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Comparative study of nanoparticle-mediated transfection in different GI epithelium co-culture models

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Abstract

Oral nonviral gene delivery is the most attractive and arguably the most challenging route of administration. To identify a suitable carrier, we studied the transport of different classes (natural polymer, synthetic polymer and synthetic lipid-polymer) of DNA nanoparticles through three well-characterized cellular models of intestinal epithelium (Caco2, Caco2-HT29MTX and Caco2-Raji). Poly(phosphoramidate-dipropylamine) (PPA) and Lipid-Protamine-DNA (LPD) nanoparticles consistently showed the highest level of human insulin mRNA expression and luciferase protein expression in these models, typically at least three orders of magnitude above background. All of the nanoparticles increased tight junction permeability, with PPA and PEI having the most dramatic transepithelial electrical resistance (TEER) decreases of ($35.3 \pm 8.5\%$) and ($37.5 \pm 1.5\%$) respectively in the first hour. The magnitude of TEER decrease correlated with nanoparticle surface charge, implicating electrostatic interactions with the tight junction proteins. However, confocal microscopy revealed that the nanoparticles were mostly uptaken by the enterocytes. Quantitative uptake and transport experiments showed that the endocytosed, quantum dot (QD)-labeled PPA-DNA nanoparticles remained in the intestinal cells even after 24 h. Negligible amount of quantum dot labeled DNA was detected in the basolateral chamber, with the exception of the Caco2-Raji co-cultures, which internalized nanoparticles 2 to 3 times more readily compared to Caco2 and Caco2-HT29MTX cultures. PEGylation decreased the transfection efficacy by at least an order of magnitude, lowered the magnitude of TEER decrease and halved the uptake of PPA-DNA nanoparticles. A key finding was insulin mRNA being detected in the underlying HepG2 cells, signifying that some of the plasmid was transported across the intestinal epithelial layer while retaining at least partial bioactivity. However, the inefficient transport suggests that transcytosis alone would not engender a significant therapeutic effect, and this transport modality must be augmented by other means in vivo to render nonviral oral gene delivery practical.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jconrel.2012.01.041.

Keywords

Oral gene delivery; Nonviral gene transfer; DNA nanoparticle; GI epithelium models; Caco2; Transcytosis

1. Introduction

Oral nonviral gene delivery is especially attractive, due to the ease of administration and greater patient compliance. It is also arguably the most challenging route of administration. The complex transport barriers, the extreme pH conditions and the presence of multiple enzymes in the gastrointestinal tract (GI) conspire to render the delivery of an intact nucleic acid extremely challenging [1,2]. There is thus a significant need to identify and optimize better gene carriers for oral gene delivery. As a first step towards this goal we conducted a systematic in vitro study of DNA nanoparticle uptake and transport in relevant intestinal epithelium cell culture models.

In addition to endocytosis by GI enterocytes, DNA nanoparticles can traverse the intestinal epithelium via paracellular and transcellular (transcytosis) pathways [3]. Specialized cell culture constructs in which intestinal epithelial cells are cultured on a transwell membrane and induced to differentiate to a continuous cell layer separating two compartments, are used to quantify the transport of orally administered biotherapeutics and deduce possible pathways. The differentiated Caco2 cells, Caco2-HT29MTX and Caco2-lymphocyte co-cultures are well-characterized cellular models of normal intestinal epithelium and M-cells respectively. The homogeneous Caco2 transwell culture is the simplest in vitro model used to elucidate the transport of drugs and particles from the apical to basolateral surface [4,5]. Consisting solely of absorptive cells linked by tight junctions to form an impermeable cell sheet, it mimics the polarity of the intestinal epithelium and exhibits the key drug and nutrient transporters. The addition of mucus-secreting HT29MTX cells more closely mimics the in vivo environment, where enterocytes are interspersed by goblet cells, disrupting the tight junctions. The mucus secreted on the apical surface forms an additional barrier and may entrap nanoparticles, hindering their uptake or enhancing their retention. Thus, the Caco2-HT29MTX is in some ways a more relevant model [6,7]. Lastly, the M-cell model consists of Raji lymphocytes added to differentiate the Caco2 monolayer into a distinctive phenotype specialized for the uptake of antigens and particulate matter [8,9]. Due to its relevance to oral vaccination, this model has been used in several comparative studies to study microparticle uptake. In this study we seek to compare the DNA nanoparticle uptake, transport and transfection efficacy in all three models.

