Chitosan-based therapeutic nanoparticles for combination gene therapy and gene silencing of in vitro cell lines relevant to type 2 diabetes

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Glucagon like peptide 1 (GLP-1), a blood glucose homeostasis modulating incretin, has been proposed for the treatment of type 2 diabetes mellitus (T2DM). However, native GLP-1 pharmacokinetics reveals low bioavailability due to degradation by the ubiquitous dipeptidyl peptidase IV (DPP-IV) endopeptidase. In this study, the glucosamine-based polymer chitosan was used as a cationic polymer-based in vitro delivery system for GLP-1, DPP-IV resistant GLP-1 analogues and siRNA targeting DPP-IV mRNA. We found chitosans to form spherical nanocomplexes with these nucleic acids, generating two distinct non-overlapping size ranges of 141–283 nm and 68–129 nm for plasmid and siRNA, respectively. The low molecular weight high DDA chitosan 92–10–5 (degree of deacetylation, molecular weight and N:P ratio (DDA–Mn–N:P)) showed the highest plasmid DNA transfection efficiency in HepG2 and Caco-2 cell lines when compared to 80–10–10 and 80–80–5 chitosans. Recombinant native GLP-1 protein levels in media of transfected cells reached 23 ng/L while our DPP-IV resistant analogues resulted in a fivefold increase of GLP-1 protein levels (115 ng/L) relative to native GLP-1, and equivalent to the Lipofectamine positive control. We also found that all chitosan–DPP-IV siRNA nanocomplexes were capable of DPP-IV silencing, with 92–10–5 being significantly more effective in abrogating enzymatic activity of DPP-IV in media of silenced cells, and with no apparent cytotoxicity. These results indicate that specific chitosan formulations may be effectively used for the delivery of plasmid DNA and siRNA in a combination therapy of type 2 diabetes.

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1. Introduction

Given the rise of sedentary lifestyles and obesity combined with genetic predisposition, type 2 diabetes mellitus (T2DM) represents one of the most prevalent metabolic diseases. Type 2 diabetes is the most common form of diabetes, accounting for 90% of all diabetes worldwide. T2DM is characterized by insulin resistance in peripheral tissues, insulin deficiency and impaired glucose homeostasis initiating a debilitating process, which in turn, promotes β-cell dysfunction that further increases morbidity, mortality and reduces treatment efficacy (Meier and Nauck, 2008). Antidiabetic treatments include sulphonyurea (Tolbutamide (T2D)), meglitinide (Repaglinide (T2D)) and Phenformin (T2D), thiazolidinediones (T2D) and α-glucosidase inhibitors (Acarbose®). Unfortunately, these treatment modalities, even when combined, are frequently constrained by safety, tolerability, weight gain, edema and gastrointestinal intolerance (Drucker et al., 2010; Kalaitzidis et al., 2009; Montanya and Sesti, 2009; Nauck et al., 2009; Ng et al., 2010; Norris et al., 2009; Pratley et al., 2010; Truitt et al., 2010; Wajcberg and Tavaria, 2009). In addition, as the disease progresses and β-cell function declines, efficacies of current treatments diminish (Turner et al., 1999). Recently, the discovery of the incretin effect has provided a new avenue of treatment using a class of therapeutics capable of controlling T2DM with minimal adverse effects.

The incretin hormone glucagon-like peptide-1 (GLP-1) enhances glucose-dependent stimulation of insulin secretion, suppresses abnormally elevated glucagon secretion, delays gastric emptying, and promotes satiety and weight loss, as well as promoting β-cell growth and inhibiting β-cell apoptosis in animal models (Nauck et al., 2002; Holst et al., 1987; Creutzfeldt et al., 1996). Multiple mammalian studies, including human, have demonstrated insulinotropic responses to exogenous administration of GLP-1, particularly GLP-1 (7–36) NH2 and GLP-1 (7–37) (Guttiak et al., 1992; Nauck et al., 1993a,b; Thorens and Waeder, 1993).
However, it has been demonstrated that the half life of GLP-1 is very short and that less than 10% of administrated GLP-1 are intact and biologically active only a few minutes after injection (Deacon et al., 1995). The short half-life of GLP-1 results mainly from rapid enzymatic degradation by dipeptidyl peptidase IV (DPP-IV) that cleaves the His7:Ala8:Glu9 sequence at the N-terminal region of GLP-1 (Hansen et al., 1999). DPP-IV, also known as CD26, is a transmembrane glycoprotein, which forms a homodimer with a molecular mass of about 250 kDa (Duke-Cohan et al., 1996). It is expressed on a plethora of cells including lymphocytes, endothelial cells, and enterocytes among others. DPP-IV plays an important role in processing peptides such as hormones, chemokines and neuropeptide Y (Mentlein, 1999). Therapeutic approaches for enhancing incretin action include degradation-resistant GLP-1 receptor agonists, and inhibitors of dipeptidylpeptidase-IV (DPP-IV) activity (incretin enhancers). For example, the incretin mimetic GLP-1 resistant agonist exenatide 4 (Bayetta™ from Eli Lilly and Amylin) has an extended half life of 60 to 90 minutes and reduces fasting and postprandial glucose concentrations, reduces plasma glycosylated hemoglobin (HbA1c) and provides mild weight loss in phase III clinical trials (DeFronzo et al., 2005; Eng, 1992; Chen and Drucker, 1997). Moreover, in a phase II clinical trial, Albilutide (Syncria® from GlaxoSmithKline) a GLP-1 receptor agonist with an extended half life (6–8 days) showed a clinically significant absolute reduction of HbA1c of 0.8% at week 16 (Rosenstock et al., 2009). Albilutide was developed as a fusion of human albumin to two copies of a DPP-IV resistant GLP-1 analog (Rosenstock et al., 2009; Baggio et al., 2004; Bush et al., 2009; Matthews et al., 2008). Orally administered DPP-IV inhibitors, such as sitagliptin (Januvia® from Merck) and vildagliptin (Galvus® from Novartis), also reduce HbA1c by 0.5–1.0%, with few adverse effects and no weight gain (Ahren et al., 2004; Herman et al., 2005). Reported adverse effects were mild to moderate with the most common being dizziness, nausea, headache (Pratley et al., 2006), severe allergic and dermatologic reactions (VanDeKoppel et al., 2008; WebMD, 2011) and infrequent hypoglycemia (Ristic et al., 2005).

