher specific activity than its human homologue(*214*), must be taken into account when considering human applications of this strategy.

A previous report by Bowman *et al* (2008) has examined the oral administration of FVIII DNA in chitosan nanoparticles to deliver human FVIII, resulting in levels of 2-4% in hemophilic mice(*195*). These levels are comparable to those reported in this study given the higher activity of canine over human FVIII transgene. Of interest, Bowman *et*

al reported a peak of FVIII delivery 3 weeks after treatment(*195*), in contrast to the transient FVIII expression reported here. The difference in kinetics may be attributed to nanoparticle formulation and method of administration. Nevertheless, the transient kinetics that peaks one day post-treatment may be amenable to daily re-administration regimen for the treatment of hemophilia A.

The transient nature of FVIII activity agrees with previous studies of oral administration of chitosan DNA nanoparticles (*191, 194*). A number of hypotheses may explain the transient nature of cFVIII expression. Transgene silencing via inhibition of the viral CMV promoter *in vivo* (*215*) or the presence of CpG dinucleotides (*203, 216, 217*) might have contributed to the loss of FVIII activity. Also, hFVIII cDNA is known to contain intrinsic sequences that inhibit its own transcription (*87*), and decrease RNA accumulation (*218*), although canine factor VIII does not display the same transcriptional down regulation as its human homologue. A further explanation for the rapid loss of cFVIII activity could be the rapid turnover of cells in the intestinal epithelium, where cFVIII expression mainly occurred. It takes as little as 48 hours for the cells to migrate from the base of the crypt where they are formed, to the tip of the villus (*219, 220*). Thus, FVIII production could have ceased as early as 2 days post treatment and cleared from plasma or soon thereafter given its short half-life. Also, loss of plasmid by the transfected cells would explain a transient FVIII expression.

Repeated administration of nanoparticles resulted in the restoration of transient FVIII activity. The presence of FVIII was confirmed with the associated detection of cFVIII antigen following re-administration. However, this transgene expression was

absent a week following final administration. The absence of FVIII accumulation may be attributed to blood-sampling related trauma. Furthermore, chitosan nanoparticle batch variation may contribute to the week-to-week variations in transgene expression. Nevertheless, these results indicate the potential of re-administration of nanoparticles to provide sustainable FVIII levels for the treatment of hemophilia. In contrast, a previous report of declining FVIII levels were not rescued following re-administration(*195*).

The antigenic nature of FVIII complicates conventional treatment through the generation of inhibitors(*100*) and subsequent loss of FVIII activity(*221*). Interestingly, while therapeutic levels were achieved in this study, inhibitors were undetectable for at least 5 weeks. The sustainable activity of plasma FVIII further suggests the absence of neutralizing antibodies. It is noted that the cross species formation of antibodies (i.e. mouse anti-canine FVIII antibodies) is different than the formation of autologous antibodies (i.e. mouse anti-murine FVIII antibodies)(*222*).

Moreover, non-neutralizing antibodies to the relatively large FVIII transgene were also undetectable. Although, it has been suggested that immune tolerance to coagulation factors may be achieved by mucosal exposure(*223*). Chitosan itself is biocompatible, biodegradable and has low cellular toxicity while it has been frequently used as an adjuvant in gene therapy strategies as well as genetic vaccine design (*190, 224*). The immune response implications of chitosan-mediated gene delivery require further investigation before implementation for human application.

Together these data suggests that chitosan-mediated oral gene delivery may provide favourable kinetic profile of FVIII transgene expression and phenotypic

correction that is amenable to re-administration. Long-term studies are required to assess the potential of chitosan nanoparticles as an alternative treatment for hemophilia A. Nevertheless, these findings suggest that administration of chitosan nanoparticles may be an effective treatment for hemophilia A without inducing immune response complications.

Chapter 4

Aim II: Examine the immune response modulation of orally administered chitosan nanoparticle-mediated hFVIII gene delivery in hemophilia A mice

Hemophilia A is genetic bleeding disorder treated with protein replacement therapy involving frequent infusions or recombinant or plasma-derived FVIII. A major challenge in hemophilia management is the formation of neutralizing antibodies (inhibitors), which reduces the effectiveness of treatment while increasing the risk of morbidity and mortality. In a third of patients, exogenous FVIII is recognized as a foreign protein and initiates a humoral immune response to facilitate its clearance from circulation. The formation of neutralizing antibodies is associated with FVIII gene mutations including missense mutations, deletions and inversions which prevent self-tolerance to functional FVIII(*225*).

Immune tolerance induction (ITI) protocols employed to overcome inhibitors involve daily administration of large amounts of FVIII. The exact mechanism involved in generating immune unresponsiveness is unknown. FVIII tolerance achieved using this technique may take months or years to achieve and has a success rate of 60-80%. This highly invasive treatment may demand use of a central venous catheter which presents risk of infection and thrombosis. ITI protocols are not a practicable option for the majority of haemophiliacs that do not have access to FVIII products or finances to cover

the $1,000,000 treatment cost. Taken together, a cost effective strategy for prophylactic tolerance induction is desirable for hemophilia patients.

Tolerance to self-antigens is primarily accomplished through central tolerance involving negative selection of autoreactive T cells in the thymus. Although the central tolerance mechanism is efficient, it cannot eliminate all self-reactive lymphocytes. Peripheral tolerance mechanisms limit the activation of self-reactive T cells that have left the thymus. Tolerance can also be achieved towards food antigens taken orally through the gut mucosa. Indeed, the gut associated lymphoid tissue (GALT) is the largest immune system in the body and presents a tolerogenic environment that absorbs diverse foreign antigens without adverse reactions and protects against pathogens.

Mucosal tolerance has been explored as a means to induce prophylactic tolerance to coagulation factors(*226-228*). Mucosal administration of FVIII C2 subunits was shown to provide tolerance to the C2 domain but not to full length FVIII. A novel approach by Verma et al, demonstrated bio-encapsulated FIX protein produced in transplastomic plants was able to suppress FIX inhibitor formation in hemophilia B mice(*228*). However, it remains to be determined if this prokaryotic system can express the larger FVIII protein and its associated post-translational modifications.

Oral gene therapy may provide tolerogenic presentation of functional proteins at the mucosal surface(*229*). Chitosan-mediated oral delivery has been demonstrated in the delivery of a variety of genes(*190, 191, 193-196*). Recently, we have reported the use of a chitosan-mediated oral gene delivery strategy to provide sustainable therapeutic levels of FVIII in hemophilic mice(*196*). The kinetics of expression was found to be transient and

amenable to daily administration. Interestingly, this mucosal gene delivery system did not produce detectable levels of neutralizing (inhibitors) or non-neutralizing antibodies. The current study further examines the immune modulation of mucosal FVIII gene delivery in hemophilia A mice. We show that prior exposure to chitosan nanoparticle containing human FVIII DNA suppresses the antibody response to VIII and provides long term tolerance.

4.1 Materials & Methods

Plasmid vectors

Plasmids pcDNA/BDDFVIII, MFGBDDhFVIII, pcDNA-IVS-hFVIII, pSP64-hFVIII, pBIISK-BDDhFVIII and pSBT/mCAGFVIII were prepared using a MaxiPrep Kit (Qiagen, Mississauga, Ontario, Canada).

Chitosan/DNA nanoparticle formulation

Chitosan/DNA nanoparticles were formed by complex coacervation method described previously (*190*). Chitosan (CL 213; degree of deacetylation >84%; Novamatrix, Drammen, Norway), and plasmid DNA dissolved in 50 mM Na_2SO_4 was heated to 55°C. The chitosan and DNA solutions were mixed and vortexed for 20 s. Nanoparticles were formulated at an N:P ratio of 3:1.

***In vitro* transfection of HEK 293 cells**

HEK 293 cells were cultured in 12-well plates with α-MEM, 10% fetal bovine serum, 1% Pen/Strep. Cells were transfected with 0.5 pmol hFVIII plasmid formulated in

lipofectamine and examined 48 h post-transfection. FVIII secretion was assayed by human FVIII ELISA (Affinity Biologicals, Canada).

Animal Procedures

Hemophilia A mice (C57BL/6$^{FVIII-/-}$) were fed chitosan nanoparticles containing 100 µg human FVIII DNA via gavage using a 20G needle (Popper & Sons, New Hyde Park, NY). Blood samples were collected via orbital plexus in citrate. Recombinant human FVIII (Bayer Healthcare Pharmaceuticals) was given by weekly i.p. injection at 0.75 U. All experiments were conducted in adherence to the Animal Ethics Guidelines of McMaster University.

Human FVIII antibody ELISA

A modified ELISA was developed to detect antibodies to human FVIII expression in mice. Briefly, wells were coated first with goat anti-human FVIII capture antibody (Affinity Biologicals, Hamilton, Ontario, Canada), followed by normal human plasma and finally mouse plasma samples. Bound mouse anti-hFVIII antibodies were detected with anti-mouse IgG antibodies (Promega, USA).

Bethesda assay

Plasma samples were examined for human FVIII inhibitors using a modified Bethesda assay(*211*). Briefly, samples were mixed with equal volumes of buffered normal human plasma pH 7.4 and incubated for 2 h at 37°C. Bethesda Units were determined using aPTT assay by comparing test samples with human FVIII deficient control plasma activities.

Statistics

Data analysis performed with GraphPad Prism 5 statistical software package. A value of $p<0.05$(*) was considered significant while $p<0.01$(**) and $p<0.001$(***) were considered highly significant.

4.2 Results

a. Chitosan/DNA nanoparticle formulation for the delivery of human FVIII

A human FVIII (hFVIII) plasmid was selected to replace the canine FVIII used previously to enable challenge with injected recombinant human FVIII (rhFVIII). The transgene expression of a number of human FVIII plasmids were examined *in vitro* (Figure 4.0). HEK 293 cells were transfected with lipofectamine containing 0.5 pmol of pcDNA/BDDFVIII, MFGBDDhFVIII, pcDNA-IVS-hFVIII, pSP64-hFVIII, pBIISK-BDDhFVIII or pSBT/mCAGFVIII. Media containing no plasmid or transfection agent was used as control. Expression of hFVIII was highest for pcDNA-IVS-hFVIII, which contains the human IVS2 intronic sequence and is used in the remainder of this study.

An optimized formulation of chitosan nanoparticles was used to compensate for the lower activity of human FVIII relative to canine FVIII. The formation of chitosan nanoparticles is dependent on the electrostatic interactions between the positively charge amine groups on chitosan and the negatively charged phosphate backbone of DNA. The previous formulation consisted of chitosan chloride complex with DNA at an N:P charge ratio of 4:1 incorporating 50 mM sodium sulphate and 25 mM sodium acetate-acetic acid.

The optimized formulation used in the following studies consists of chitosan chloride salt 213 (CL213) at an N:P ratio of 3:1 with 50 mM sodium sulphate only. This optimized formulation was shown to increase transfection 4-fold (see Chapter 6).