By applying the in vitro models to the transport of DNA nanoparticles, this study aims to identify the best nanoparticle formulation for oral gene delivery and deduce the fate of the nanoparticles following intestinal epithelial uptake. As a prelude to understanding how the composition of the gene carrier affects uptake, three different classes of gene carrier are chosen: natural polymer (chitosan), synthetic polymer (poly(phosphoramidate-dipropylamine and polyethyleneimine), and synthetic lipid-polymer complex. Chitosan nanoparticles have been previously successfully used in oral gene delivery [10] and vaccination [11]. It is particularly attractive as it is mucoadhesive and enhances paracellular permeability [12,13] by opening the tight junctions between epithelial cells. PPA is a carrier that has proven to be as effective as Lipofectamine and PEI in various cell lines but with lower cytotoxicity [14]. PEI served as the positive control, although it proved to be too cytotoxic to the enterocytes. Lipid-Protamine-DNA (LPD) nanoparticles have been used extensively in vitro and in vivo with great success to deliver DNA [15] and siRNA [16] systemically. In addition, the PEGylated analogs of chitosan, PPA [17] and LPD are also

evaluated. Although PEGylation enhances colloidal stability by preventing aggregation, it diminishes cellular uptake. It is important to study how PEG chains will affect uptake and transport of nanoparticles.

Oral gene delivery is also unique as the polarity of the GI enterocytes implies that they can secrete the transgene product via the apical and/or basolateral surface. For an optimal therapeutic effect systemically, basolateral secretion into the underlying capillaries is preferred. Human insulin is used as a model therapeutic gene to evaluate the benefits of different modifications to the plasmid, and then to determine the directionality of protein secretion. The oral delivery of insulin plasmid is attractive for the therapy of diabetes mellitus, as constitutive background level of insulin secretion can satisfy basal needs, supplementing insulin injections that meet the postprandial demand [18,19]. As the gene expression is expected to be low, there is a need to work with the most potent plasmid in order to achieve the best results in vivo. Consequently, effort is expended to optimize the insulin plasmid in this study.

This study compares the efficacy of some of the most efficient gene delivery systems available in the field, in widely accepted in vitro GI epithelium models. In doing so, it hopes to shed light on the fate of DNA nanoparticles and thus facilitate future work in designing efficient non viral oral gene delivery systems.

2. Materials and methods

2.1. Materials

2.1.1. Cell lines—The human colon carcinoma Caco2, Burkitt's lymphoma (B lymphocyte) Raji and HepG2 cell lines were obtained from the American Type Culture Collection (ATCC) (Rockville, MD). The mucus-secreting colorectal adenocarcinoma HT29MTX [20] was a gift from Dr. Thécla Lesuffleur (INSERM U843, Paris, France). Caco2 cells from passages 32 to 45, HT29MTX cells from passages 12 to 16 and HepG2 cells from passages 81 to 86 were used for the transport studies.

2.1.2. Cell culture reagents and chemicals—Eagle's minimum essential media with 2 mM L-glutamine and Earle's BSS, supplemented with 20% fetal bovine serum and 1% penicillin–streptomycin, was used to culture Caco2 cells; Eagle's minimum essential media with 2 mM L-glutamine and Earle's BSS, supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin, was used to culture HT29MTX cells; Raji cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, 1% penicillin–streptomycin and glucose. All cell culture supplies were purchased from Gibco™ Invitrogen (Carlsbad, CA).

High molecular weight chitosan of 390,000 Da and 83.5% deacetylation was a gift from Vanson Chemicals (Redmond, WA). N-hydroxyl succinimide polyethylene glycol (NHS-PEG) was purchased from Nektar Therapeutics (Huntsville, AL). Poly(phosphoramidate-dipropylamine) (PPA, Mw = 41,000) and 56% grafting efficiency and PPA-PEG (Mw = 51,000) were synthesized as previously described [21]. The cationic lipid and protamine was a gift from Dr. Leaf Huang (University of North Carolina, Chapel Hill, NC). Branched polyethyleneimine (PEI) of 25,000 Da was purchased from Sigma (St Louis, MO).