Major drawbacks of these incretin enhancers are their low patient compliance as they still require repeated administration by subcutaneous means, high production cost; complex production processes and induce adverse side effects. An alternative mean of sustaining native GLP-1 activity can be achieved through the endogenous expression of recombinant GLP-1 and/or DPP-IV gene silencing. Most gene delivery methods to date use viral vectors (Alexander et al., 2008; Robbins and Ghivizzani, 1998). Although some of these approaches have been remarkably successful in life-threatening diseases (Alexander et al., 2008; Robbins and Ghivizzani, 1998), viral vectors are associated with safety issues (Daniel and Smith, 2008; Lowenstein et al., 2007). Hence non-viral delivery systems have received growing attention to achieve a high level of cellular transfection in a safe and effective manner.

Chitosan is a copolymer of β-(1→4)-glucosamine and N-acetyl-D-glucosamine derived by partial deacetylation of chitin from crustacean shells (Illum, 1998). Protonation of amine groups on chitosan glucosamine monomers is facilitated at slightly acidic pH below chitosan pKa of ~6.5; thus conferring cationic properties enabling chitosan to interact with anionic components such as cell surface macromolecules and nucleic acids (Ma et al., 2009, 2010). Chitosan formulations with specific degrees of deacetylation (DDA) and number average molecular weight (Mn) can be obtained through appropriate processing (Muzzarelli, 1977; Roberts, 1992). These two parameters have a major influence on chitosan biological and physicochemical properties (Ma et al., 2009; Ma et al., 2010; Zhang and Neau, 2001; Lavertu et al., 2006; Huang et al., 2005; Kopling-Hoggard et al., 2004; Strand et al., 2005, 2010). Using a library of different chitosan formulations (DDA–Mn–N:P), we have previously demonstrated the efficacy of some of these tunable formulations for gene delivery (Lavertu et al., 2006; Jean et al., 2009; Nimesh et al., 2010; Alameh et al., 2010; Jean et al., 2011). We also found that specific chitosans should be used for specific biological applications ranging from therapeutic protein delivery to immunization (Lavertu et al., 2006; Jean et al., 2009; Nimesh et al., 2010; Jean et al., 2011).

Here we investigate the ability of specific chitosan formulations (92–10–5, 80–10–10 and 80–80–5) at low N:P ratios to deliver native GLP-1 and DPP-IV resistant GLP-1 analogues in vitro for augmented bioavailability of GLP-1 in DPP-IV expressing cell lines. Increased bioavailability is achieved by engineering a Ser8 and/or Tyr9 modified GLP-1 using site directed mutagenesis and/or by silencing the DPP-IV serine protease. These modified GLP-1 sequences confer resistance to degradation by the alteration of the His:Ala:Glu DPP-IV substrate/cleavage specificity (Green et al., 2004). Also, we perform physico-chemical characterization of these specific formulations for complexation and efficient delivery of siRNA targeting the DDP-IV serine protease in HepG2 cell line. Characterization of chitosan/siRNA nanoparticles included size and zeta potential determination using dynamic light scattering (DLS) and environmental scanning microscopy (ESEM), stability and nuclease protection assays, uptake determination by flow cytometry (FACS) and confocal microscopy as well as the determination of silencing efficiency by quantitative real time PCR (qPCR). The results presented show great promises for a combined therapy — GLP-1 encoding pDNA and DPP-IV targeting siRNA — using these same and specific chitosan formulations.

2. Material and methods

2.1. GLP-1 plasmid constructs

The construction of a plasmid expressing GLP-1 was engineered in the eukaryotic pVax1® plasmid (Cat #V2620-20, Invitrogen) driven by a cytomegalovirus promoter. A furin cleavage site was fused between a Kozak sequence and the GLP-1-coding region (amino acids 7–37) to generate an active form of GLP-1 terminated by a bovine growth hormone (BGH) poly A signal (Fig. 1). GLP-1(7–37) sequences were generated by PCR and PCR-based mutagenesis techniques and cloned into a pVax1 plasmid using a restriction enzyme based strategy. First, the GLP-1 sequence was amplified

![Fig. 1. Native GLP-1 and DPP-IV resistant GLP-1 analog constructs. A Kozak sequence, start codon and the furin recognition site were added upstream and a stop codon downstream of the sequence coding for GLP-1 (7–37). The GLP-1 nucleic acid sequence was modified to produce DPP-IV resistant variants pVax1-Ser8-GLP-1 and pVax1-Tyr9-GLP-1 (encircled codons). Restriction endonuclease sites for HindIII and Xhol added to each end allowed the cloning of the GLP-1 (7–37) sequence inside the eukaryotic plasmid pVAX1. Transcription was driven by the presence of a cytomegalovirus promoter (pCMV) with the Bovine Growth Hormone Polyadenylation Signal (BGHPA) located downstream to stabilize the transcribed mRNA. The choice of these mutations (Ala8→Ser8 and Glu9→Tyr9) is based on the rational of modifying the GLP-1N-terminal DPP-IV recognition/cleavage site – His7:Ala8:Glu9 – in order to enhance resistance.](image-url)
using a polymerase chain reaction on the proglucagon cdna using a specific set of primers (rv-glpl-1(7–37) tctctggcctttct; fw-glpl-1(7–37) catgctcaagggacc; fw-[ser8] glpl-1(7–37) catgcttatgggacc). in order to generate dpp-iv resistant glpl-1 variants, the forward primer was modified to incorporate either ser or tyr at residues 8 and 9, respectively (fig. 1). the amplified products were cloned between hindiii and xhol sites in pvax1© and all plasmid constructs were confirmed by dna sequencing using gene specific primers.

2.2. siRNA and dsODN as a structural model of siRNA

siRNA sequences targeting the DPP-IV gene sequence for knock-down studies were synthesized using a novel RNA synthesis chemistry, the 5'-silyl-2'-Orthoester protecting groups (2'-ACE) Scaringe et al., 1998 combined with a standard phosphoramidite solid-phase technology and are available in the On Target Plus® catalogue (L-004181-00, Dharmacon, Thermo Scientific, Dharmacon RNAi Technologies, Lafayette, USA). The siRNA sequences have a dual strand modification pattern to reduce off target effects caused by both strands. Mock siRNA were also used as a negative control. Mock siRNA is a non-targeting siRNA (Dharmacon, D-001710-01-05) designed to have minimal targeting of known genes in human, mouse and rat cells.

double stranded oligodeoxynucleotides (dsODN, 21 bp) encoding the same sequences and mimicking siRNA physicochemical properties were used for nanoparticle characterization. The dsODN sequences were synthesized, using the phosphoramidite chemistry (Integrated DNA Technologies, Coralville, USA) and used for size and zeta potential determination, nanoparticle stability and protection assays. For confocal microscopy and FACS analysis, 6-carboxyfluorescein (6-FAM) 5' labeled dsODN were used (Integrated DNA technologies, Coralville, USA).