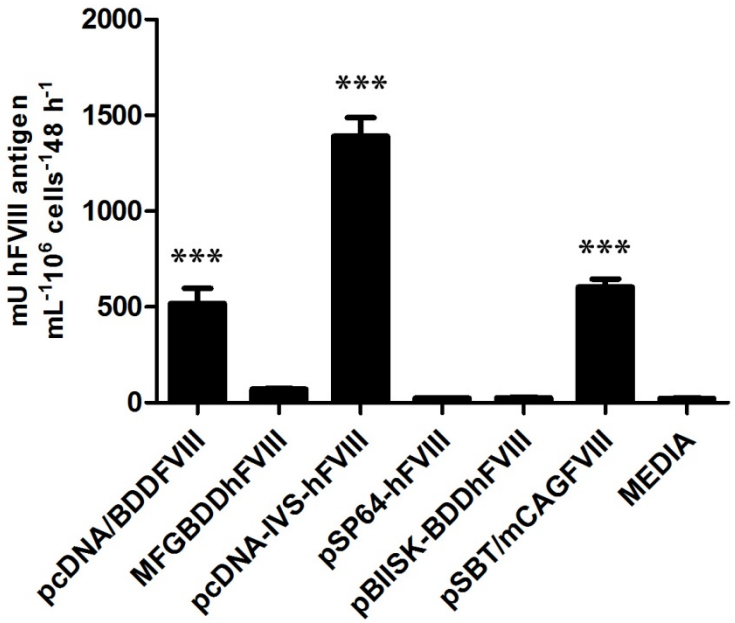

Figure 4.0: *in vitro* expression of hFVIII plasmids. HEK 293 cells were transfected with lipofectamine containing either pcDNA/BDDFVIII, MFGBDDhFVIII, pcDNA-IVS-hFVIII, pSP64-hFVIII, pBIISK-BDDhFVIII or pSBT/mCAGFVIII. Media containing no plasmid DNA or transfection agent was used a control. FVIII expression was determined by human FVIII ELISA following 48 hours transfection. Bars represent mean±SEM. ***$p<0.001$, compared with control.

b. Immune response modulation following oral chitosan/hFVIII administration

Next we examined the potential for immune response modulation following orally administered chitosan-hFVIII nanoparticles. Hemophilia A mice (n=14) were fed nanoparticles containing 100 µg hFVIII DNA once weekly for a total of four weeks

(Figure 4.1A-B). A subgroup of these mice (n=8) were subsequently immunized with 0.75 U rhFVIII once a week for a total of four weeks, which represent weeks 5, 6, 7 and 8 post-treatment (Figure 4.1B). Blood plasma samples were examined for both neutralizing (inhibitors) and non-neutralizing antibodies by anti-hFVIII ELISA. Consistent with our previous findings, hFVIII antibodies were undetectable following oral administration of chitosan nanoparticles (Figure 4.1A, 4.1E). Furthermore, subsequent immunization with rhFVIII did not produce hFVIII antibodies that were statistically significant from controls (Figure 4.1B, 4.1E). This is in contrast to immunization of naïve hemophilia A mice, which produced high antibody titres (Figure 4.1C, 4.1E). Taken together, these results suggest that pre-treatment with orally administered nanoparticles provides protection against antibody formation following rhFVIII challenge.

The modulation of pre-existing antibodies following oral nanoparticle administration was also examined. Hemophilia a mice (n=11) were immunized with weekly i.p. injections with 0.75 U rhFVIII for a total of four weeks (Figure 4.1C, 4.1D). A subgroup of mice (n=5) were subsequently fed chitosan nanoparticles containing 100 µg hFVIII DNA on weeks 5, 6, 7 and 8 (Figure 4.1D). Immunized mice produced high antibody titres that persist for up to 8 weeks (Figure 4.1E). Treatment with nanoparticles did not significantly reduce these antibody titres; although, the average antibody titre was lower (Figure 4.1D, 4.1E). It will be interesting to determine if this downward trend continues over time as a result of oral nanoparticle administration.

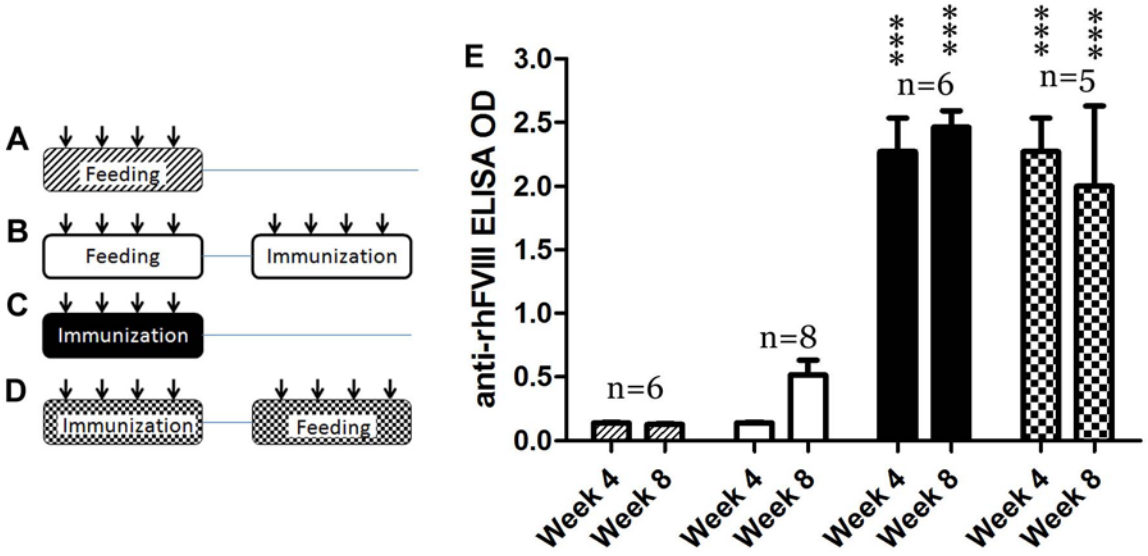

Figure 4.1: hFVIII antibody response modulation. (A) Hemophilic mice fed chitosan nanoparticles containing 100 µg hFVIII DNA once a week for a total of four weeks. (B) Mice fed chitosan nanoparticles during the first four weeks followed by weekly immunizations with 0.75 U rhFVIII on weeks 5, 6, 7 and 8. (C) Hemophilia A mice were given weekly i.p. injections of 0.75 U rhFVIII for a total of four weeks. (D) Mice given weekly rhFVIII injections over the first four weeks followed by oral nanoparticle administration on weeks 5, 6, 7, and 8. Plasma samples were taken on weeks 4 and 8. Weekly administration regiment for hemophilia A mice indicated by arrows. (E) Anti-human FVIII ELISA performed on plasma samples to detect both neutralizing (inhibitors) and non-neutralizing human FVIII antibodies. Bars represent mean±SEM. ***$p<0.001$, compared with control (A).

c. Oral nanoparticle administration induce long-term tolerance to hFVIII in naïve hemophilia A mice

The prophylactic tolerance induced in naïve mice was further examined over a 16-week period. A group of hemophilia A mice (n=5) were fed chitosan nanoparticles containing 100µg hFVIII DNA weekly for a total of eight weeks. Consistent with previous findings, the repeated oral administration of chitosan nanoparticles did not produce detectable antibody or inhibitor levels as determined by anti-hFVIII ELISA

(Figure 4.2A, 4.2G) and Bethesda assay (Figure 4.2D, 4.2H), respectively. A second group of mice (n=7) were also fed chitosan nanoparticles for a total of eight weeks and subsequently immunized with 0.75 U rhFVIII on weeks 9, 10, 11 and 12. The majority of immunized treated mice did not develop detectable antibodies over the 16-week study period (Figure 4.2B, 4.2G), which correlated with inhibitor titres that were not significant from controls (Figure 4.2E, 4.2H). This further supports our previous findings that oral nanoparticle administration can induce tolerance to rhFVIII challenge. Finally, a third group of hemophilia A mice (n=8) were fed weekly for a total of twelve weeks concurrently with rhFVIII immunizations on weeks 9, 10, 11 and 12. Low level antibody titres were detected on week 12 which reduced to background by week 16 (Figure 4.2C, 4.2G). Nevertheless, inhibitor titres were not significantly different from controls (Figure 4.2F, 4.2H). These results suggest that pre-treatment with nanoparticles can induce oral tolerance to suppress antibody titres and prevent inhibitor development to FVIII challenge.

Dosing schedule
Group A (n=5) Feed (week1-8)
Group B (n=7) Feed (week1-8), Immunize (week9-12)
Group C (n=8) Feed (week1-12), immunize (week 9-12)

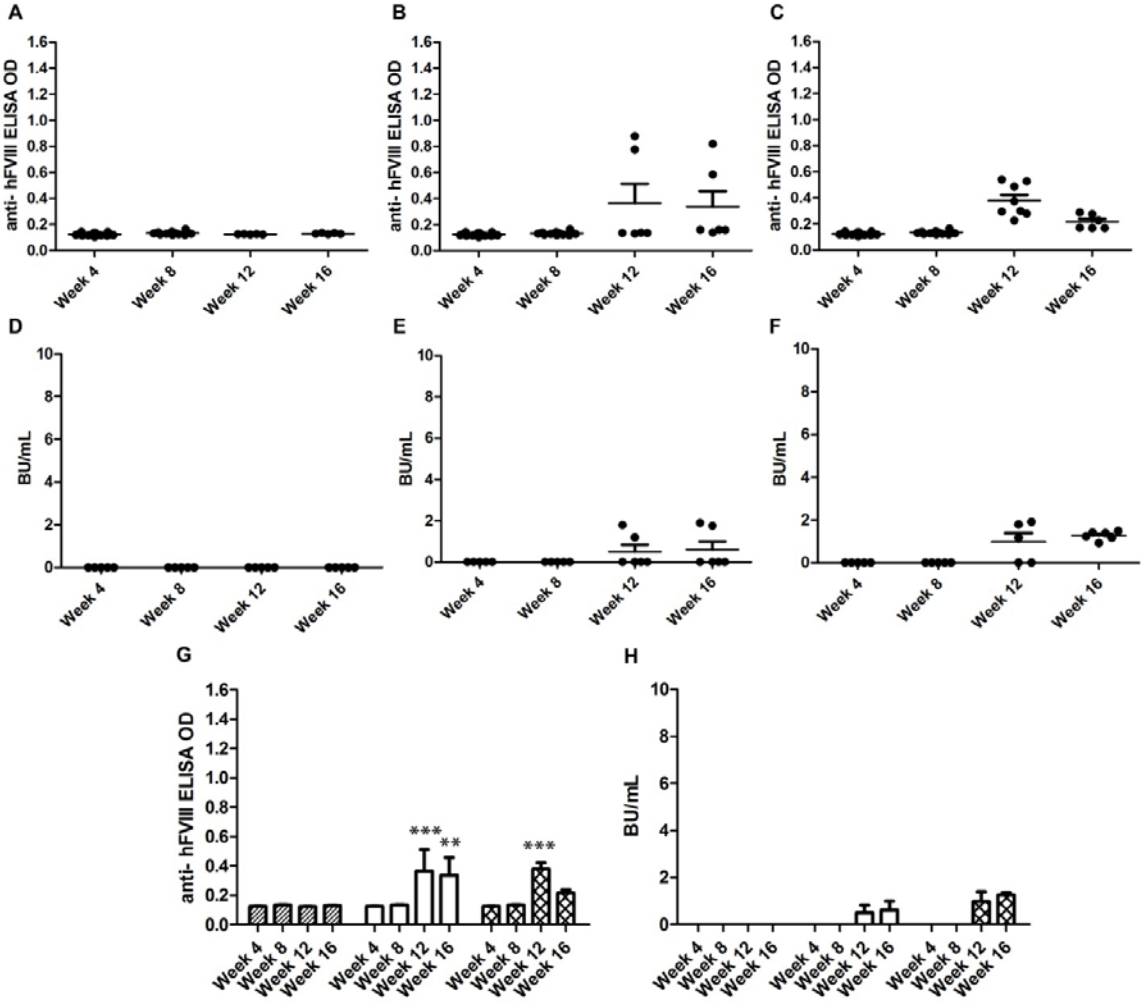

Figure 4.2: Immune tolerance in naïve hemophilia A mice. (A, D) Naïve hemophilia A mice fed chitosan nanoparticles containing 100 µg human FVIII DNA once a week for a total of eight weeks. (B, E) Mice fed nanoparticles during the first eight weeks followed by weekly immunizations with 0.75 U rhFVIII on weeks 9, 10 11 and 12. (C, F) Hemophilia A mice fed chitosan nanoparticles once a week for a total of twelve weeks and immunized with rhFVIII on weeks 9 10, 11 and 12. Anti-human FVIII ELISA (A-C) was used to detect both neutralizing (inhibitors) and non-neutralizing human FVIII antibodies, while inhibiter titres were examined by Bethesda assay (D-F). A summary of antibody response (G) and inhibitor production (H) for all groups is shown. Bars represent mean±SEM. **p>0.01, ***p<0.001, compared with control.