2.2. Methods

2.2.1. Plasmid construction—A series of plasmids expressing human insulin was used to elucidate the factors that impact gene expression of insulin. Three different insulin gene sequences were evaluated: native insulin (hINS), insulin with furin cleavage mutation (hINSFurG) and codon-optimized insulin with furin cleavage mutation (hINSFur). The native insulin gene (hINS) was purchased from Origene. Insulin gene with the furin

cleavage mutation (hINSFurG) was a gift from Genentech (San Francisco, CA) [22]. Codonoptimized insulin with the furin cleavage mutation (hINSFur) was designed and synthesized by GeneART Inc. (Regensburg, Germany). All three open reading frames were cloned into the Clontech pEGFP-C1 vector via PCR, using primers with NheI and XhoI sites. In the process, the GFP open reading frame was excised. The resulting plasmids were pC_hINS, pC_hINSFurG and pC_hINSFur. The hINSFur construct was also cloned into pCA_Luc using PCR primers with SalI and EcoRV sites. The resulting plasmid pCA_hINSFur was then digested with MscI and XmnI, treated with Klenow enzyme and blunt-ligated with the minicircle precursor p2phiC31. The final construct pMCA_hINSFur was then obtained by amplifying the resulting product as previously described [23].

2.2.2. Cell culture—Caco2 cells were expanded as per recommended by ATCC. Upon reaching 80% confluence, Caco2 cells were harvested by trypsinization, washed and resuspended in culture medium. 3×10^5 cells were then seeded onto the upper chamber of a transwell (6.5 mm diameter, 3.0 μ m pore-size membrane) (Costar, Cambridge, MA). All transport experiments were performed using transwells of polyester (PE) membranes.

The Caco2-HT29MTX co-culture protocol was adapted from [6]. Briefly, Caco2 cells and HT29-MTX cells were seeded onto the upper chamber of the transwell in a 9:1 ratio, as per the ratio of epithelial cells to goblet cells in vivo [24]. The cells were cultured for 14–16 days with media changes on alternate days using Caco2 and HT29MTX media in a 1:1 ratio. The Caco2-Raji co-culture protocol was adapted from Pringaul et al. [8]. After 14–16 days of growth, Raji B lymphocytes were added at a concentration of 10^6 cells per well to the lower chamber of the transwell. The co-culture was maintained for the next 3 days in Caco2 and Raji media in a 2:1 ratio.

For the insulin mRNA expression studies, 5×10^5 HepG2 cells were cultured in a separate 24-well plate and the co-culture was assembled just prior to transfection (Fig. 1). For luciferase activity studies, 1×10^5 HEK293 cells were used in lieu of HepG2.

2.2.3. Preparation and characterization of polymer–DNA nanoparticles—

Chitosan nanoparticles were formed as previously described in Leong et al. [25]. Briefly, 10 μ g of DNA was added to 100 μ L of 50 mM sodium sulfate and subsequently mixed with 100 μ L of chitosan solution under high-speed vortexing. The reaction was conducted at 55 °C. Chitosan–DNA nanoparticles were PEGylated by reacting with 4 μ L of 5 mM NHS-PEG for an hour at pH7.4. PPA–DNA, PPA–PEG–DNA and PEI–DNA nanoparticles were similarly synthesized by adding 100 μ L (containing 800 μ g) of polymer to 100 μ L (10 μ g) of DNA under high-speed vortexing. Lipid–Protamine–DNA nanoplexes were formed by vigorously mixing Lipid–Protamine with DNA as previously described [15]. All the nanoparticle formulations and their PEGylated analogs have been optimized in previous studies. The sizes and zeta-potentials of the nanoparticles were measured using a Zetasizer-3000 (Malvern Instruments, Southborough, MA).

2.2.4. Transfection assays—2 μ g of DNA was added to each well, in the form of polymer–DNA nanoparticles. Briefly, the cell culture media in the apical and basolateral chamber of the transwell was replaced with OptiMEM reduced serum medium, and nanoparticle suspension in PBS was added to the apical chamber for 4 h at 37 °C. The OptiMEM was then aspirated and replaced with cell culture media. Media changes were performed 10 h, 2, 4 and 6 days post-transfection. For the mRNA expression studies, the cells were lysed at days 2 and 6 post-transfection, and the mRNA was extracted using the Qiagen RNeasy Kit (Valencia, CA). The insulin mRNA expression was ascertained using a one-step reverse transcriptase polymerase chain reaction, RT-PCR, kit from Qiagen (Valencia, CA). For the protein expression assays, the cells were lysed using Glo Lysis

Buffer (Promega, Madison, WI). 50 μ L of the cell lysate was reacted with 50 μ L of Bright Glo reagent (Promega, Madison, WI) and the luminescence was measured by a plate reader (BMG Labtech, Cary, NC). The luminescence reading was then normalized to the total protein content of each sample, as determined using a BCA Assay (Pierce).