2.3. Preparation of chitosan/pVax-GLP-1 plasmid, chitosan/DPP-IV siRNA and chitosan /DPP-IV dsODN nanoparticles

Chitosans with specific average molecular weight (Mn) and degree of deacetylation (DDA) were prepared using chemical degrada-dation methods and characterized using CPC and NMR as described previously (Lavertu et al., 2003, 2006; Nguyen et al., 2009a,b). They were dissolved overnight on rotary mixer at 0.5% (w/v) in hydrochloric acid using a glucosamine: HCl ratio of 1:1. Chitosan solutions were then diluted with deionized water to reach the desired amine (glucosamine monomers) to phosphate ratio (N:P ratio) when 100 μl of chitosan would be mixed with 100 μl of plasmid or siRNA or dsODN. Diluted chitosan solutions were then diluted with a 0.2 μm syringe filter. Nanoparticles were prepared by adding 100 μl of sterile diluted chitosan solution to 100 μl of pvax1-GLP-1 (0.33 μg/μl) and its variants or to 100 μl of DPP-IV siRNA at 0.05 μg/μl or at 0.33 μg/μl for DPP-IV dsODN at room temperature, pipetting rapidly up and down. The 0.05 μg/μl concentration was used for silencing using siRNA whereas the higher concentration of siRNA mimicking ODN (0.1 μg/μl) was used for better DLS signal and visibility on gels. The mixed solutions were incubated 30 min for nanoparticles formation prior to analyses or treatment of cells. The recombinant pvax1/GLP-1 vectors, the DPP-IV siRNA and the DPP-IV dsODN sequences were complexed with each of three distinct and well characterized formulations of chitosan [92–10–5, 80–10–10 and 80–80–5 (DDA– Mn– N:P)] to create therapeutic nanoparticles that were tested in vitro for gene transfection, knockdown efficiency and complex formation/stability/nuclease-protection.

2.4. Nanoparticle size and surface charge analysis

The size of chitosan/pVax1-GLP-1, chitosan/pVax1-GLP-1 DPP-IV-resistant analogues and chitosan/dsODN-DPP-IV nanoparticles were determined by dynamic light scattering (DLS) using a Malvern Zetasizer Nano ZS (Malvern, Worcestershire, UK). Samples were measured in triplicate using the refractive index and viscosity of pure water in calculations. The zeta potential was measured in duplicate with laser Doppler velocimetry at 25°C on the same instrument, and with the viscosity and dielectric constant of pure water used for calculations. For both of the above measurements, nanoparticles were diluted 1:10 in 10 mM NaCl.

2.5. Scanning electron microscopy

An environmental scanning electron microscope (ESEM, Quanta 200 FEG, FEI Company Hillsboro, OR, USA) was used to image chitosan/pVax1-GLP1 and chitosan/dsODN-DPP-IV nanoparticles to obtain shape and size after complexation. Nanoparticles were sprayed on silicon wafer substrate, and then sputter-coated with gold (Agar Manual Sputter Coater, Marivac Inc.). Observations were performed at 20 kV in the high vacuum mode.

2.6. Nanoparticle formation, stability and protection against nuclease attack

Chitosan/dsODN-DPP-IV nanoparticles were incubated following complex formation for 30 min, 4 h or 20 h at pH 6.5 in 2-(N-morpholino)ethanesulfonic acid buffer (MES 1X) or at pH 8 in Tris–acetate–EDTA buffer (TAE 1X) in order to assess their stability as a model for complexes formed with siRNA. The formation and stability of the chitosan/dsODN-DPP-IV nanoparticles was then analyzed by polyacrylamide gel electrophoresis (13% w/v, pH 6.5 and pH 8). To assess protection against nuclease attack, chitosan/ dsODN-DPP-IV nanoparticles were incubated with 0.5, 1, 2, 5 or 10 units of DNase 1 in 20 μl of MES–MgCl2 buffer (20 mM MES, 1 mM MgCl2, pH 6.5) for 30 min at 37°C. The reactions were then stopped by adding 2 μl of EDTA (50 mM) and chitosan was digested with Streptomyces griseus chitosanase (Sigma–Aldrich, Oakville, ON) for 90 minutes at 37°C. The integrity of the DPP-IV dsODN after nuclease attack of the complexes was then analyzed by agarose gel electrophoresis (4%, pH 8) prepared with TAE buffer (1X). Electrophoresis was carried out at a constant voltage of 100 V for 45 min (HorizonTM 11.14 BRL, Life Technologie Inc). DNA bands were visualized following ethidium bromide (EtBr) staining under a UV transilluminator (Bio-Vision 3000, Montréal Biotech, Montréal, QC, Canada) and captured images were analyzed using Vision-Capt software (V.15.06, Vilber Lourmat, Paris, France). Relative amounts of dsODN-DPP-IV (%) were determined by compari-sion of integrated signal intensity of DNase treated samples versus non-treated samples. Chitosan was visualized by Coomassie blue staining under white light on the same instrument.

2.7. In vitro transfection

2.7.1. Cell culture

HT-29, HepG2 and Caco-2 cell lines (ATCC, Manassas, VA) were cultured at 37°C and 5% CO2. HT-29 cells were cultured in McCoy's media with 2.2 g/l sodium bicarbonate and supplemented with 10% fetal bovine serum (FBS) (Cedarlane Laboratories, Burlington, ON). HepG2 and Caco-2 cells were cultured in Minimum Essential medium (MEM). Caco-2 medium was supplemented with 1.5 g/l sodium bicarbonate, 1 mM sodium pyruvate and 0.1 mM of non-essential amino acids and 10% FBS. HepG2 medium was supple-mented with 8% FBS. These cell types were chosen since they express DPP-IV and represent model cell lines for diabetes
of 0.0081

for siRNA transfection, HepG2, Caco-2 and HT-29 cell lines were plated in 24 well plates at 300,000 cells/well, whereas for siRNA transfection, HepG2, HT-29 and Caco-2 cells were plated in 96 well plates at 25000 cells/well. The cells were transfected the next day at 50% confluency.