d. Immune response modulation in the presence of pre-existing FVIII inhibitors

Next we examined if repeated administration of nanoparticles can reduce antibody (neutralizing and non-neutralizing antibodies) and inhibitor (neutralizing antibodies) titres in hemophilia A mice with pre-existing antibodies. A group of hemophilia A mice (n=7) were immunized i.p. with 0.75 U rhFVIII over a four week period followed by oral administration of nanoparticles on weeks 5, 6, 7, 8, 9, 10, 11 and 12. Antibody (Figure 4.3B, 4.3G) and inhibitor (Figure 4.3E, 4.3H) titres peaked on week 4 and declined steadily on weeks 8, 12 and 16. Indeed there is a significant decrease in the antibody response by weeks 12 and 16. This would suggest that repeated administration of nanoparticles contribute to the reduction of antibody and inhibitor titres. However, this reduction may be explained by the natural decline of antibody and inhibitor titres shown in a control group of hemophilia A mice (n=5) immunized for four week (Figure 4.3A, 4.3G and Figure 4.3D, 4.3H). A comparison of these two groups did not reveal any significant differences over the 16 week study period (Figure 4.3G, 4.3H). This data indicate that oral nanoparticle administration may not suppress antibody and inhibitor titres in hemophilia A mice with pre-existing antibodies.

We further examined if repeated nanoparticle administration could limit the increase in antibody and inhibitor titres in hemophilia A mice with pre-existing antibodies while receiving continuous immunizations. A group of hemophilia A mice (n=8) were immunized weekly with 0.75 U rhFVIII for sixteen weeks and were fed nanoparticle containing 100 µg hFVIII from weeks 5 to 16. The antibody titres increased on weeks 8, 12 and 16 (Figure 4.3C, 4.3G) while inhibitor levels reached a plateau by week 8 (Figure

4.3F, 4.3H). This data suggests that oral administrations of nanoparticles are unable to suppress the antibody response to repeated immunizations with the presence of pre-existing antibodies. The inhibitor titre was not suppressed on week 8, however the plateau reached on weeks 8, 12 and 16 may be attributed to repeated nanoparticle administration.

Dosing schedule:
Group A (n=5) Immunize (week1-4)
Group B (n=7) Immunize (week1-4), Feed (week5-16)
Group C (n=8) Immunize (week 1-16), Feed (week 5-16)

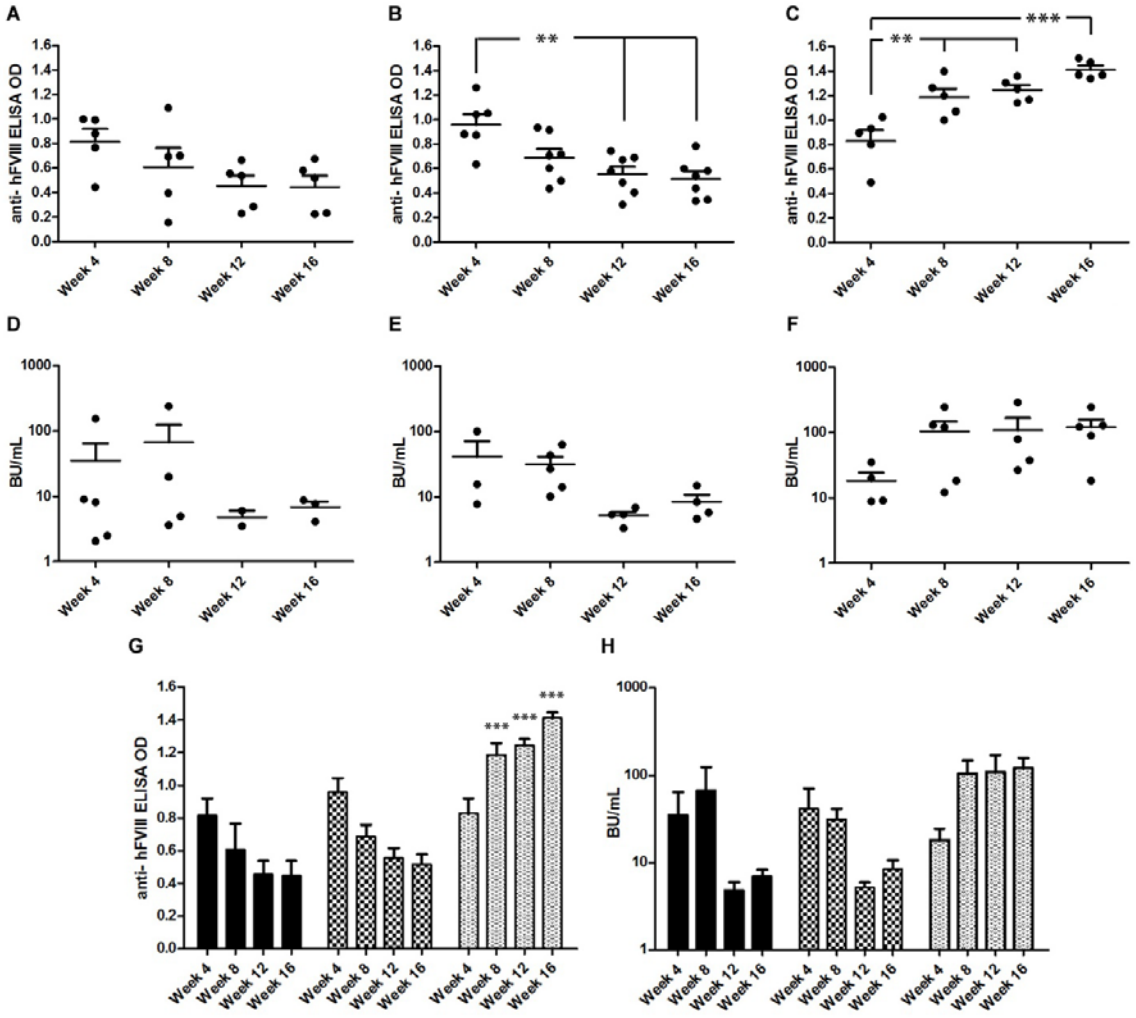

Figure 4.3: Oral tolerance in hemophilia A mice with pre-existing hFVIII antibodies. (A, D) Hemophilia A mice given immunized weekly i.p. injections of 0.75 U rhFVIII for a total of four weeks. (B, E) Mice were given four weekly rhFVIII injections followed by weekly oral nanoparticle administration on weeks 5-16. (C, F) Hemophilia A mice immunized weekly with rhFVIII for a total of 16 weeks and fed nanoparticles on weeks 5-16.Plasma samples were taken on weeks 4, 8, 12 and 16. Anti-human FVIII ELISA performed on plasma samples to detect both neutralizing (inhibitors) and non-neutralizing human FVIII antibodies (A-C). Inhibitors were examined by Bethesda assay (D-F). A summary of antibody response (G) and inhibitor production (H) for all groups is shown. Bars represent mean±SEM. **p<0.01, ***p<0.001, compared with control.

e. Adoptive transfer of splenocytes from tolerized mice into naïve recipient mice

To determine if oral administration of chitosan nanoparticles result in the production of regulatory T cells that mediate tolerance, splenocytes from tolerized mice were tested for their ability to transfer tolerance to naïve recipient mice. Hemophilia A mice donor mice (n=5) were fed nanoparticles containing 100 µg hFVIII DNA weekly for four weeks (Figure 4.4B). Splenocyte were isolated and injected intravenously into naïve hemophilia A mice (n=5). Recipient mice were challenged i.p. with 0.75 U rhFVIII on days 1, 7, 14 and 21 following splenocyte transfer (Figure 4.4B). Splenocyte transfer protected naïve recipient mice from high titre antibody development (Figure 4.4D, 4.4E) compared with controls (Figure 4.4A, 4.4C, 4.4E). The apparent one-week delay in the development of antibodies in tolerized mice may be attributed to the passive immunity provided by splenocyte transfer against immunization on day 1 but not on days 7, 14 and 21. Taken together, the suppression of hFVIII antibody titres suggests that T regulatory cells mediate suppression and may be involved the development of tolerance.

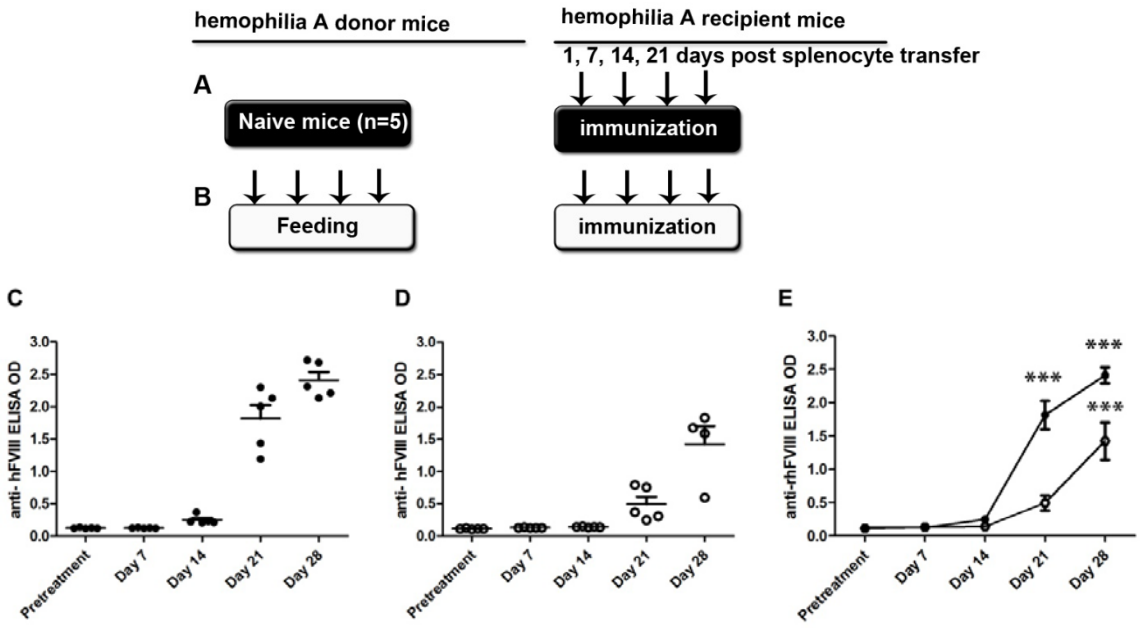

Figure 4.4: Adoptive transfer of splenocytes. Hemophilia A mice donor mice fed nanoparticles containing 100µg hFVIII DNA once a week for a total of four weeks. Splenocytes were isolated and injected into naïve hemophilia A recipient mice. Twenty four hours following adoptive transfer recipient mice were challenged with i.p. injection of 0.75 U rhFVIII on days 1, 7, 14 and 21. Anti-human FVIII ELISA performed on plasma samples to detect both neutralizing (inhibitors) and non-neutralizing human FVIII antibodies. (A, C, E) Control mice received splenocytes from naïve mice. (B, D, E) Mice received splenocytes from nanoparticle-fed donor mice. Bars represent mean±SEM. ***$p<0.001$, compared with control.