2.2.5. Transmembrane permeability studies—The transepithelial resistance (TEER) across the intestinal epithelium is a measure of paracellular ion movement. A drop in TEER is interpreted as an increase in tight junction permeability. The TEER of each cellular insert was determined by means of a voltohmmeter—EVOM manufactured by World Precision Instruments (Sarasota, FL). All transport studies were conducted after equilibration to room temperature.

2.2.6. Transport of nanoparticles into and across intestinal epithelia—In order to track the nanoparticles, DNA was labeled with 605 nm quantum dots (Invitrogen, Carlsbad, CA) prior to nanoparticle formation. 5 μ L of quantum dots was diluted 10 fold and reacted with biotinylated plasmid for 10 min at room temperature. The labeled DNA was then used to form nanoparticles as described above. Nanoparticle suspension containing 5 μ g of DNA was added to each type of intestinal epithelial transwell culture. At various time points, media was collected from the basolateral chamber, while the intestinal epithelial cells were washed with PBS and lysed. The cell lysate and media samples were analyzed using inductively-coupled plasma mass spectrometry. The cadmium content of the samples was used as a measure of quantum dots (and hence nanoparticles) uptaken by the cells, and transcytosed into the basolateral chamber.

2.2.7. Immunohistochemistry—Nanoparticle suspension containing 605 nm quantum dot labeled DNA was added to each type of intestinal epithelial transwell culture. After 4 h, the cells were fixed in 4% formaldehyde. The cellular inserts were washed in PBS thrice, and 10% normal goat serum was added to suppress non-specific binding of antibodies for 15 min. Tight junctions were visualized with FITC-conjugated mouse monoclonal anti-ZO-1 antibody (Zymed, San Francisco, CA). Cell nuclei were also stained using DAPI (Molecular Probes Invitrogen, Carlsbad, CA). The intestinal epithelial monolayer and transwell membrane were separated from the insert and mounted on cover glass for visualization with an LSM 510 inverted confocal microscope (Carl Zeiss, Thornwood, NY).

2.2.8. Transmission electron microscopy—Nanoparticles used for transmission electron microscopy (TEM) transport studies were synthesized using pGeneGrip Gold 10 nm colloidal gold-conjugated plasmid DNA (Genlantis, San Diego, CA). 9×10^6 Caco2 cells were seeded in 35 mm tissue culture dishes and cultured for 16 days prior to transfection. Prior to transfection, the culture medium was changed to OptiMEM. A 6 μ g DNA dose of nanoparticles was added for 4 h at 37 °C, then fixed in a 4% formaldehyde, 2% glutaraldehyde solution overnight at 4 °C. The cells were post-fixed in 1% osmium tetroxide and stained en bloc with 0.5% uranyl acetate, then dehydrated with a graded series of ethanol. Samples were embedded in Polybed 812 resin, thin-sectioned, and picked up on 150 mesh copper grids. After further staining with 1% uranyl acetate and 0.4% lead citrate, grids were examined on a FEI Tecnai G² Twin instrument under 80 kV.

3. Results

3.1. Transgene expression of optimized insulin plasmid constructs

A mutated version of insulin where cell-specific proconvertase enzymatic sites were replaced with ubiquitous furin cleavage sequences, approximately doubled the secretion of mature insulin by Caco2 cells (Fig. 2A). The increase in insulin levels could be attributed to

improved proteolytic conversion of proinsulin via constitutive secretory pathways. Codon optimization of the mutant gene did not have a significant impact on protein expression. The inclusion of the cytomegalovirus (CMV) intron A enhancer resulted in a 25-fold increase in insulin expression initially (Fig. 2B), after which the plasmid construct pC_hINSFur had baseline values of insulin expression. With the intron A enhancer, pCA_hINSFur still retained detectable levels of insulin secretion a fortnight post-transfection.