2.7.2. Transfection with chitosan/pVax1-GLP-1, chitosan/pVax1-GLP-1 DPP-IV resistant analogues and chitosan/siRNA-DPP-IV therapeutic nanoparticles

For in vitro transfection, DMEM high glucose (HG) was prepared with 0.976 g/L of 2-(N-morpholino)ethanesulfonic acid (MES) and 0.84 g/L of sodium bicarbonate (NaHCO₃) and supplemented with 10% FBS. This complete transfection media was equilibrated over-night at 37 °C, 5% CO₂ before pH adjustment to 6.5 with 1 N sterile HCl and distributed in a ghost plate of either 24 wells (500 μl/well) or 96 wells (100 μl/well). Chitosan/pVax1-GLP-1, chitosan/pVax1-GLP-1 DPP-IV resistant analogues and chitosan/dsODN-DPP-IV or chitosan/siRNA-DPP-IV were prepared as described above; 30 minutes before use. For pDNA based transfections, nanoparticles were incubated in DMEM HG media, at a final concentration of 2.5 μg pDNA/well. For siRNA transfection performed in a 96 well plate, a 100 μl siRNA solution at a concentration of 0.05 μg/μl was used for siRNA complexation with chitosan at a 1:1 ratio (v/v). Following complexation, nanoparticles were incubated in a ghost plate containing DMEM-HG media, at a final concentration of 0.135 μg/well of siRNA; equivalent to 100 nM or to a concentration of 0.00135 μg/μl. For dsODN transfection performed in a 24 well plate, a 100 μl dsODN solution at a concentration of 0.05 μg/μl was used for dsODN complexation with chitosan at a 1:1 ratio (v/v). Following complexation, nanoparticles were incubated in a ghost plate containing DMEM-HG media, at a final concentration of 0.81 μg/well of dsODN; equivalent to 600 nM or a concentration of 0.0081 μg/μl. All plates containing nanoparticles were equilibrated for 10 min before transfection at 37 °C, 5% CO₂. Medium over cells was aspirated and replenished with the appropriate volume of the equilibrated transfection medium at pH 7.5 containing plasmid based nanoparticles or dsODN or siRNA based nanoparticles at a final concentration of 2.5 μg/well, 0.81 μg/well and 0.135 μg/well, respectively. Cells were incubated with either plasmid based nanoparticles or dsODN or siRNA based nanoparticles for 48 h and 24 h, respectively before analysis. Lipofectamine™ and DharmaFECT™ were used as positive controls for pDNA and dsODN/siRNA transfections, respectively. Both untreated cells and naked pVax1-GLP-1 and empty pVax1 were used as negative controls for pDNA transfections whereas untreated cells and uncompelled siRNA treated cells were used as negative controls for siRNA transfections.

GLP-1 concentration was determined 48 h post transfection by a specific anti-biologically active GLP-1 ELISA Kit (Linco research, St. Charles, MO) whereas DPP-IV knockdown was assessed by the evaluation of its enzymatic activity 48 h post-transfection as well as qRT-PCR for mRNA levels 24 h post-transfection (see below). For the former, chitosan/siRNA-DPP-IV treated cells were incubated for 1 h with 200 μL of Gly-Pro p-nitroanilide hydrochloride (Sigma–Aldrich, St-Louis, MO, USA). For pVax1-GLP-1 nanoparticle transfections, plasmid DNA was labeled with fluorescein (488 Ex, 520 Em) using Mirus Label IT Kit (Mirus, Madison, WI, USA) according to the manufacturer protocol at a ratio of 0.20/1 (μl of labeling/μg of pVax1-GLP-1) resulting in a final labeling efficiency of 1 fluorescein unit per 225 DNA base pairs. Internalisation of dsODN-DPP-IV was done using dsODN-DPP-IV prelabelled by the manufacturer (Integrated DNA technologies, Coralville, USA) with 6-Carboxyfluorescein (6FAM) (488 Ex, 520 Em) at the 5’ extremity. For confocal imaging, cells were seeded 24 h prior to transfection in 35 mm glass bottom culture dishes (MatTek, Ashland, MA, USA) using 500 μl of complete medium at 40,000 cells/dish. Transfection was performed as previously described using 2.5 μg of pVax1-GLP-1 or dsODN-DPP-IV /well. Prior to imaging, cell membranes were stained for 5 minutes at 37 °C with 2.5 μg/ml of CellMaskTM Deep red (cat # C10045, Invitrogen, Carlsbad, CA, USA). In addition to the assessment of internalization and general intracellular nanoparticle distribution, complexation of DNA with chitosan was also qualitatively assessed by colocalisation. The latter is assessed by the occurrence of yellow pixels resulting from the spatial overlap of red (chitosan pseudocolor) and green pixels (pVax1-GLP-1 or dsODN pseudocolor) from two separate channels.

2.9. FACS analysis

The cellular uptake of pVax1-GLP-1 and dsODN-DPP-IV was determined by transfecting HepG2 cell lines with nanoparticles formed with fluorescein or 6FAM labeled pVax1-GLP-1 and dsODNs, respectively. After 24 h of incubation with the nanoparticles, cells were treated with chitosanase for 60 min to eliminate any cell surface-associated complexes left from the transfection, as described previously (Alameh et al., 2010). Afterwards, cells were washed twice with PBS, trypsinized, and resuspended in PBS. The analysis of cell uptake was performed using a BD Canto flow cytometer (Becton Dickinson, San Jose, CA, USA). For each sample, 20,000 events were counted and to exclude cell debris, dead cells and aggregated cells, a collection gate was established using a dot plot of the forward light scatter against the side scatter. The cells were excited using a 488 nm laser line and the detection was made using a 530/30 nm band pass filter. Non-transfected cells were used as negative controls to distinguish positive cells from auto-fluorescence.

2.10. Quantitative PCR analysis of DPP-IV mRNA knockdown

2.10.1. RNA extraction and assessment methods (yield, purity and integrity)

RNA extraction was performed using the RNA XS® extraction kit from Macherey-Nagel (Biolyx, Montréal, QC, Canada) according to
the manufacturer’s protocol following chitosanase treatment, as previously described (Alameh et al., 2010). Total RNA was quantified and RNA integrity measured using the Agilent BioAnalyzer 2100 (Agilent Technologies, Mississauga, ON, Canada) following the manufacturer’s protocol. RNA integrity was evaluated by the ratio of 28S/18S ribosomal RNA (rRNA) Skrypina et al., 2003 and the RNA integrity number (RIN). The RIN is a relative measure of RNA quality based mainly on electrophoretic trace analysis. The BioAnalyzer 2100 automatically computes RIN, and as a reference, an ideal non degraded RNA sample has an RIN of 10.