4.3 Discussion

Oral or mucosal tolerance involves the induction of antigen-specific antibodies in external secretions (IgA) and systemic unresponsiveness initiated at mucosal surfaces such as the gut associated lymphoid tissue (GALT). This mucosally induced tolerance provides a mechanism to suppress the systemic immune response to external antigens that gain access to the body and avoid allergic or other harmful immunologic reactions to injected antigens. This is in contrast to classical ITI protocols achieved through repeated

systemic administration of large doses of antigen to induce lymphocyte deletion, anergy or deletion. These ITI protocols can increase systemic antigen-specific IgG production and is associated with the requirement for frequent venous access and high product costs.

Mucosal tolerance induction using proteins is not economical considering the large quantities of FVIII required(*227, 230*). Furthermore, breast milk containing FVIII proteins does not suppress inhibitor development in young hemophiliacs(*231*). This suggests that preserving the conformational relevance of FVIII may be important for inducing tolerance. In this study we examined the use of chitosan-mediated gene therapy delivered orally to modulate the systemic immune response to exogenous human FVIII protein in hemophilia A mice.

Our previous work demonstrated sustainable canine FVIII expression and phenotypic correction in hemophilic mice following oral gene therapy. Of particular interest, both antibody and inhibitor development was not detected despite plasma FVIII levels of >100 mU/mL(*196*). Consistent with these finding, human FVIII-specific antibodies were not produced to nanoparticle treatment containing human FVIII DNA.

Here we show that prior oral administration of chitosan nanoparticles containing human FVIII DNA in hemophilia A mice suppressed systemic antibody formation following challenge with recombinant FVIII protein compared with immunized controls. Inhibitor formation is believed to be T helper dependant. FVIII expression in the gut-associated lymphoid tissue may recruit $CD4^+$ $Foxp3^+$ regulatory T cells to facilitate peripheral tolerance mechanisms that may involve immune suppressive cytokines IL-10 and TGF-β. This systemic immune tolerance could be transferred to naive mice. The

transient FVIII-specific tolerance observed during transfer may be attributed to the immunization protocol used, which consisted of four weekly i.p. injections of rhFVIII without adjuvant and low engraftment of regulatory T cells.

Prophylactic mucosal tolerance to human FVIII was long-lasting. Tolerized mice did not produce systemic FVIII-specific antibody response for at least 8 weeks following immunization protocols. Low level antibody response in a subset of tolerized mice may be attributed to batch variation or protein sequence differences between mucosal presented FVIII transgene and recombinant FVIII used for immunization.

In contrast, the oral administration of nanoparticles had limited immunomodulatoy effect against pre-existing inhibitors. The continuous decline in antibody titres over 16 weeks with repeated nanoparticle administration emulated the trend observed in the control group following discontinued immunization. This suggests that nanoparticle treatment did not contribute to the regression of antibody titres in mice with pre-existing antibodies. Although, mucosal tolerance is believed to suppress antigen-presenting cell activation and may not affect clearance of long-lived plasma cells and circulating antibodies (*232*). Indeed, studies have shown that regulatory T cells can modulate effector T cell function (*233*).

Furthermore, mucosal expression of hFVIII did not illicit a prime-boost response characteristic of DNA vaccines. In this study, prior mucosal gene therapy suppressed development of antibodies to immunization while FVIII gene expression did not boost antibody titres in mice with pre-existing antibodies. The CpG motif present on bacterial plasmid DNA in an unmethylated form can amplify the immune response to FVIII by

activation of innate immune response through interaction with Toll-like receptors(*234*). Alternatively, it has been suggested that CpG may contribute to the development of regulatory T cells that may redirect the immune response towards mucosal IgA production instead of systemic antibodies (*235*).

Clinically, it is unlikely to suspend protein replacement therapy during tolerance induction protocols. To better recapitulate clinical practice, nanoparticles were administered contemporaneously with weekly rhFVIII immunization. However, concurrent nanoparticle treatment did not suppress continuous immune response activation for 12 weeks, although inhibitor titres appeared to plateau. Mucosal expression levels of FVIII transgene may be insufficient to alter effector T cell function through mechanisms of anergy, deletion or stimulate the development of anti-idiotypic antibodies.

Inhibitor development is a significant challenge to clinical management of hemophilia. Oral gene therapy may provide long-term prophylactic tolerance in paediatric patients without need of immunosuppressive agents or risk of insertional mutagenesis. Further investigation into the mechanism of prophylactic tolerance induction is warranted.

Chapter 5

Aim III: Demonstrate chitosan nanoparticle-mediated FIX gene delivery for the treatment of hemophilia B

Hemophilia B is a monogenetic bleeding disorder attributed to a deficiency of functional factor IX that inflicts 1 in 25,000 males. Current treatment involves protein replacement therapy with recombinant or plasma-derived FIX which is invasive, expensive and inaccessible to haemophiliacs in developing nations. Inhibitor formation to FIX products, occurs in ~3% of patients, is associated with severe allergic anaphylactic reactions and increased morbidity(*236*). Hemophilia B is classified by the level of normal plasma FIX activity as severe (<1%), moderate (1-5%) or mild (5-50). This broad therapeutic window is beneficial for the application of gene therapy strategies. Although the therapeutic threshold is 25-50 fold greater than hemophilia A treatment.

Viral vectors are efficient gene delivery agents that have demonstrated efficient FIX expression in a variety of studies (*131, 136, 147-149, 151, 159, 237-239*). Nevertheless, viral vectors present safety concerns including oncogenesis, genotoxicity which limit their clinical application. In contrast, non-viral gene therapy strategies offer a safe alternative treatment for genetic disorders.

Recently we have developed a non-viral oral gene therapy strategy for the treatment of hemophilia A (*196*). This ‘gene pill’ strategy provides sustainable therapeutic FVIII expression in hemophilic mice. Furthermore, mucosal transgene expression provided prophylactic tolerance to subsequent challenge with recombinant

FVIII. In this study chitosan-mediated oral FIX gene therapy is examined in the treatment of hemophilia B. We show that oral gene therapy provides transient therapeutic human FIX transgene expression in hemophilia B mice.

5.2 Materials & Methods

Plasmid vectors

Plasmids coding for human FIX including CpG-IXi, MFG-IXi and pLnmβ-IXi along with a luciferase reporter gene pGL3CMV-Luc (Promega Corporation, Madison, WI) were prepared using a MaxiPrep Kit (Qiagen, Mississauga, Ontario, Canada).

Chitosan/DNA nanoparticle formulation

Chitosan/DNA nanoparticles were formed by complex coacervation method described previously (*190*). Chitosan (CL 213; degree of deacetylation >84%; Novamatrix, Drammen, Norway), and plasmid DNA dissolved in 50 mM Na_2SO_4 was heated to 55°C. The chitosan and DNA solutions were mixed and vortexed for 20 s. Nanoparticles were formulated at an N:P ratio of 3:1.

***In vitro* transfection of HEK 293 cells**

HEK 293 cells were cultured in 12-well plates with α-MEM, 10% fetal bovine serum, 1% Pen/Strep. Cells were transfected with hFVIX plasmid formulated in lipofectamine and examined 25, 48 and 72 h post-transfection. FIX secretion was assayed by human FIX ELISA (Affinity Biologicals, Canada).

Reported gene expression was examined in HEK 293 cells transfected with chitosan nanoparticles formulated with luciferase reporter gene. Luciferase assay (Promega, USA) was detected on a microplate luminometer (Applied Biosystems, USA).

Animal Procedures

Hemophilia B mice (C57BL/6$^{FIX-/-}$) were fed chitosan nanoparticles containing 100 µg human FIX DNA via gavage using a 20G needle (Popper & Sons, New Hyde Park, NY). Blood samples were collected via orbital plexus in citrate. All experiments were conducted in adherence to the Animal Ethics Guidelines of McMaster University.

Statistics

Data analysis performed with GraphPad Prism 5 statistical software package. A value of $p<0.05$(*) was considered significant while $p<0.01$(**) and $p<0.001$(***) were considered highly significant.

5.3 Results

a. Selection FIX plasmid

The transgene expression of three FIX plasmids was examined *in vitro* using lipofectamine formulated with CpG-IXi, MFG-IXi and pLnmβ-IXi. Transgene expression was assessed 24 hr, 48 hr and 72 hr post transfection (Figure 5.0). The CpG-IXi plasmid provided the highest FIX transgene expression followed by pLnmB-IXi and MFG-IXi at all time points examined (Figure 5.0). The reduction in expression on 72 hr seen for all nanoparticle formulations may be attributed to the confluency of cells.

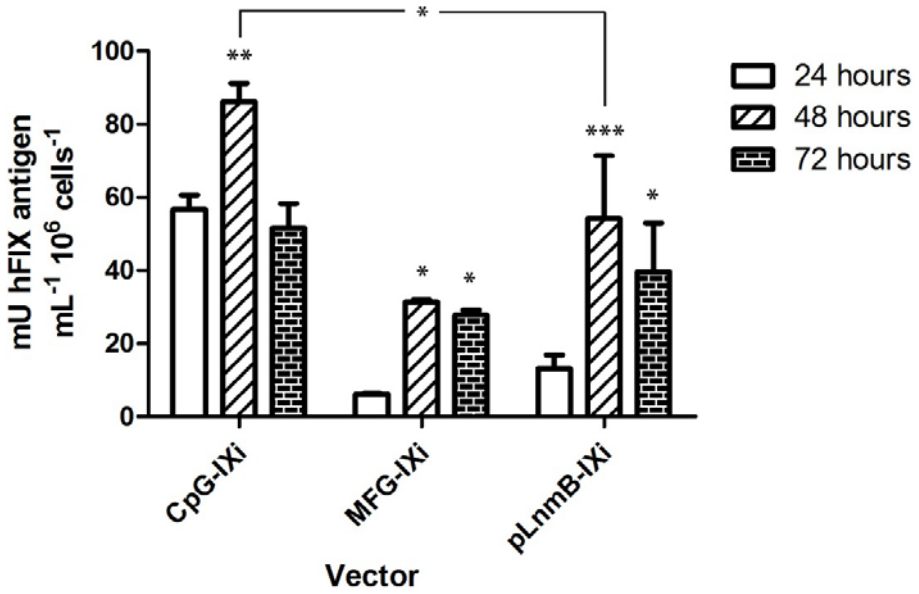

Figure 5.0: *in vitro* expression of hFIX. HEK 293 cells were transfected with lipofectamine containing CpG-IXi, MFG-IXi or pLNMβ-IXi. Media samples were taken at 24 hr, 48 hr and 72 hr post transfection and analyzed by hFIX ELISA. Bars represent mean ± SEM. *p<0.05, **p<0.01, ***p<0.001.

The transfection efficiency of chitosan nanoparticles formulated with CpG-IXi, MFG-IXi and pLnmβ-IXi were also examined *in vitro*. HEK 293 cells were transfected with nanoparticles containing CpG-IXi, MfG-IXi or pLnmB-IXi. Nanoparticles are constructed thorough electrostatic interactions between the positively charged amine of chitosan and the negatively charged phosphate backbone of DNA. The nanoparticles used in this study were formulated at an N:P charge ratio of 3:1 using 50 mM sodium sulphate. Interestingly, nanoparticles formulated with CpG-IXi performed better than either MFG-IXi or pLnmβ-IXi (Figure 5.1). This trend is identical to that found with lipofectamine where the order of greatest transgene expression was CpG-IXi>pLnmB-IXi>MFG-IXi (Figure 5.1). This trend may be partly attributed to plasmid size as CpG-IXi is the smallest (4905bp) followed by MFG-IXi (8700bp) and pLnmβ-IXi (12726bp).