The minicircle constructs improved initial gene expression 5-fold in the absence of intron A, for a given molar concentration of plasmid (Fig. 2B). In the presence of intron A, the minicircle did not have much of an advantage initially, though expression levels were more than double that of pCA_hINSFur at 2 weeks. Comparing minicircle constructs with and without intron A, the enhancer only increased insulin secretion by 3.5 to 6-fold in the first week. Although the improvement in insulin gene expression by the minicircle was less significant compared to other proteins reported, the constructs conferred additional advantages by minimizing gene silencing and also enabled a higher molar dose of plasmid to be delivered for a given mass of DNA. The latter enabled less gene carrier to be used, resulting in cost savings as well as reduced carrier-associated toxicity. All p-values were less than 0.001.

pMCA_hINSFur showed the highest secreted insulin expression and the interplay of the different plasmid elements was nonadditive. Increasing the amount of plasmid administered did not result in a linear amplification in protein expression. In the Caco2 transwell model (in the absence of the underlying HepG2 cells), insulin secretion by the enterocytes was bi-directional (data not shown), as observed by the comparable amounts of insulin detected in the apical and basolateral chambers.

3.2. Biophysical characteristics of polymer–DNA nanocomplexes

As shown in Table 1, the hydrodynamic diameters of non-PEGylated nanoparticles were approximately 120 nm, while the PEG versions were slightly larger. In general, PEGylation decreased the surface charge. Since the size of nanocomplexes synthesized from different polymers was comparable, differences in transport characteristics could be attributed to functional groups or surface properties.

3.3. Transfection efficacy of polymer–DNA nanocomplexes on intestinal cells

Based on statistical (two-way ANOVA) analysis of the data, the carrier, culture system and interaction of the two are important parameters influencing transgene expression. Among all the gene carriers screened, PPA and LPD consistently had the highest level of human insulin mRNA expression in all three models at 2 and 6 days post-transfection (Fig. 3A). This was further verified by protein expression using the luciferase reporter assay (p-values <0.001 compared to background and chitosan) (Fig. 3B). LPD resulted in higher gene expression 2 days post-transfection, but PPA had 2-fold higher luciferase activity at days 6 and 10 (Figure S1). PEGylation decreased the transfection efficacy for PPA and LPD by at least an order of magnitude. Chitosan–PEG was the exception, suggesting that conjugation of PEG to free amines on the chitosan destabilized the complex, permitting more efficient unpacking [26,27]. The positive control PEI proved to be too cytotoxic, as reflected by the negligible amounts of total mRNA collected.

Comparing different cell culture systems, it was not possible to conclude any statistical significance in luciferase expression in Caco2 and Caco2-Raji culture systems. However, Caco2-HT29MTX culture had significantly lower luciferase activity (p-values <0.01).

3.4. Transport of polymer–DNA nanocomplexes across intestinal cells and gene expression in underlying HepG2 cells

Human insulin mRNA expression was detected in all the underlying HepG2 cells (Fig. 3A), implying that intact plasmid DNA transcytosed across the intestinal epithelium and retained at least partial bioactivity. This key finding corroborates literature findings in vivo [10] where the gene of interest was detected in the liver. The insulin mRNA expression levels in HepG2 cells shared the same trend as the Caco2 monolayer. PEGylated nanoparticles also showed lower expression levels, which signify decreased transcytosis as a result of PEG shielding. Although the mechanism of transport is unknown, the transfection efficacy in underlying HepG2 cells appears to correlate with the magnitude of TEER change, as well as uptake and expression in the Caco2 monolayer (Fig. 4A). When the luciferase minicircle plasmid was used for gene delivery, LPD and LPD-PEG appeared to be the best gene carriers for inducing gene expression in underlying HepG2 cells. However, in general, luciferase expression in the underlying HEK293 cells was too low for meaningful conclusions to be drawn.

All the nanoparticles increased tight junction permeability (Fig. 4A), with PPA and PEI having the most dramatic TEER decreases of $(35.3 \pm 8.5)\%$ and $(37.5 \pm 1.5)\%$ respectively in the first hour. While the tight junction modulating capabilities of chitosan have been well studied [28,29], this is the first study of DNA nanoparticles showing the same effects. The drop in TEER was not due to cytotoxicity of PPA as determined by the WST-1 assay (Fig. 4B). However, the TEER decline for PEI can be ascribed to the leaky nature of the monolayer resulting from the death of 25% of the cells at the dose of $0.1 \mu\text{g}/\mu\text{L}$ of PEI delivered. PEGylation decreased the magnitude of TEER change, implicating that PEG might shield interactions with tight junction components. Such interactions were most likely electrostatic in nature, since the magnitude of TEER decrease correlated with nanoparticle surface charge. LPD nanoparticles, despite having the highest zeta potential, were outliers possibly because the lipid content modulated interactions with the cell membrane.