2.10.2. Reverse transcription
Total RNA was reverse transcribed in a final volume of 20 μl using the First Strand cDNA Transcription Kit with oligo dT primers (Roche Diagnostics, Laval, QC, Canada). Endogenous control (TBP (TATA binding protein) and HPRT (hypoxanthine guanine phosphoribosyl transferase)) expression levels were determined using prevalidated TaqMan Gene Expression Assays (Applied Biosystems (ABI), Carlsbad, CA, USA). DPP-IV mRNA (target detection) reactions for 384 well plate formats were performed using 1.5 μl of cDNA samples (25-50 ng), 5 μl of the Fast Universal qPCR MasterMix (Applied Biosystems (ABI), Carlsbad, CA, USA), 2 μl of each primer and 1 μl of a Universal Probe Library (UPL) probe (#71) in a total volume of 10 μl. For endogenous control assessment, reactions were performed using identical volume of cDNA, Fast Universal qPCR Master Mix, 0.5 μl of the TaqMan Gene Expression Assay (20X) and 2.5 μl of water in a total volume of 10 μl.

2.10.3. Gene expression assays
The DPP-IV mRNA expression level was determined using assays designed with the Universal Probe Library from Roche (Roche applied science, Laval, QC, Canada). Endogenous control (TBP (TATA binding protein) and HPRT (hypoxanthine guanine phosphoribosyl transferase)) expression levels were determined using prevalidated Universal Probe Library from Roche (Roche Diagnostics, Laval, QC, Canada, Cat # 04896866001) as described by the manufacturer protocol. Samples were stored at −20 °C.

2.10.4. Detection and analysis
The ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems, Carlsbad, CA, USA) was used to detect the amplification level and was programmed with an initial step of 3 min at 95 °C, followed by 45 cycles of: 5 s at 95 °C and 30 s at 60 °C. All reactions were run in triplicata and the average values of Ct’s (threshold cycle) were used for quantification. TBP (TATA binding protein), HPRT (hypoxanthine guanine phosphoribosyl transferase), were used as endogenous controls. The relative quantification of target genes was determined using the delta delta cycle threshold (ΔΔCt) method (Livak and Schmittgen, 2001). Briefly, the Cycle threshold (Ct) values of target genes were normalized to an endogenous control gene (endoC) (ΔΔCt = Cttarget − CtendoC) and compared with a calibrator: ΔΔCt = ΔCt(target) − ΔCt(calibrator). Relative expression (RQ) was calculated using the sequence detection system (SDS) 2.2.2 software (Applied Biosystems, Carlsbad, CA, USA) using the RQ = 2−ΔΔCt formula.

2.11. Statistical analysis
The measurement data were collected and expressed as means values ± standard deviation (s.d.) and were analyzed with Statistica 9.0 software (STATSOFT; Statistica, Tulsa, OK, USA). Statistical significance was determined by one-way ANOVA followed by Tukey post hoc test. Differences were considered significant * at p < 0.05 and highly significant ** at p < 0.01.

3. Results
3.1. Characterization of chitosan nanoparticles
All formulations of pDNA–chitosan nanoparticles were in the range of 141–283 nm, (Table 1) whereas the formulations of dsODN–chitosan had a smaller size range between 68–129 nm as measured by DLS (Table 1) and ESEM (Fig. 2). The excess chitosan in all formulations resulted in positively charged nanoparticles as shown by zeta potential (Table 1). The zeta potential of the shorter dsODN complexed with chitosan was lower (14–21 mV) than for pDNA chitosan complexes (23–35 mV). These size and zeta potential differences between the pDNA and siRNA based nanoparticles may be due to the nucleic acid sequence length.

3.2. Nanoparticle stability and nuclease protection assay
Nanoparticle stability was evaluated at 0.5, 4 and 20 h after complexation at two different pH. When nanoparticles are unstable, dsODNs are released and migrate (Fig. 3A). Also, since chitosan is in excess, its free and soluble component migrates (Fig. 3B), consistent with previous analyses by Field-Flow Fractionation (Ma et al., 2010). At pH 6.5, chitosan/dsODN-DPP-IV complexes with N: P ratios of 1 and above formed to completely bind dsODN. These complexes were stable up to 20 h following nanoparticle formation (Fig. 3A). In contrast, at pH 8, chitosan did not bind dsODN completely at N:P ratio of 1 but did when in excess at N:P ratio of 10, consistent with the lower ionization state of chitosan at pH 8 given the chitosan pKa of ~6.5. For effective gene expression and/or inhibition, nucleic acids entrapped in the delivery vehicle should be protected from degradation by enzymes such as serum nuclease (Quong and Neufeld, 1998). The ability of chitosan-based nanoparticles to protect dsODN-DPP-IV sequences was assessed using a DNAse I protection assay against chitosan complexed with dsODN-DPP-IV, as a model for siRNA. Upon incubation with DNAse, naked dsODN, DPP-IV (control) was completely degraded (Fig. 4, lane C3). In contrast, dsODN-DPP-IV recovered from nanoparticles remained intact after DNAse I treatment up to 2 U of DNAse I per μg of DNA, which is 4× the amount required to entirely digest naked dsODN (Fig. 4). The protection is considerable and accounts for approximately 70% of the nucleic acid load when using 2 units of DNAse I per μg of dsODN complexed. The assessment of protection has been performed using image analysis software (Vision-Capt software V.15.06, Vilber Lourmat, Paris, France). The ability of these formulations to protect pVax-GLP-1 was also assessed.

Table 1
Size (average) and zeta potential values obtained by dynamic light scattering (DLS) for chitosan/pVax-GLP-1 and chitosan/dsODN-DPP-IV nanoparticles (mean ± s.d.; n = 3). The size on chitosan based nanoparticle is reported as intensity based (average) as calculated by the Malvern Zetasizer Nano series.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Chitosan Size (nm)</th>
<th>Zeta potential (mV)</th>
<th>Polydispersity index (PDI)</th>
</tr>
</thead>
<tbody>
<tr>
<td>pVax1-GLP-1</td>
<td>92–10–5</td>
<td>235 ± 48</td>
<td>32.0 ± 3.4</td>
</tr>
<tr>
<td></td>
<td>80–10–10</td>
<td>163 ± 22</td>
<td>26.7 ± 3.9</td>
</tr>
<tr>
<td></td>
<td>80–80–5</td>
<td>246 ± 30</td>
<td>31.1 ± 1.3</td>
</tr>
<tr>
<td>dsODN-DPP-IV</td>
<td>92–10–5</td>
<td>78 ± 10</td>
<td>18.2 ± 2.0</td>
</tr>
<tr>
<td></td>
<td>80–10–10</td>
<td>87 ± 8</td>
<td>16.2 ± 1.9</td>
</tr>
<tr>
<td></td>
<td>80–80–5</td>
<td>111 ± 18</td>
<td>19.5 ± 2.2</td>
</tr>
</tbody>
</table>
and showed a similar protection profile when compared to dsODN-DPP-IV (Jean et al., 2011).