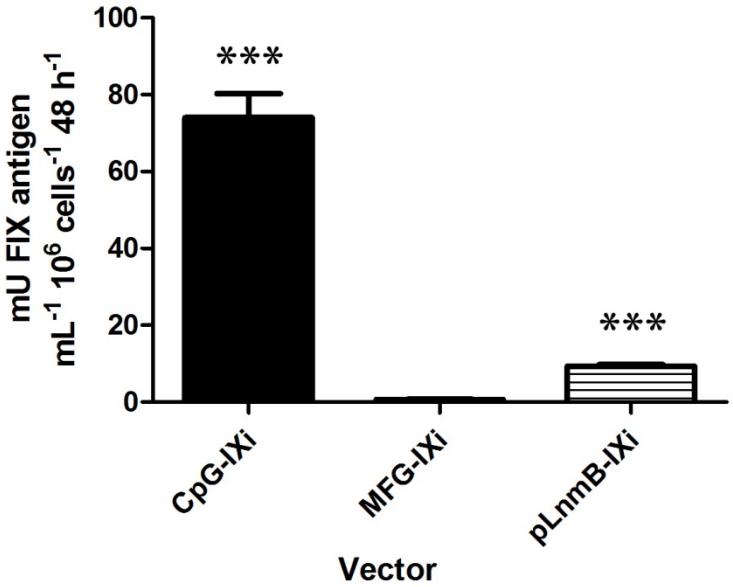

Figure 5.1: *in vitro* transfection with chitosan nanoparticles containing hFIXi. HEK 293 cells were transfected with chitosan nanoparticles formulated with 50 mM sodium sulphate containing 2.5 pmol of CpG-IXi, MFG-IXi or pLnmβ-IXi. Samples take 48 hr following transfection and analyzed by hFIX ELISA. Bars represent mean±SEM. ***p<0.001.

The higher expression of formulations containing the CpG-IXi plasmid may be attributed its intrinsic properties. The CpG-IXi plasmid is devoid of CpG dinucleotides that are commonly found unmethylated in bacteria DNA but present at a low frequency in mammalian DNA. These unmethylated CpGs may illicit an immune response that can limit gene delivery by rapid loss of transgene expression. The combination of a cellular promoter, elongation factor 1 alpha, with a CpG reduce backbone is believed to provide an increase expression lifetime and lower immune response.

b. FIX transgene expression in hemophilia B mice

The transgene expression kinetics of FIX delivery in hemophilia B mice was examined. Hemophilia B mice (n=16) were fed chitosan nanoparticles containing 100 µg hFIX. Blood plasma samples were collected on days 1, 2, 3, 4 and 14 post-treatment. Untreated hemophilic mice were used as controls. The hFIX activity reached peak expression on day 1 post-treatment and regressed back to baseline by day 3 post-treatment (Figure 5.2). The detection of hFIX activity on day 2 post treatment may be attributed to the longer half-life circulating FIX 18-24 than hFVIII. Nevertheless, the *in vivo* activity observed was low.

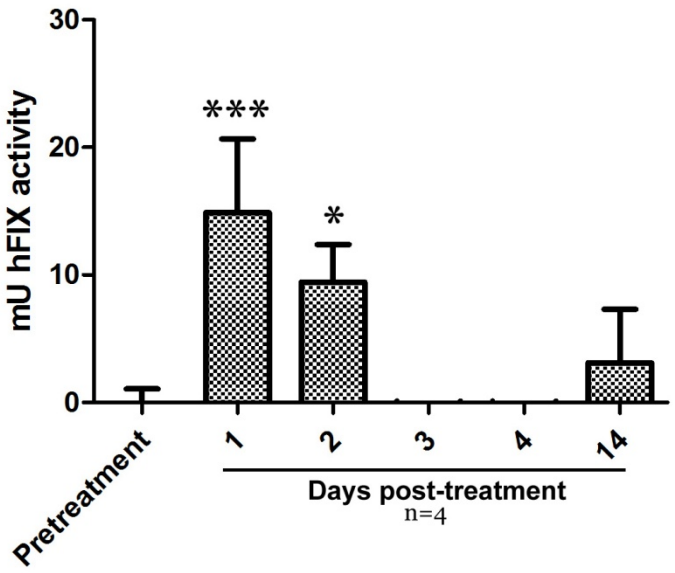

Figure 5.2: Plasma hFIX activity following oral administration in hemophilia B mice. Mice were fed chitosan nanoparticles containing 100 µg hFIX. Plasma samples were examined in days 1, 2, 3, 4 and 14. Bars represent mean±SEM. ***$p<0.001$, *$p<0.05$ compared with untreated hemophilic mice.

c. Batch variation

The low activity of circulating FIX may be attributed to the chosen batch used in nanoparticle formulation. The transfection efficiency of the new chitosan used in this study was compared to expression levels from the previous chitosan batch (Figure 5.3). Nanoparticles were prepared with both chitosan batches containing luciferase plasmid and applied to HEK 293 cells. Surprisingly, the transfection efficacy of new chitosan was 7-fold lower than the old chitosan, which may be a consequence of the higher deacetylation percent, >90% and >84% respectively. The higher deacetylation percent increases the charge density of chitosan and allows for tighter binding with DNA and prevent its release. The kinetics of *in vivo* hFIX expression will be re-evaluated using the old chitosan batch.

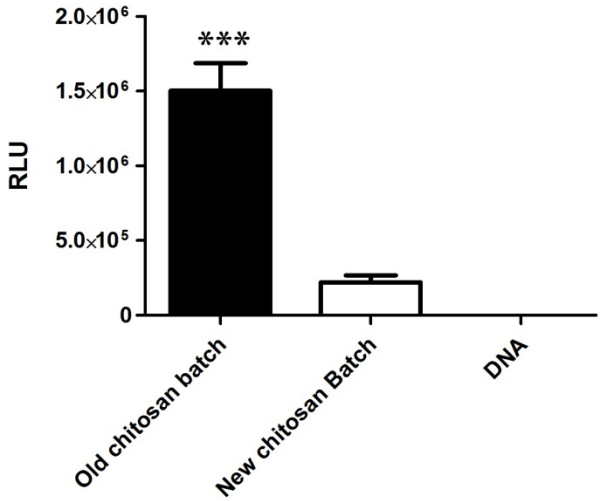

Figure 5.3: *in vitro* evaluation of chitosan batch variation. HSK 293 cells were transfected with luciferase DNA contained in old chitosan batch, new chitosan batch or naked plasmid. Lipofectamine transfected cells were used as a control ($1.25x10^7 \pm 1.2x10^6$ RLU). Bars represent mean±SEM. ***$p<0.001$.

5.3 Discussion

Chitosan nanoparticle-mediated oral gene delivery has been shown to provide therapeutic FVIII transgene expression and phenotypic correction(*196*). Here we apply this gene delivery strategy for the delivery of clinically relevant human FIX expression in hemophilia B mice.

Human FIX plasmids were evaluated *in vitro* for transgene expression. The CpG-IXi plasmid provided higher FIX expression than MFG-IXi or pLNMβ-IXi. The smaller size of CpG-IXi may facilitate entry into cells and/or dissociation from the gene delivery vehicle.

Oral administration of chitosan nanoparticles containing human FIX DNA provided detectable FIX transgene expression. This transient expression provided FIX plasma activity in treated mice with average values of >14mU and >9.5 mU FIX activity at 24 and 48 hours post treatment, respectively. This level of FIX activity is sufficient to convert a severe hemophilia disorder to a moderate phenotype. This level of expression is significant considering the 25-50 fold higher physiological levels of FIX compared with FVIII (5µg/mL vs. 100-200ng/mL). Indeed, on a molar basis, this level of expression may be greater than in our previous report for FVIII expression (>100mU) (*196*).

A number of factors may contribute to the therapeutic FIX expression reported here. The FIX gene is relatively small with lower requirement for post-translational modifications. This CpG-free plasmid is devoid of all bacterial CpG dinucleotides that could potentially stimulate the immune response through TLR9 receptors. Furthermore, removal of CpG bacterial backbone has been attributed to longer half-life of FIX (*240,*

241). However, the transient expression presented here may be attributed to the rapid turnover of cells in the intestinal epithelium. It takes as little as 48 hours for the cells to migrate from the base of the crypt where they are formed, to the tip of the villus (*219, 220*).

The transfection efficiency is susceptible to batch variation that can have unique distribution of chain lengths and deacetylation frequency (*180, 181*). Batch variation can modulate FIX expression by 7-fold. Further optimization of nanoparticle formulation may be necessary for clinical applications. Fungal-derived chitosan have been reported to provide source material with batch to batch reproducibility(*161*). Moreover, concentrated nanoparticles may provide enhanced gene expression required for hemophilia B correction (*242-244*).

Here we have demonstrated that therapeutically relevant expression of FIX can be achieved by oral gene therapy using chitosan nanoparticles. These findings show that oral gene therapy is a potential alternative treatment for hemophilia B.

Chapter 6

Aim IV: Optimize and characterize transfection efficiency of Chitosan/DNA nanoparticle formulations

Gene therapy strategies have been devised to provide effective cell-mediated delivery of therapeutic proteins.(*245, 246*) The central idea of gene therapy involves the transfer of genetic material, coding for a protein therapeutic, into somatic cells either outside the body (*ex vivo*)(*85, 127, 247-249*) or by direct administration to the body (*in vivo*)(*250-252*). Viral vectors are efficient tools for transferring genetic material to host cells. However, viral-mediated gene delivery presents concerns of potential oncogenicity, toxicity and immunogenicity. Alternatively, non-viral gene delivery provides a safer and cost effective strategy, albeit lower transfection efficiency.

Chitosan has been explored as a versatile non-viral gene delivery agent (*6, 190-193, 253*). Chitosan is biodegradable, biocompatible, mucoadhesive cationic polysaccharide with low cytotoxicity. Chitosan, derived from the shells of crustaceans and fungal cell walls is composed of β-(1, 4)-linked D-glucosamine and N-acetyle-D-glucosamine. The primary amines groups, with a pKa value of 6.5, facilitate interactions with negatively charged DNA and cell membranes.

Nanoparticles are formed through electrostatic interaction between positively charged amine group on chitosan and the negative phosphate backbone of DNA denoted as the N:P ratio. This binding induces condensation of DNA, protects against DNase

digestion, and enhances cell attachment and intracellular delivery. Chitosan nanoparticles can be internalized by macropinocytosis and caveolae-mediated endocytosis. Improving chitosan nanoparticle transfection may require optimization of cell penetration, intracellular unpacking or nuclear localization. A number of factors influence transfection efficiency including charge ratio and electrolytes(*189*).

Recently, we have demonstrated that orally administered chitosan nanoparticles can provide therapeutic *in vivo* FVIII transgene expression for the treatment of hemophilia A mice(*196*). Nanoparticles were formulated with 25 mM acetate-acetic acid and 50 mM sodium sulphate at an N:P charge ratio of 4:1. FVIII DNA was protected from gastrointestinal degradation to provide therapeutic expression of >100mU FVIII and phenotypic correction. Indeed, modest FVIII is sufficient to provide therapeutic correction in hemophilia A. Nevertheless, optimization of nanoparticle formulation may enhance transgene expression for human application. Furthermore, treatment of hemophilia B requires 25-50 fold higher transgene expression to achieve similar results shown for hemophilia A.

In this study, chitosan nanoparticle formulations with DNA are investigated to improve transfection efficiency. The detection of a luciferase pseudo-gene will be used to systematically optimize nanoparticle formulations. We show that transfection efficiency can be enhanced by modulating the charge ratio and electrolyte content of nanoparticle formulations.