3.5. Transport of quantum dot (QD) and gold-labeled nanocomplexes into and across intestinal cells

Using QD to label the DNA in the nanoparticles, the intracellular localization of the nanoparticles can be studied using confocal microscopy. The cadmium content present in QD (as detected by mass spectrometry) can also be used as an indirect measure of the amount of DNA present in different compartments at various timepoints. Transmission electron microscopy shows that the nanoparticles internalized by the epithelial monolayer remain intact within the cell.

The QD-labeled nanoparticles were mostly internalized by the Caco2 cells and did not co-localize with the ZO-1 staining (Fig. 6A). This suggests that the positively charged nanoparticles did not strongly interact with the tight junction proteins. Thus, the presence of bioactive plasmid in the underlying HepG2 cells is most likely attributed to transcytosis. To complement the confocal microscopic observations and to further elucidate the trafficking mechanism of nanoparticles through the epithelium, TEM of gold-labeled nanoparticles revealed an abundance of intact particles throughout the intracellular compartment of Caco2 cells (Fig. 5). In accordance with the insulin transgene and luciferase expression results, delivery of PPA resulted in the greatest number of internalized nanoparticles, whereas PEGylation of PPA reduced both aggregation and internalization. Relative to PPA, uptake of chitosan nanoparticles was significantly diminished.

Mass spectrometry analysis (Fig. 6B) of the cells and the media in the basolateral chamber showed that the endocytosed labeled PPA–DNA nanoparticles remained internalized by the

intestinal cells, even after 24 h. Negligible amount of cadmium in the basolateral chamber, with the exception of the Caco2 Raji co-cultures (Fig. 6C), demonstrated that very few plasmids either free or in the form of nanoparticles, were transported to the basolateral chamber. Hence, paracellular transport of the nanoparticles did not occur despite the increase in transmembrane permeability. Caco2-Raji cultures internalized nanoparticles 2 to 3 times more readily compared to Caco2 and Caco2-HT29MTX cultures. PEGylation decreased the uptake of PPA-DNA nanoparticles by at least a factor of 2. However, transcytosis was more efficient for PPA-PEG nanoparticles.

4. Discussion

The homology of the polarized Caco2 cell culture model to human intestinal enterocytes makes it an attractive in vitro model to evaluate intestinal transport [30,31]. It is a homogeneous cell system consisting solely of absorptive cells linked by tight junctions, forming an impermeable cell sheet. In vivo, the intestinal epithelium is often interspersed by mucus-secreting goblet cells, which disrupt the tight junctions. However, the mucus on the apical surface of the cell sheet adds an additional transport barrier. In order to mimic this, mucus-producing HT29MTX cells are added to Caco2 in a co-culture model. A 9:1 ratio is used in this study, since goblet cells make up approximately 10% of the epithelial cells in the human gut. A third intestinal cell culture model is also evaluated based on its similarity to the M-cells in Peyer's patches. The infiltration of lymphocytes into the Caco2 monolayer triggers the differentiation into an M-cell phenotype. Since M-cells are specialized for transport of antigens and intact nano- and microparticles to the underlying immune cells, this is an appropriate model for studying transcytosis of particulate therapeutics. Caco2-Raji cultures uptake nanoparticles two to three times more readily compared to Caco2 and Caco2-HT29MTX cultures. Furthermore, Caco2-Raji co-cultures indirectly demonstrate transcellular transport of the QD conjugated plasmid. This corroborates previous studies in which the transcytosis rate across Caco2 cell layer in the absence of lymphocytes is extremely low [9] and in which the M-cell model demonstrates higher transcytosis rates of approximately five times more chitosan-DNA nanoparticles across the cell layer compared to normal enterocytes [32]. Although the uptake of nanoparticles by Caco2-Raji is significantly higher than Caco2 and Caco2-HT29MTX cultures, luciferase protein expression in the former is comparable to Caco2 and significantly higher ($p < 0.01$) than Caco2-HT29MTX 2 days after transfection. This suggests that mucus secretion is an obstacle to efficient transfection, hindering nanoparticle uptake. Furthermore, although the Caco2-Raji cultures are capable of transcytosing the nanoparticles, expression of insulin mRNA in the underlying HepG2 layer is much lower than Caco2 and Caco2-HT29MTX. This may be attributed to the function of Peyer's patches in breaking down particulate matter and presenting the debris to underlying immune cells. Thus targeting M-cells for nanoparticle-mediated gene delivery not ideal. Furthermore, intact nanoparticles were detected in and around vesicles in the intracellular domain of enterocytes by TEM, indicating that particles internalized from the apical surface remain available for exocytosis to the basolateral compartment. Generally, higher rates of uptake by Caco2 cells corresponded to increased transport to the basolateral chamber, presumably via transcytosis, and transgene expression. It is also interesting to note that although PPA nanoparticles are more readily uptaken, PPA-PEG nanoparticles are more readily transported across the monolayer. This perplexing phenomenon has also been observed in vivo [33]. We postulate that since transport is size-dependent, PEGylated nanoparticles are less likely to aggregate and thereby favoring transcytosis.