3.3. Confocal microscopy and FACS analysis of HepG2, Caco-2 and HT-29 dsODN-DPP-IV and pVax1-GLP-1 transfected cell lines

Confocal microscopy data showed that dsODN-DPP-IV was internalized in HepG2, Caco-2 and HT-29 cell lines (Fig. 5A, B, and C). However, HT-29 cells exhibited a reduced internalization rate compared to HepG2 and Caco-2. We also found tha chitosan/dsODN-DPP-IV were internalized within 4 h post transfection (data not shown) and that these nanoparticles were mostly decomplexed by 24 h post-transfection as seen by the lack of colocalization between chitosan and nucleic acids (Fig. 5). Chitosan/dsODN-DPP-IV showed qualitatively similar decomplexation behavior compared to pVax1-GLP-1 nanoparticles (Fig. 5D). These results are consistent with previous observations of chitosan-EGFPLuc plasmid internalization studies (Nimesh et al., 2010). FACS analysis of HepG2 cells showed a high uptake of pVax1-GLP-1 and dsODN-DPP-IV with all formulations tested (Fig. 6). Chitosan nanoparticles were significantly more internalized compared to both negative and positive controls; Lipofectamine™ and DharmaFECT® for pVax1 and dsODN-DPP-IV, respectively.

3.4. Therapeutic protein production following in vitro pVax1-GLP-1 transfection into HepG2 cells

HepG2 cells were used to evaluate in vitro transfection efficiency. Naked (without chitosan) pVax1 and pVax1-GLP-1 both served as negative controls with pVax-1/Lipofectamine™ as positive control to monitor transfection and expression efficiency. Naked pVax1-GLP-1 showed an expression level similar to that of the GLP-1 lacking vector (pVax1) indicative of poor transfection efficiency (~2.5 ng/l GLP-1). Chitosan delivered pVax1-GLP-1 show a considerable increase in GLP-1 expression levels several fold higher than the negative control (Fig. 7). Chitosan-based nanopar-

Fig. 2. ESEM images of spherical chitosan/pVax1-GLP-1 and chitosan/dsDNA-DPP-IV nanoparticles. (A), (B) and (C) chitosan/pVax1-GLP-1 complexed with chitosan 92–10–5, 80–10–10 and 80–80–5, respectively. (D), (E) and (F) chitosan/dsODN-DPP-IV nanoparticles complexed with chitosan 92–10–5, 80–10–10 and 80–80–5, respectively.
articles using the specific 92–10–5 formulation reached transfection levels of 23 ng/l GLP-1 protein in media, similar to the commercially available liposome (Lipofectamine™/positive control). GLP-1 analogues showed a further fivefold increase in GLP-1 concentration to 115 ng/l GLP-1 when compared to the native GLP-1 delivered using chitosan (Fig. 7). Chitosan formulations 80–10–10 and 80–80–5 produced lower GLP-1 expression levels when compared to 92–10–5, most probably due to lower uptake especially for the 80–80–5 formulation; consistent with our FACS data (Fig. 6).

3.5. Inhibition of DPP-IV following in vitro DPP-IV siRNA transfection in HepG2

Chitosan specific formulations – 92–10–5, 80–10–10 and 80–80–5 – were assessed for the delivery of siRNA–DPP-IV and silencing of DPP-IV in HepG2 cells. Quantitative real time PCR showed inhibition of the DPP-IV coding mRNA by the chitosan systems at levels similar to that of the positive control DharmaFECT™ (72% DPP-IV mRNA inhibition) with chitosan 92–10–5 being the most efficient (Fig. 8).

3.6. DPP-IV enzymatic activity in chitosan/siRNA-DPP-IV transfected cell lines

Following demonstration of gene silencing, DPP-IV activity in three different cell lines was assessed using the highly DPP-IV specific Gly-Pro p-nitroanilide hydrochloride enzymatic assay (Fukasawa et al., 1981; Lin et al., 1998). Our results show that DPP-IV activity was reduced to 56% in HepG2 and Caco-2 cell lines with chitosan 92–10–5 achieving the most significant reduction, to levels similar to that of the positive control DharmaFECT™ (Fig. 9). DPP-IV activity reduction in HT-29 cell line by the chitosan systems was however not significant, which could be explained by the low uptake efficiency of these cells as observed by confocal...
microscopy (Fig. 5C), FACS analysis (data not shown), and silencing of DPP-IV enzymatic activity (Fig. 9). Silencing efficiency of DPP-IV mRNA using qPCR showed that HT-29 cell line is difficult to transfect using these specific chitosan/siRNA-DPP-IV formulations (Alameh et al., 2010) in agreement with low uptake and silencing efficiencies observed in this report.

4. Discussion

GLP-1, an incretin hormone secreted by the intestinal endocrine L cells augments insulin release from islet β-cells postprandially
nism of the insulinotropic effects of GLP-1 (Schirra et al., 1998). In infusion of exenatide in healthy humans, with only 53% sequence neutralization and autoimmunity. For example, intravenous a recombinant peptide – have the potential of inducing immune- Moreover, Exenatide – a synthetic xenopeptide – and NN2211 – synthetic peptides produce many side effects including nausea, vomiting, diarrhea and headaches (VanDeKoppel et al., 2008). Although effective, these clinical trials with these compounds indicate that, Exendin-4 and Exendin-4 analogues were constructed downstream of a furin cleavage site resulting in an enhanced substrate cleavage. In general, our results show that chitosan 92–10–5 formulation achieved the highest transfection efficiency in HepG2 cells – a DPP-IV expressing cell line – comparable to the commercially available liposome – Lipofectamine™ – used as a positive control (Fig. 7). Ser8 and Tyr9 modification of GLP-1 yielded a further fivefold increase in GLP-1 concentration likely due to GLP-1 accumulation following half-life extension in agreement with previous reports (Deacon et al., 1998; Ritzel et al., 1998; Siegel et al., 1999). An alternative to synthetic or recombinant peptide administration for the control of T2D is the administration of GLP-1 or DPP-IV resistant GLP-1 analogue encoding genes. Gene therapy approaches have been shown to lower blood glucose levels effectively in murine models of severe T2D (Jean et al., 2011; Choi et al., 2010; Kumar et al., 2007; Lee et al., 2007; Soltani et al., 2007). Although both plasmid and viral based systems have been used to express GLP-1 receptor agonist (GLP-1R), these systems yielded short term expression to date (Kumar et al., 2007; Lee et al., 2007; 2008; Parsons et al., 2007). Recently, double stranded adeno-associated virus vector (dsAAV) has been used for long term delivery of GLP-1 both in vitro and in vivo (Choi et al., 2010). The results of this study showed a long term antiadibeticogenic effect for more than 120 days in db/db mice. However, one drawback to AAV vectors is their low packaging capabilities when compared to other viral or non viral vectors (Duan et al., 2001). Despite their safety, reflected in the lack of T-cell mediated immune priming (Jooss and Chirmule, 2003), recent reports indicate that humoral immune responses to AAV depend on the route of administration and on the promoter used upstream of the gene of interest, thus limiting its reinvigoration (Jooss and Chirmule, 2003).