6.1 Materials & Methods

Plasmid vectors

Plasmid coding for the luciferase reporter gene pGL3CMV-Luc (Promega Corporation, Madison, WI) was prepared using a MaxiPrep Kit (Qiagen, Mississauga, Ontario, Canada).

Chitosan/DNA nanoparticle formulation

Chitosan/DNA nanoparticles were formed by complex coacervation method described previously (*190*). Chitosan (CL 213; degree of deacetylation >84%; Novamatrix, Drammen, Norway), was formulated with DNA. The chitosan and DNA solutions were heated to 55°C mixed and vortexed for 20 s. Distinct formulations of nanoparticles were prepared by altering the N:P ratio, salt concentration and chitosan type. Solutions were prepared at various N:P ratios including 1:1, 2:1, 3:1, 4:1, 5:1, 6:1. 7:1, 8:1, 9:1 and 10:1. Salt content examined include 25 mM acetate-acetic acid and 50 mM Sodium sulphate, 25 mM acetate-acetic acid only, 50 mM sodium sulphate only, and no salts. Chitosan salt types examined include chloride bound (CL 213, CL 113) and glutamate bound (G 213, G 113) forms.

Transmission Electron Microscopy (TEM)

A Formvar coated Cu^{++} grid was covered with a 10 μL solution of nanoparticles and left for approximately 1 min. Grids were air-dried, then viewed in a Jem-1200EX Transmission Electron Microscope (JOEL, Tokyo, Japan) operating at 80 KV.

Particle size and zeta potential

The particle size of nanoparticles is determined using a dynamic laser light scattering system (Brookhaven instruments, Holtsville, NY) with a HeNe laser with 635nm wavelength. Scattered light was detected at a 90° angle. Sample are diluted to 2 mL volume and tested for 5 minutes. Particle formulations with various N:P ratios are prepared in triplicate and measured. Particle surface charge was examined using zeta potential analyzer (Brookhaven Instruments, Holtsville, NY).

***In vitro* transfection of HEK 293 cells**

HEK 293 cells were cultured in 12-well plates with α-MEM, 10% fetal bovine serum, 1% Pen/Strep. Cells were transfected with Luciferase plasmid formulated in lipofectamine, chitosan nanoparticles or as naked plasmid and examined 24, 48 or 72 h post-transfection. Expression of eGFP was determined by fluorescent microscope (Leica Microsystems, Germany); Luciferase assay (Promega, USA) was detected on a microplate luminometer (Applied Biosystems, USA) and normalized by protein content determined by Bradford Assay (Pierce, USA); FVIII secretion was assayed by canine FVIII ELISA (Affinity Biologicals, Canada).

Nuclease and proton protection assay

Chitosan nanoparticles were incubated with DNase I (Fermentas, Burlington, Canada) for 15 min at 37°C. DNase was inactivated by incubation at 80°C for 10 min in the presence of EDTA. Plasmid DNA was released from chitosan nanoparticles following incubation for 4 h in the presence of 0.06 U chitosanase enzyme (Sigma, Oakville, Canada). To determine protection against proton digestion, HCl was used to decrease the

nanoparticle solution pH to approximately 1-2 for 1 h and then neutralized with NaOH. Entrapped DNA was released with chitosanase and DNA migration was examined using 1% agarose gel electrophoresis.

Statistics

Data analysis performed with GraphPad Prism 5 statistical software package. A value of $p<0.05$(*) was considered significant while $p<0.01$(**) and $p<0.001$(***) were considered highly significant.

6.2 Results

a. Size and morphology of nanoparticles

Chitosan nanoparticles are formed through electrostatic interactions between the positively charged amine groups on chitosan and negatively charged phosphate backbone of DNA. This chitosan-DNA interaction influences the size, morphology and transfection ability of the resulting nanoparticles. The morphology of nanoparticle formulations prepared at an N:P ratio of 4:1 and different salt contents were compared by Transmission Electron Microscopy (TEM). Nanoparticles containing 25 mM sodium acetate-acetic acid and 50 mM sodium sulphate induce assembly of globular aggregates into large networks (Figure 6.0A). Chitosan nanoparticle formulated with 25 mM sodium acetate-acetic acid resulted in spherical particles (Figure 6.0B). In contrast, nanoparticles formed in the absence of salt produced small discrete globular particles that do not appear to aggregate (Figure 6.0C). These globular structures are consistent over the range of charge ratios examined from 1:1 to 7:1 (Figure 6.1A-G).

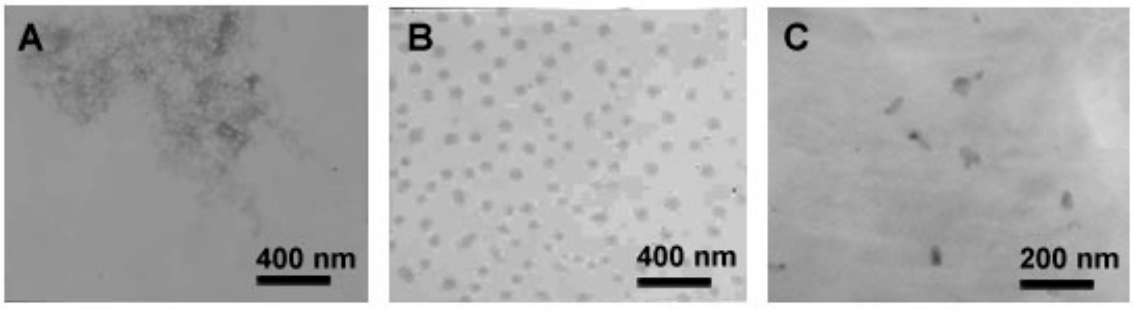

Figure 6.0 Salts affect chitosan/DNA morphologies. Transmission electron microscopy images of Chitosan (CL 213)-DNA (Luciferase) combined at 4:1 charge ratio. Chitosan (CL 213) nanoparticle formulated with (A) 25 mM sodium acetate-acetic and 50 mM sodium sulphate (B) 25 mM sodium acetate-acetic acid only or (C) in the absence of salts.

The size of chitosan nanoparticle formulated in the absence of salts is dependent on the charge ratio. Dynamic light scattering of these nanoparticles revealed increasing particle diameter as a function of N:P ratio (Figure 6.2A). These particles had a size range of about 140 nm to 270 nm. This suggests that at higher charge ratios more chitosan accumulates on the surface of each nanoparticle.

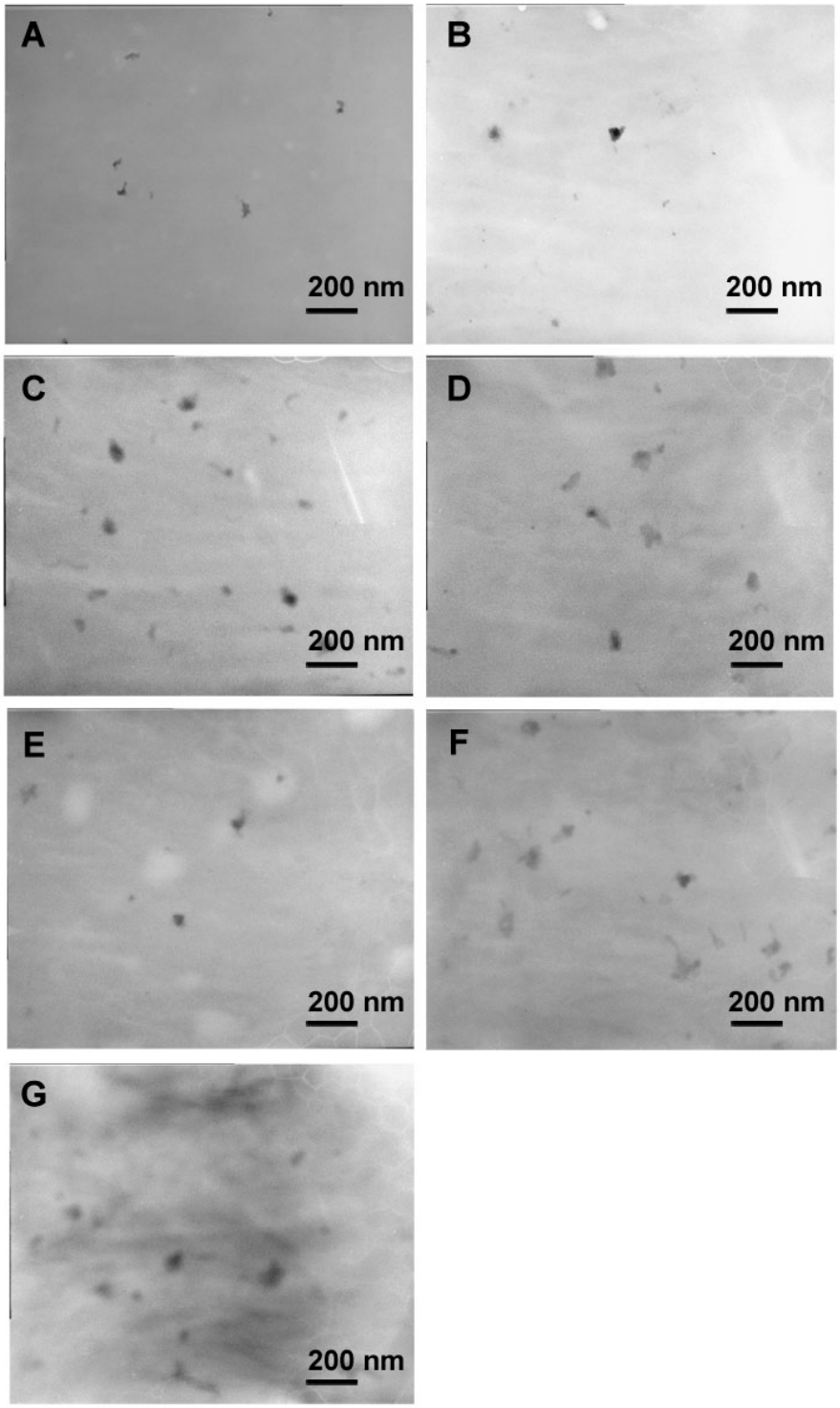

Figure 6.1: Morphology of Chitosan/DNA nanoparticles particles. Chitosan (CL 213) nanoparticle formulations containing luciferase plasmid in the absence of salt at N:P charge ratios from 1:1 to 7:1.

b. Surface charge of nanoparticles

The surface charge of chitosan nanoparticles may influence its interactions with cells. The surface charge of chitosan nanoparticles is negative at low N:P ratio of less than 2:1 but becomes positive with the addition of more chitosan (Figure 6.2B).