Using the above cell culture models, this study demonstrates that the bulk of the nanoparticles uptaken will remain in the intestinal epithelial cells. This result implies that enterocytes will most likely produce the gene of interest delivered orally. Fortunately, in the

case of insulin, secretion, as dictated by the signal sequence of the protein, is bi-directional. Thus the insulin secreted into the basolateral space can diffuse into the lamina propria and from there into the underlying capillary or lymph vessels. Since insulin is not normally produced by enterocytes, in this study, an engineered human insulin construct modified to be constitutively secreted by a variety of cell types is further enhanced to augment gene expression. Although the mutation produces higher levels of bioactive insulin compared to the native sequence, codon optimization does not have any impact. During the optimization process, the codon adaptation index is improved from 0.84 to 0.94, while the GC content is marginally raised from 63% to 67%. Since the gene is relatively short (333 bp), the optimization program replaces only a handful of rare codon sequences with more bountiful ones. In other words, the starting gene sequence is relatively efficient in terms of codon usage and mRNA stability. Neither does it contain any cryptic splice donor sites nor RNA instability motifs that can potentially result in lower translation efficiencies of the mRNA transcript. Of the factors studied, the addition of CMV intron A enhancer has the greatest impact (25-fold increase) on protein production. Literature studies have reflected that the magnitude of enhancement by intron A is transgene dependent [34]. Likewise, minicircle constructs also have a varying effect on gene expression depending on cell type and gene sequence (unpublished data). Thus, codon optimization, addition of intron A and utilization of minicircle constructs will increase transgene expression, but the quantitative levels of enhancement will vary with the transgene.

Of the gene carriers screened, LPD has higher gene expression initially but PPA is more efficient over time. The slower unpacking kinetics of polymer–DNA nanoparticles [26] may confer the advantage of more sustained transgene expression. PEGylation decreases the transfection efficacy. This is ascribed to the shielding effect of PEG which decreases interactions with the cell surface and diminishes cellular uptake. Chitosan–PEG is the exception. Conjugation of PEG to free amines on the chitosan can potentially destabilize the complex, permitting more efficient unpacking and hence higher transfection efficiency. The most impactful discovery in this comparative study is that transcytosis of bioactive plasmid across the intestinal epithelial monolayer occurs, even in the presence of a mucus layer. This is the first in vitro study to demonstrate this phenomenon. However, the low levels of transgene expression could only be detected by the sensitive PCR technique, in a highly optimized environment for nanoparticle uptake. The implication is that transcytosis alone is not sufficient for a therapeutic effect and future work will have to involve the design of strategies to improve the fraction of intact nanoparticles or bioactive unpacked plasmid transported. The transport of intact microparticles by enterocytes to the underlying lamina propria, mesenteric lymph node, liver and spleen has been observed in vivo [35–38]. Nanoparticle transport across the intestinal epithelium in this study is most likely to be transcellular since the size of the intact polymer–DNA nanoparticle (120 nm) is significantly larger than the pore size [39] and also because most of the nanoparticles do not associate with the tight junctions. The same conclusion was also reached in other studies [40]. This is in contrast to chitosan–protein (insulin) nanoparticles, which co-localize with tight junctions [12]. The unpacking of the chitosan–insulin nanoparticles may occur in the GI lumen, and the interaction of the free chitosan with the tight junction proteins results in transient openings, allowing the small protein molecule to be transported paracellularly.