In this study, we report the first pVax1-GLP-1/GLP-1 analogue – and siRNA-DPP-IV– in vitro delivery using polymer based systems. GLP-1 synthetic substitution of the Alanine (Ala) to serine (Ser) amino acid at position 8 as well as the substitution of glutamine (Glu) to Tyrosine (Tyr) amino acid at position 9 resulted in an enhancement of DPP-IV resistance both in vitro and in vivo as well as an increase in insulin levels in the case of Ser8 in vivo (Deacon et al., 1998; Ritzel et al., 1998; Siegel et al., 1999). For gene therapy purposes, and to our knowledge, we report for the first time the design and the construction of several GLP-1 expression vectors encoding native GLP-1 (7–37) and its mutated DPP-IV resistant analogues Ser8 and Tyr9 (Green et al., 2004; Deacon et al., 1998; Ritzel et al., 1998; Siegel et al., 1999) using site specific mutagenesis for gene therapy purposes. Native GLP-1 and DPP-IV resistant GLP-1 analogues were constructed downstream of a furin cleavage site resulting in an enhanced substrate cleavage. In general, our results show that chitosan 92–10–5 formulation achieved the highest transfection efficiency in HepG2 cells – a DPP-IV expressing cell line – comparable to the commercially available liposome – Lipofectamine™ – used as a positive control (Fig. 7). Ser8 and Tyr9 modification of GLP-1 yielded a further fivefold increase in GLP-1 concentration likely due to GLP-1 accumulation following half-life extension in agreement with previous reports (Deacon et al., 1998; Ritzel et al., 1998; Siegel et al., 1999) (Fig. 7). Our FACS data demonstrated no significant differences in uptake efficiency between tested nanoparticles using different chitosans to deliver either pVax1-GLP-1 or DPP-IV ODN (Fig. 6). This result of high cell uptake with all chitosans (Fig. 6) tested but much higher protein expression using the specific 92–10–5 formulation (Fig. 7) is consistent with our recent confocal live-cell imaging study (Thibault et al., 2010) and isotothermal titration calorimetry study (Ma et al., 2009) showing that an intermediate stability of chitosan-nucleic acid binding is achieved by this 92–10–5 system, leading to optimal intracellular release characteristics not found with other systems. These findings are also consistent with previous in vivo studies showing that an intermediate stability of chitosan-nucleic acid binding is achieved by this 92–10–5 system, leading to optimal intracellular release characteristics not found with other systems. These findings are also consistent with previous in vivo
assessments showing higher GLP-1 and growth factor expression when using chitosan 92–10–5 as a delivery system (Jean et al., 2009, 2011). Confocal microscopy of pVax1-GLP-1 transfection/release kinetics here also reveals higher transfection/release efficiency of the 92–10–5 nanoparticles (Fig. 5) when compared to 80–10–10 and 80–80–5 nanoparticles. Transfection efficiency was shown previously to be dependent on chitosan DDA, Mn and N:P ratio (Lavertu et al., 2006; Koping-Hoggard et al., 2004; Strand et al., 2010) consistent with our findings here. Mechanistically, higher DDA results in increased degradation ability of chitosan thus a better protection of pDNA against nucleases whereas complex instability can occur at low Mn and/or DDA due to reduced binding affinity (Ma et al., 2009). However lower DDA or Mn can also permit DNA dissociation from chitosan, as required to access transcriptional machinery (Strand et al., 2005; Strand et al., 2010; MacLaughlin et al., 1998). The N:P ratio plays an important role in transfection efficiency where it is believed that excess polyca-

In transfection efficiency where it is believed that excess polycations in solution are responsible of the endosomal escape via the proton sponge effect (Boeckle et al., 2004; Clamme et al., 2003). Although, controversial, the exact mechanism of endosomal escape remain elusive with a mandatory requirement for free chitosan to achieve efficient transfection. In this perspective, Thibault et al., (2011), tested the hypothesis that low transfection efficiency at low N:P ratios (<2) can be rescued to high transfection efficiency by the addition of free chitosan (Thibault et al., 2011). Indeed, the addition of free chitosan rescued low transfection efficiency in Human Embryonic Kidney cells (HEK293) and found that N:P ratios above 5 do not enhance cell uptake or endosomal escape (Thibault et al., 2011).

Nanoparticle formulations used in this study to efficiently deliver GLP-1 or siRNA–DPP-IV therefore combined intermediate to high DDA levels – 80% and 92%– with low to intermediate molecular weights – 10 and 80 kDa – with most favorable N:P ratios to achieve an optimal intermediate stability profile, simultaneously providing nuclease protection and intracellular disassembly for efficient endosomal escape and high transfection efficiencies.

One important feature of gene delivery systems is the ability to condense/release nucleic acid sequences and protect them from the extracellular matrix nuclease rich environment during nanoparticle residence time at the cell surface. dsODN–DPP-IV were protected from nuclease degradation for up to 2 U of DNAseI/μg of DNA for all chitosan formulations described in this report (Fig. 4). pVax1-GLP-1 protection showed an identical profile to dsODN nanoparticles (Jean et al., 2011). Additionally, and in contrast to a previous study by Liu et al. (2007), our results show that low Mn chitosan (10 kDa) can effectively complex and compact dsODN into stable particles (Figs. 3 and 4). The discrepancy observed between these two studies is explained by our data comparing pH of 6.5–8 in our study and the use of pH 7.9 only in Liu’s report (Liu et al., 2007). Complexation between chitosan and siRNA or dsODN depends on electrostatic interactions between the polyanion and polycation species; a pH near chitosan pKa of 6.5 results in sufficient charge density for siRNA binding consequently explaining the observed increase in stability compared to pH 8. Temporal stability is another important feature of an efficient gene delivery system. Complexation of chitosan to dsODN–DPP-IV showed a temporal stability at pH 6.5 for a minimum up to 20 h post complexation at room temperature. However at pH 8, as used in previous publications, we found complexes to be unaltered, at N:P ratio of 1 and above, most probably due to chitosan deprotonation.