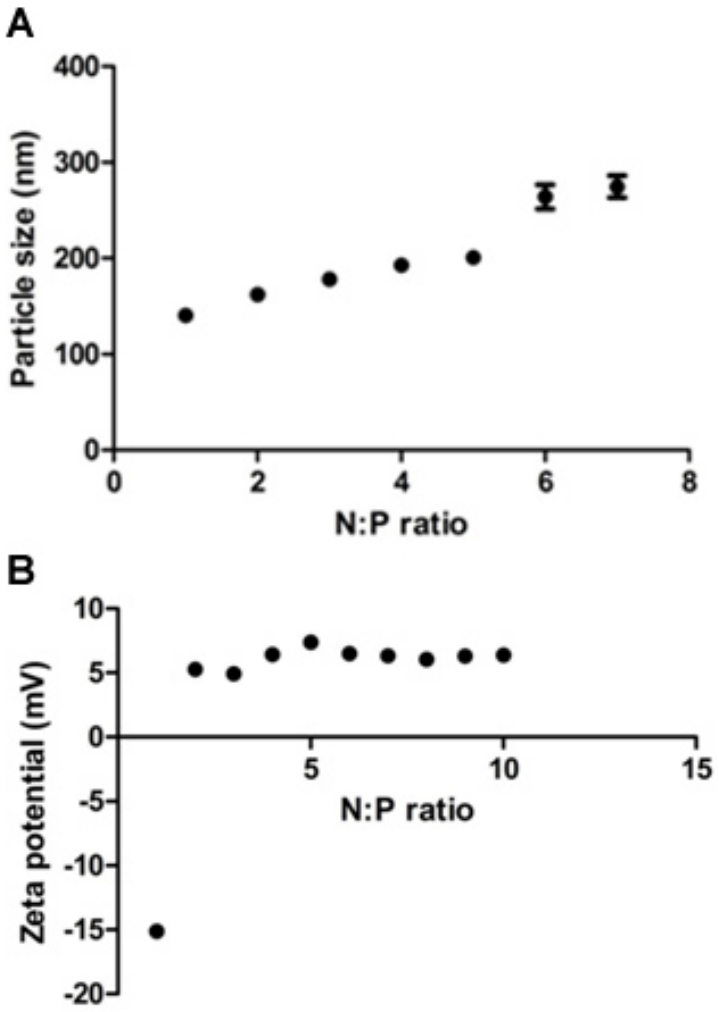

Figure 6.2: Particle size of chitosan nanoparticles. Chitosan (CL 213) nanoparticles were formulated based on charge ratio between the positively charge amine groups on chitosan and the negative phosphate backbone of DNA (N:P ratio). The particle size of nanoparticles formulated at charge ratios ranging from 1:1 to 10:1 were examined by (A) dynamic light scattering and (B) zeta potential.

c. Stability of DNA against proton and enzymatic degradation

The stability of the nanoparticle formulations is examined to determine the degree of DNA protection. Stable nanoparticles are required to bind tightly to DNA and prevent degradation before the target cells can be transfected. A gel shift assay was performed to determine if all DNA is bound to chitosan. The formulations examined include the use of 25 mM sodium acetate-acetic acid and 50 mM sodium sulphate, 25 mM sodium acetate-acetic acid only, 50 mM sodium sulphate only, and without addition of salts (Figure 6.3Aa-d). Complete DNA binding was observed in all nanoparticle formulations (Figure 6.3A). A charge ratio (N:P) of 1:1 or greater was sufficient to completely bind DNA.

The oral delivery of chitosan–mediated gene therapy requires protection of DNA from degradation conditions in the stomach and intestine. The conditions of the stomach are simulated by exposing nanoparticles to pH of 2 for 30 min at 37°C using hydrochloric acid. Insufficient chitosan coating allows exposed DNA to be degraded. Alternatively, excessive amounts of chitosan may prevent release of DNA and impede transfection. The stability of N:P ratio from 1:1 to 1:7, formulated without salts, indicated that a charge ratio > 2:1 is required for DNA protection (Figure 6.3B). Similar results were achieved for formulations containing 25 mM sodium acetate-acetic acid and 50 mM sodium sulphate, 25 mM sodium acetate-acetic acid only and 50 mM sodium sulphate only (data not shown)

Chitosan nanoparticles were also subjected to DNase I to mimic enzymatic digestion in the ileum. Nanoparticles formulated at a charge ratio of >2:1 provided

sufficient DNA protection against enzymatic degradation, which was independent of the salt formulation (data not shown).

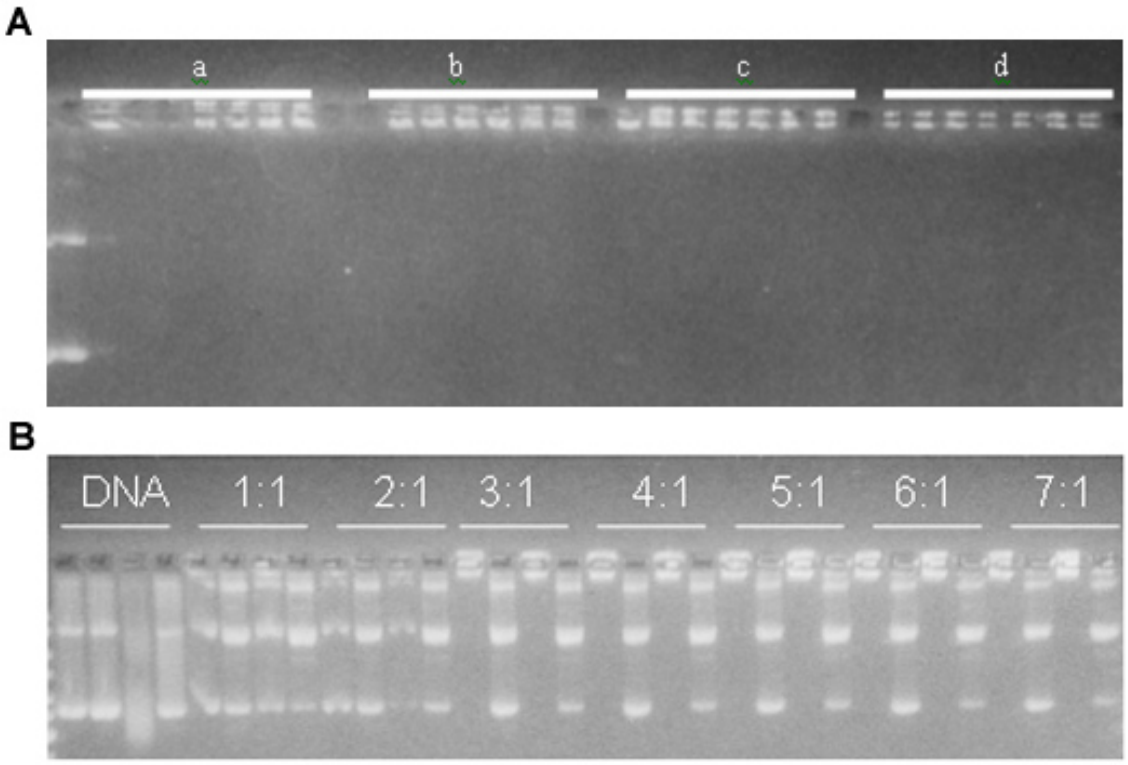

Figure 6.3: Chitosan DNA stability. (A) Gel shift assay determined if DNA completely complexed with chitosan (CL 213). Lane 1, Luciferase DNA. Lane beneath white lines represent N:P ratios 1:1, 2:1, 3:1, 4:1, 5:1, 6:1 and 7:1 in (a.) absence of salt, (b.) 25mM sodium acetate-acetic acid (c.) 50mM sodium sulphate (d.) 25mM sodium acetate-acetic acid and 50mM sodium sulphate. (B) Stability of chitosan/DNA nanoparticles exposed to acid degradation. Each sample set run under four conditions. Lane 1: sample; Lane 2: sample exposed to chitosanase to release bound DNA. Lane 3: Samples exposed to pH 2 for 30min at 37ºC. Lane 4. Samples exposed to pH 2, neutralized and digested with chitosanase.

d. Effect of electrolyte concentration & N:P charge ratio

The influence of charge ratio and salt content on nanoparticle transfection efficiency was examined. Chitosan nanoparticles containing the luciferase plasmid were

formulated at N:P ratios of 1:1, 2:1, 3:1, 4:1, 5:1, 6:1, 7:1, 8:1, 9:1 and 10:1 using both 25 mM sodium acetate-acetic acid and 50 mM sodium sulphate, 25 mM sodium acetate-acetic acid only, 50 mM sodium sulphate only or no salts. The results indicate that maximum transfection occurred at a charge ratio of 3:1 (Figure 6.4A). Furthermore, formulations containing only 50 mM sodium sulphate provided enhanced transfection while formulations including 25 mM sodium acetate-acetic acid suppressed transgene expression

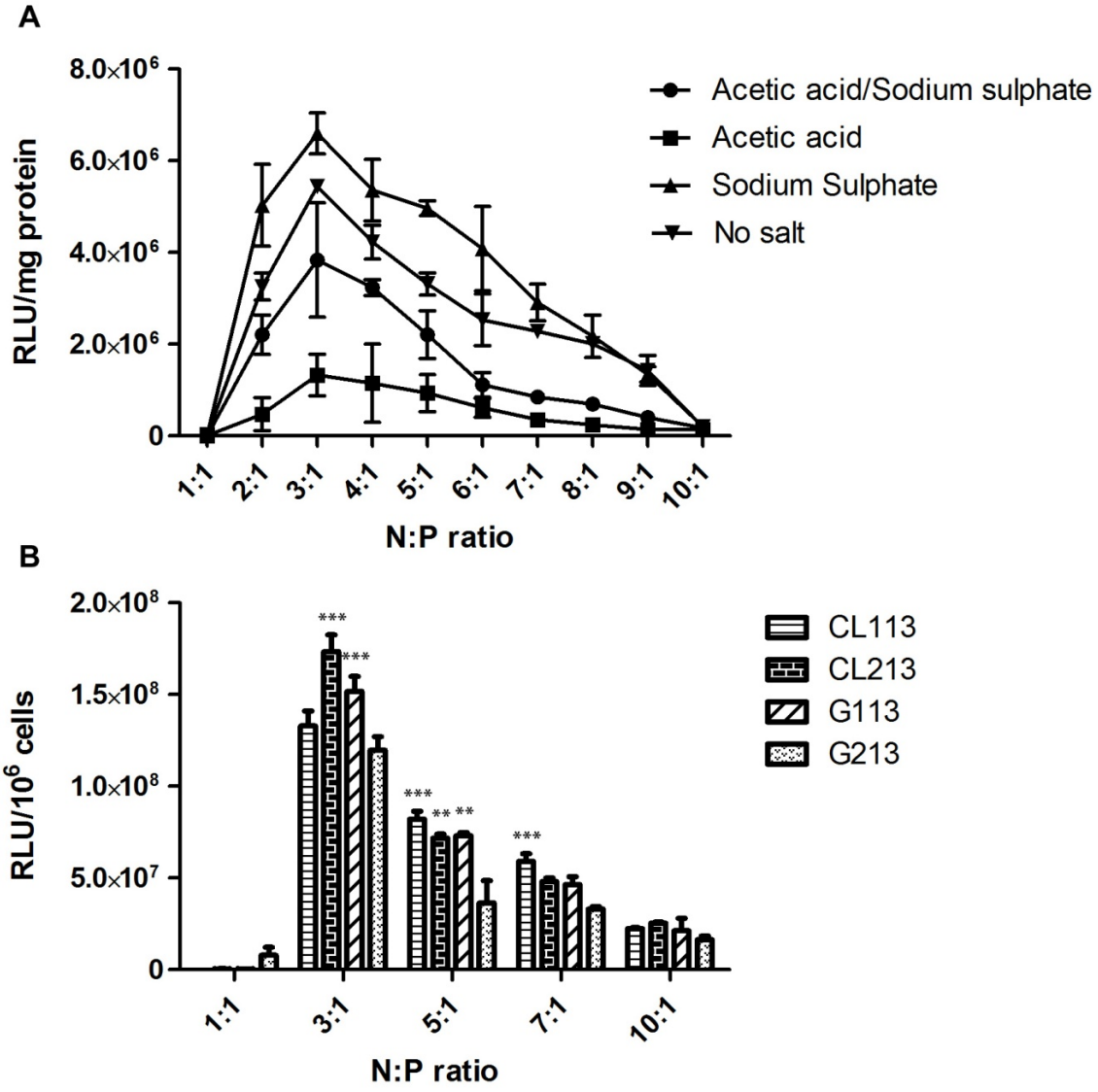

Figure 6.4: Effect of salts and charge ratio on nanoparticle transfection efficiency. (A) Chitosan nanoparticles were formulated at charge ratios of 1:1 to 10:1 using 10 µg of luciferase DNA. Chitosan (CL 213) nanoparticle were formulated in four different salt solutions with either 25 mM sodium acetate and 50 mM sodium sulphate, 25 mM sodium acetate-acetic acid only, 50 mM sodium sulphate only or no salts. (B) Transfection efficiency of nanoparticles formulated with four different chitosan forms. Nanoparticles formulated with chitosan chloride salt of molecular weight 113 kDa (CL 113) or 213 kDa (CL 213) and glutamic acid salt form of molecular weight 113 kDa (G113) or 213 kDa (G213). Transfection of HEK 293 cells with nanoparticles containing 10 µg luciferase plasmid were examined 24 hr post transfection. **p<0.01, ***p<0.001 compared to G213

e. Effect of chitosan type

The type of chitosan used in the formulation of nanoparticles may influence transfection efficiency. All pervious experiments have utilized a chitosan chloride salt with a molecular weight of 213 kDa (CL 213). The influence of chitosan type on transfection efficiency was examined with chitosan chloride salt 213 (CL 213), chitosan chloride salt 113 (CL113), chitosan glutamate salt 213 (G213) and chitosan glutamate 113 (G113). Nanoparticles were formulated using these four different chitosan salts in 50 mM sodium sulphate at charge ratios of 1:1, 3:1, 5:1 7:1 and 10:1. Consistent with our previous findings the optimal charge ratio was determined to be 3:1 for all chitosan salt forms (Figure 6.4B). Furthermore, the chitosan chloride (CL 213) provided the highest transfection, while G 213 provided the lowest transgene expression.