5. Conclusion

The expression levels of a transgene delivered orally via polymer–DNA nanoparticles are determined by the gene carrier used, the plasmid design, and the constitution of the intestinal epithelium. The Caco2-HT29MTX co-culture model most accurately mimics in vivo GI histology. However, for the most part, nanoparticle transport follows the same trends as in the simpler Caco2 model, except with lower uptake and transfection efficiencies. This is

attributed to the presence of mucus which interferes with endocytosis. Caco2-Raji transports the nanoparticles across the monolayer more efficiently, but degrades the plasmid *en route*, resulting in lower protein expression levels. The bulk of the nanoparticles are endocytosed by the enterocytes, which can then synthesize and secrete the protein of interest into the basolateral hepatic portal circulation. Importantly, a small fraction of bioactive plasmid is also transcytosed into the basolateral space to be uptaken by other cell types. However, this transport is very inefficient, as the low-level expression is only detected via PCR, under optimized conditions for nanoparticle uptake.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Fig. 1. Cell culture models of intestinal epithelia. Caco2 cells differentiate and form an impermeable monolayer after 16 days. Caco2-HT29MTX co-cultures secrete mucus on the apical surface. Caco2-Raji co-cultures further differentiate into M-cells when Raji lymphocytes are added 14 days after the Caco2 monolayer has formed.

Fig. 2.

Insulin protein expression. (A) Protein expression by different insulin sequences. The substitution of cell-specific proconvertase cleavage sites with ubiquitous furin shows appreciable increase in insulin secretion, although codon optimization does not. (B) Plasmid elements affect insulin protein expression. The addition of intron A has the most significant impact on insulin secretion (25-fold increase) for a given molar concentration of plasmid. Using minicircle constructs also facilitates insulin secretion 5-fold.

Fig. 3. Bioactivity of nanoparticles. (A) mRNA expression of a minicircle insulin plasmid by monolayer and underlying HepG2 cells, two and six days post-transfection. The expression of the control housekeeping gene (beta-actin) is consistent for all the samples. (B) Luciferase expression by intestinal epithelial monolayer and underlying HEK293 cells, two days post-transfection. PPA and LPD show the highest expression levels in both the intestinal epithelial monolayer and underlying cells. PEGylation decreases expression in the intestinal epithelial monolayer cells.

Fig. 4. DNA nanoparticle effect on monolayer permeability. (A) DNA nanoparticles increase intestinal epithelial tight junction permeability, as measured by decreases in transepithelial electrical resistance (TEER). PPA and LPD nanoparticles exert the most dramatic effect, while PEGylation mutes the decline. (B) Cytotoxicity of polymers as measured by WST-1 assay, after 1 h exposure to varying concentrations of polymer. PEI is the most cytotoxic, killing 25% of the cells at the concentration of 0.1 mg/mL used in the nanoparticle formulation. Chitosan and PPA are not cytotoxic at 0.02 mg/mL and 0.08 mg/mL, concentrations used in their respective nanoparticle formulations.

Fig. 5. Internalization of intact gold-labeled nanoparticles. TEM images showing the uptake and intracellular localization of intact nanoparticles and aggregates, which appear as dark black spots. PPA nanoparticles (A) are internalized most readily and PEGylation (B) results in diminished uptake and aggregation. Internalized chitosan nanoparticles (C) were less abundant, but also observed. Presumably, a fraction of those particles internalized via the apical surface are trafficked and exocytosed through the basolateral membrane.

Fig. 6.

Nanoparticle uptake by monolayer. (A) Projection of a Z-stack confocal image of Chitosan–DNA nanoparticles uptaken by Caco2-Raji cells. Closer examination of the individual stacks reveals that the QD-labeled nanoparticles (red) did not co-localize with tight junctions (green), suggesting minimal or weak interactions despite the decrease in TEER. PPA–DNA nanoparticles (B) uptaken and (C) transcytosed by monolayer cells as determined by inductively coupled plasma mass spectrometry. The cadmium (Cd) content correlates with the amount of DNA present in the QD-labeled nanoparticles. In Caco2 and Caco2-HT29MTX monolayer cultures, the nanoparticles are endocytosed and there is negligible transport across the monolayer. Caco2-Raji endocytoses PPA–DNA nanoparticles 3 times more readily and is the only culture to demonstrate significant transport across the epithelial layer. PEGylation decreases nanoparticle endocytosis.

Table 1

Physical characteristics of polymer–DNA nanoparticles.

	Gene carrier	Size (nm)	Zeta potential (mV)
Chitosan (CS)		116.9±36.3	14.8±0.5
Chitosan–PEG (CS–PEG)		207.3±66.8	−4.98±1.4
Poly(phosphoramidatedipropylamine) (PPA)		119.3±20.9	23.4±2.7
PPA–PEG		127.7±14.4	11.6±4.3
Lipid-Protamine (LPD)		130.9±5.6	57.9±1.9
LPD–PEG		146.3±2.6	44.7±1.3
Polyethyleneimine (PEI)		121.4±7.6	25.7±3.5