Transfection of three DPP-IV expressing cell lines using the aforementioned chitosans to complex siRNA targeting the DPP-IV mRNA resulted in similar cell uptake as observed with pVax1-GLP-1 when used for therapeutic protein production nanoparticles. In both cases, chitosan formulation 92–10–5 was most efficient in terms of GLP-1 expression and DPP-IV silencing capability (Figs. 7–9). DPP-IV ODN showed high cell uptake, nearly identical to the pVax1-GLP-1 profile as determined by flow cytometry. Silencing efficiency was also highest when using chitosan 92–10–5, supporting the notion that efficient nucleic acid delivery requires a fine balance between protection and release of cargo following internalization (Nimesh et al., 2010). Here we found HT-29 cells to exhibit low uptake and low silencing, pinpointing a cell line dependency of uptake (Mao et al., 2001; Douglas et al., 2008) (Figs. 6–8). Differences in transfection efficiencies between HepG2, Caco-2 and HT-29 cell line are probably due to different internalization mechanisms and intracellular trafficking of the vectors. Transfection efficiency has also been shown to be dependent on different endocytic pathways (Douglas et al., 2008); supporting our interpretation of low uptake and gene silencing efficiency in HT-29 cells.

### Table 2
Safety and performance criteria’s for the development of effective non-viral gene delivery systems.

<table>
<thead>
<tr>
<th>Categories</th>
<th>Performance criteria¹ ¹</th>
</tr>
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<tbody>
<tr>
<td>Physical and chemical properties</td>
<td>Reproducible assembly of functional complexes at 10 mg nucleic acid scale</td>
</tr>
<tr>
<td></td>
<td>Assemblies less than 300 nm, PDI (&lt;0.3) and no less than 80% incorporation efficiency</td>
</tr>
<tr>
<td></td>
<td>No Aggregation in 50% mouse/human serum</td>
</tr>
<tr>
<td></td>
<td>Chemical stability of the assembly for &gt;30 days</td>
</tr>
<tr>
<td></td>
<td>Preferably amenable to freeze-drying without any loss of its performance criteria’s</td>
</tr>
<tr>
<td></td>
<td>&gt;50% reduction in target mRNA by target specific siRNA at concentrations &lt;100 nm in 10% serum containing media</td>
</tr>
<tr>
<td></td>
<td>&lt;10% reduction target mRNA by control siRNA at concentrations &lt;10 nm in 10% serum containing media</td>
</tr>
<tr>
<td></td>
<td>Expression of the construct in the case of transgene delivery using plasmid DNA as a backbone vector</td>
</tr>
<tr>
<td></td>
<td>&gt;5-fold window between target gene silencing/transgene expression ICSO and ICSO for reduction in viability</td>
</tr>
<tr>
<td></td>
<td>Activity at least relevant 3 cell lines to the delivery system under evaluation</td>
</tr>
<tr>
<td></td>
<td>&gt;50% reduction in target mRNA levels by target siRNA and &lt;10% reduction in target mRNA levels in target issue at 1 mg/kg dose by control siRNA by 24–48 h</td>
</tr>
<tr>
<td></td>
<td>Demonstration of RNAi-mediated target mRNA cleavage by 5’-RACE</td>
</tr>
<tr>
<td></td>
<td>&lt;10-fold cytokine induction (TNFα, IFNγ, IL6) and &lt;10-fold increases in ALT and AST at 3 mg/kg siRNA dose</td>
</tr>
<tr>
<td></td>
<td>No effect on body weight and normal blood clinical chemistry and hematology data</td>
</tr>
<tr>
<td></td>
<td>Lack of immunogenicity and neutralizing antibodies against pDNA expressed construct following plasmid based nanoparticle injection</td>
</tr>
<tr>
<td></td>
<td>Lack of auto-antibodies and anti-DNA antibodies following plasmid based nanoparticle injection</td>
</tr>
<tr>
<td></td>
<td>Absence of genomic integration following plasmid based nanoparticle injection</td>
</tr>
<tr>
<td></td>
<td>No effect on body weight and normal blood clinical chemistry and hematology data</td>
</tr>
</tbody>
</table>

¹ If the delivery platform incorporates a targeting moiety evidence should be provided for targeting by (a) using cell line with differential expression of targeted receptor and (b) using assemblies with “active” and “inactive or mutant” targeting moieties.

¹¹ Performance criteria’s described here are both for siRNA and plasmid DNA delivery systems. These criteria’s were taken from Merk Inc and the FDA.
Chitosan 92–10–5 reduced DPP-IV mRNA levels by ∼72% in HepG2 cells (Fig. 8) resulting in a ∼56% decrease of DPP-IV enzymatic activity 48 h post-transfection (Fig. 9). The observed reduction in DPP-IV enzymatic activity may be explained by the fact that wild type DPP-IV has a half life of approximately 50 h (Fan et al., 1997). DPP-IV is a ubiquitously expressed serine protease playing a major role in protein processing, thus its complete inhibition may be deleterious for future therapies of chronic diseases such as T2DM. Taken together, our study demonstrates clearly that specific chitosan nucleic acid formulations can achieve high levels GLP-1 expression and DPP-VI silencing in vitro, suggesting promise for a combined delivery of both GLP-1 and siRNA-DPP-IV to enhance glycemic control in type 2 diabetes. Although in vitro and in vivo performance criteria’s requirement differs no consensus on such performances is found in the literature. For in vivo performance, safety remains the major issue with available guidance’s from the FDA for the development of gene and cell therapy products (CBER, 2007) therefore the development of non-viral drug delivery systems for in vivo use should take into account physico-chemical criteria’s, cell based assay criteria’s and most importantly in vivo performance and safety criteria’s (Table 2). Our system meets many of these criteria’s and has already been demonstrated efficient in vivo (Jean et al., 2009, 2011). However a thorough characterization of safety and performance criteria’s should be performed before preclinical studies.

5. Conclusion

In this report we have shown the ability of specific chitosan formulations to deliver GLP-1 analogues and DPP-IV targeting siRNA, both of which can contribute to increased bioavailability of GLP-1. Nanoparticles formed by chitosan binding to these nucleic acids were spherical with a size range between 64–283 nm being larger for plasmid DNA compared to dsODN based complexes. These specific chitosan formulations efficiently protected and delivered nucleic acid into cells, with DPP-IV resistant GLP-1 analogues reaching 115 ng/L in media; a fivefold increase when compared to the native GLP-1. In addition, DPP-IV gene silencing using chitosan formulation 92–10–5 reached levels similar to those of the commercially available lipoplex DharmaFECT®, but with the advantage of reduced toxicity. The versatility of these specific formulations to deliver plasmid DNA and siRNA render their use promising as a combined in vivo therapy for the control of type 2 diabetes.

Acknowledgements

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