f. Production of concentrated nanoparticles

A concentrated oral dose of chitosan nanoparticles may provide higher *in vivo* transgene expression. To this end, the effect of freeze-drying nanoparticles on transfection efficiency was examined *in vitro*. Chitosan nanoparticles formulated with luciferase were freeze-dried and re-suspended in the same volume. HEK 293 cells were then transfected with either freeze-dried nanoparticles, freshly prepared nanoparticles, or nanoparticles prepared 30 days in advance. Interestingly, freeze-dried nanoparticles showed a 50% reduction in transgene expression (Figure 6.5). Furthermore, un-concentrated nanoparticles maintained their transfection potential for at least 30 days after preparation.

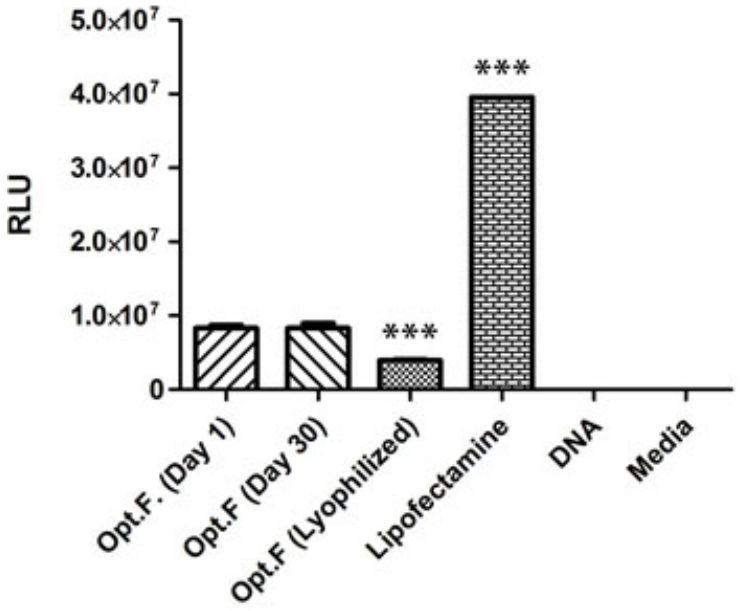

Figure 6.5: Transfection efficiency of chitosan nanoparticle formulations. Chitosan nanoparticles were formulated with 10 µg luciferase plasmid. Nanoparticles were lyophilized to examine the effect of concentrating nanoparticles. Bars represent mean ± SEM. ***p<0.001.

6.3 Discussion

We have previously demonstrated oral FVIII gene delivery using chitosan nanoparticles in hemophilia A mice. The nanoparticles were formulated with chitosan chloride (CL 213) in 25 mM acetate-acetic acid and 50 mM sodium sulphate at an N:P charge ratio of 4:1. The purpose of this study was to optimize this chitosan nanoparticle formulation to increase transfection efficiency.

The morphology of nanoparticle formulations was visualized by transmission electron microscopy (TEM). Gene transfection efficiencies *in vitro* and *in vivo* have been related to physical shape and stability of complexes (*254*). The presence of electrolytes

through their charge and size modulate the electrostatic interaction between chitosan and DNA to influence the morphology of the nanoparticles. Indeed, the morphologies of nanoparticle preparations were sensitive to the presence of various electrolytes. Although nanoparticle can be visualized by TEM, sample drying can influence particle properties.

Published studies suggest that the optimal nanoparticle size for effective gene transfer ranges from under 100 nm to over 1 µm(*192*, *206*, *255*). However, smaller particles, with an average size of between 150 nm to 300 nm, are typically effective (*190*, *194*, *206*). Nanoparticles prepared in this study were within this range as particle size increased proportionally with charge ratio. Nanoparticles formulated at low charge ratios (<3:1) may provide insufficient DNA protection while cellular uptake with nanoparticles formulated at higher charge ratios (>3:1) may be impeded by their increased size. Alternatively, higher charge ratios may provide additional chitosan layers and shield the negatively charged DNA from the periphery. The increased nanoparticle diameter would provide additional barriers to DNA release from the interior of the nanoparticles.

The stability of nanoparticles was investigated in this study. Nanoparticles formulated at a charge ratio greater than 1:1 provided sufficient DNA encapsulation while a charge ratio >2:1 was necessary to protect DNA from degradation by DNase and from low pH environments. This suggests that the higher charge ratios provide complete encapsulation of DNA and prevents plasmid exposure to degradation agents.

Chitosan salts used in this study are water soluble and have improved transfection efficiency compared to chitosan base(*14*). Nanoparticles formulated with chitosan chloride (CL 213) with 50 mM sodium sulphate at a charge ratio of 3:1 increased

transfection by 40% compared to the formulation used previously(*196*). Peak transgene expression was observed with nanoparticle formulated at a 3:1 charge ratio regardless of electrolyte content. Interestingly, electrolytes modulated nanoparticle properties as sodium sulphate increased transfection while acetic acid suppressed transgene expression. These electrolytes may modulate chitosan-DNA electrostatic interaction along with their packaging and release characteristics. These factors must also be considered in preparations of concentrated formulations.

Efficient gene transfection requires a balance between DNA protection and unpacking (*188, 256*). In this study, low charge ratios (<3:1) may not provide sufficient protection against DNA degradation while the reduced surface charge may impede interactions with cell membranes. In contrast, high charge ratios (>3:1) might prevent exogene unpacking and hinder expression. A number of studies have examined the methods of improving DNA unpacking to improve transfection. Inclusion of negatively charged poly-glutamic acid competes with DNA to bind chitosan and enhanced uptake(*257, 258*). Also, attachment of a phosphorylatable short peptide facilitates unpacking(*171*).

Chitosan nanoparticles are a versatile gene therapy tool that is amenable to modifications to enhance transgene expression. This modification can include solubility, targeting, endosomal escape, unpacking and nuclear localization. Here we further optimize nanoparticle formulations for the purpose of applications for hemophilia treatment.

Chapter VII

Conclusions

Viral gene therapy strategies have received great interest as a potential cure for genetic disorders. Viruses are highly evolved gene delivery vectors that present risks of insertional mutagenesis and/or immune response complications. Thus, viral vectors present safety risks that may impede its application in non-fatal disorders, such as hemophilia, where effective treatments exist.

Chitosan-mediated gene therapy presented here is an orally administered non-viral strategy that is shown to be a viable treatment option for hemophilia. Chitosan-mediated oral gene delivery of FVIII and FIX has been demonstrated for the treatment of hemophilia A and B, respectively. In addition, prophylactic treatment was discovered to suppresses inhibitor development and confer long-term tolerance for at least 8 weeks. Treatment of hemophilic patients with pre-existing inhibitors remains a challenge. However, orally delivered gene therapy treatment or prophylactic tolerance using therapeutic protein variants may achieve similar success in these patients. This therapy can potentially supersede protein replacement therapy or prevent inhibitor formation to complement conventional treatment.

Chitosan nanoparticle formulations can be modified for enhanced efficacy. Further modifications may include targeting ligands to facilitate tissue-specific expression. Also, batch variation may be addressed by controlling chitosan chain length

and charge density. Interestingly, higher expression may be achieved with coagulation factor variants including codon optimized genes.

Although chitosan-mediated oral gene delivery is not a cure for hemophilia, it is an attractive alternative to conventional treatment especially in paediatric patients. Furthermore, scaling-up to human application would be inexpensive while modest transgene product expression is sufficient for effective treatment. Taken together, chitosan-mediated oral gene delivery provides a treatment strategy that is painless, patent friendly, cost effective and potentially accessible to the majority of haemophiliacs. Furthermore, this technology may benefit other monogenic disorders including muscular dystrophy, cystic fibrosis, thalassaemia, sickle cell anaemia Pompe disease or mucopolysaccharidosis.

Aim I: Evaluate the gene delivery of FVIII by oral administration of chitosan/DNA nanoparticles in hemophilia A mice

The transient expression of FVIII has been demonstrated in hemophilia A mice. Transient phenotypic correction was also demonstrated following transgene expression *in vivo*. This transient cFVIII expression was sustainable following repeated administration of chitosan nanoparticles. Of interest, the absence of a detectable immune response may suggest immune response modulation to the FVIII transgene. These findings have been published in the Journal of Thrombosis and Haemostasis(*196*).

Aim II: Examine the immune response modulation of orally administered chitosan nanoparticle-mediated hFVIII gene delivery in hemophilia A mice

Chitosan nanoparticles can produce immune tolerance and provide protection against rhFVIII challenge. Treatment of naïve mice with nanoparticles suppressed antibody and inhibitor development. Results also indicate that oral nanoparticle administration results in the production of T regulatory cells that mediate tolerance. Furthermore, nanoparticles were unable to effectively suppress antibody or inhibitor titres in mice with pre-existing hFVIII inhibitors.

Aim III: Demonstrate chitosan nanoparticle-mediated FIX gene delivery for the treatment of hemophilia B

Preliminary results have identified the CpG-IXi plasmid for incorporation into nanoparticle formulations for the treatment of hemophilia B. *In vivo* examination showed peak expression one day post-treatment and expression for up to two days post-treatment. The expression kinetic observed for FIX delivery is consistent with results obtained for FVIII delivery.

Aim IV: Optimize and characterize transfection efficiency of Chitosan/DNA nanoparticles formulations

Formulations of chitosan-DNA nanoparticles are being assessed based on physical-chemical properties (size, morphology, charge, stability) and transfection ability. The inclusion of salts into chitosan-DNA formulations appears to induce aggregation of

nanoparticles. The size of the nanoparticles increases with chitosan/DNA charge ratio. Also, a charge ratio of >1:1 is required to completely complex with DNA while ratios >3:1 are required protect against acid degradation. Optimal nanoparticle transfection is formulated using chitosan chloride salt 213 at a charge ratio of 3:1 in 50 mM sodium sulphate. Surprisingly, in initial experiments, concentration of nanoparticles by freeze-drying appears to decrease transgene expression *in vitro.* Taken together, the accumulated improvements in chitosan formulations will facilitate transgene expression of FVIII or FIX in hemophilic mice.

7.1 Reference

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