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Oral Nucleic Acid Therapy using Multi-Compartmental Delivery Systems

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Abstract

Nucleic acid-based therapeutics has the potential for treating numerous diseases by correcting abnormal expression of specific genes. Lack of safe and efficacious delivery strategies pose a major obstacle limiting clinical advancement of nucleic acid therapeutics. Oral route of drug administration has greater delivery challenges, since the administered genes or oligonucleotides have to bypass degrading environment of the GI tract in addition to overcoming other cellular barriers preventing nucleic acid delivery. For efficient oral nucleic acid delivery, vector should be such that it can protect encapsulated material during transit through the GI tract, facilitate efficient uptake and intracellular trafficking at desired target sites, along with being safe and well tolerated. In this review, we have discussed multi-compartmental systems for overcoming extracellular and intracellular barrier to oral delivery of nucleic acids. A nanoparticles-in-microsphere (NiMOS) based multi-compartmental system was developed and tested for in vivo gene and siRNA delivery for treating colitis in mice. This system has shown efficient transgene expression or gene silencing when delivered orally along with favorable downstream anti-inflammatory effects, when tested in a mouse model of intestinal bowel disease (IBD).

Graphical abstract

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Keywords

Oral gene therapy; plasmid DNA; small interfering RNA; multicompartamental delivery systems; nanoparticles-in-microsphere (NiMOS); inflammatory bowel disease

1. INTRODUCTION

Delivery of nucleic acids (DNA or RNA) can induce or inhibit gene expression with high specificity which can be useful in treating almost all of the human diseases, provided nucleic acids can be safely and effectively delivered at the target site of action. Gene therapy involves delivery and translation of delivered gene into therapeutic proteins for treatment of genetic or acquired diseases originating from absence or lack of specific protein expression¹. RNA interference (RNAi) is a homology dependent knockdown of target mRNA mediated by complementary RNA, resulting in suppression of target genes. Small interference RNAs (siRNA) are ~22 nucleotide long double stranded RNA molecules, capable of mediating knockdown of specific target genes based on complementary base pairing². MicroRNAs are 19 to 25 nucleotide long single stranded RNAs which play a regulatory role in the expression of a number of proteins by inhibiting mRNA translation³. Synthetic siRNAs and microRNAs can be used for treatment of diseases originating from abnormal over-expression of specific genes. For successful gene silencing, both siRNA and microRNAs need to gain access to cellular cytoplasm where they can get loaded on to the RNA induced silencing complex (RISC) and cause downstream effects^{4–6}.

To overcome different obstacles such as instability of nucleic acids in presence of degrading enzymes, high molecular weight and anionic charge associated with nucleic acid delivery^{7, 8}, researchers have developed a large number of strategies over last twenty years, and conducted a number of clinical trials, but only a very handful have progressed into clinical trials, where majority have failed due to adverse effects such as immunogenicity and oncogenic effects^{9, 10}. Recently, first gene therapy based drug and first microRNA-based drug earned approval by regulatory agencies in the Western world; however, even these approved therapies have shown immunogenic adverse effects^{11–14}. Hence, there is a tremendous need for developing safe alternatives for fully realizing potential of nucleic acid therapies meant for curing human diseases. Although significant amount of research efforts have been made for tackling challenges associated with systemic nucleic acid delivery, a very small number of researches have made successful contributions towards developing orally active nucleic acid therapies. This is because the oral route of drug delivery poses additional challenges such as nucleic acid degradation by enzymes in the GI track and low pH, limiting or diminishing local and systemic nucleic acid bioavailability. This review describes challenges, opportunities and promising strategies for enabling efficient nucleic acid delivery through the oral route¹⁵.

2. NOVEL METHODS OF ORAL GENE DELIVERY

2.1 Extracellular and intracellular barriers to oral gene delivery

Delivery of nucleic acids is a multi-step process where nucleic acids must remain stable during their transit from site of administration until they are bioavailable at the target site (extracellular barriers), must undergo suitable intracellular trafficking to avoid degradation by the endo-lysosomal pathway, and facilitate cytosolic release and incorporation in the appropriate intracellular pathways for exhibiting desired pharmacological effects (intracellular barriers). Majority of the studies have focused on optimizing cellular uptake, fewer number of studies have utilized targeted approach for tissue or cell specific delivery, very few number of studies have looked into strategies for optimizing intracellular trafficking and even fewer number of studies have tried to overcome delivery barriers associated with oral route of nucleic acid delivery. Oral route of drug administration has a number of advantages over the systemic route such as patient compliance, ease of administration, non-invasiveness, cost effectiveness, no need for sterilization and possibility of administering with different types of dosage forms such as capsules, controlled release, enteric coated, suspension or mixtures¹⁶. However, oral route is not very straightforward for nucleic acid delivery because of a number of anatomical and physiological barriers. For example, nucleic acids undergo rapid degradation at low gastric pH, undergo enzymatic degradation by endo- and exo-nucleases in the small and large intestines, and get clearance by phagocytes such as macrophages. Additionally, the physical barrier of epithelial tight junctions and negatively charged mucus along with negatively charge on cell surface prevents uptake of nucleic acids, which are also negatively charged. As a result of all of the above barriers, only a very small percent of delivered dose becomes available at the cellular sites for uptake, where additional challenges are encountered. After cellular uptake, nucleic acids have to undergo efficient intracellular trafficking where they have to bypass endo-lysosomal degradation, get released in the cellular cytoplasm and efficiently get incorporated into relevant intracellular pathways such as RISC loading (for siRNA, mRNA, microRNA) or nuclear uptake (for plasmid DNA) for ultimately producing intended pharmacological effects. Challenges associated with the oral route of nucleic acid delivery are illustrated in Figure 1.

2.2 Multi-compartmental systems for oral nucleic acid delivery

Oral nucleic acid formulations consisting of inner nucleic acid containing compartment surrounded by one or more external protective compartment(s) are under active research in the Amiji's laboratory located at Northeastern University^{17–23}. These systems were designed with the rationale that nucleic acids encapsulated within multiple compartments will remain stable during transit through the GI tract, while enabling adequate uptake or release of encapsulated material for promoting therapeutic effects. Multi-compartmental systems developed so far can be categorized into three categories, and are described below.

2.2.1 Solid-in-solid multi-compartmental systems—These systems consist of a solid internal phase encapsulated within a solid external phase. Nucleic acids can be entrapped within the solid internal phase, while the solid external phase can serve as a protective casing. External and internal phases can be selected such that nucleic acids remain protected

during their GI transit, and release encapsulated material at desired target sites. An example of this type of system is nanoparticles-in-microsphere oral system (NiMOS), where nucleic acids were first encapsulated within type-B gelatin nanoparticles, followed by encapsulation of gelatin nanoparticles within poly(ϵ -caprolactone) (PCL) based microspheres^{17, 18, 21, 22, 24, 25}. NiMOS are discussed in further detail in later sections of this review.

2.2.2 Solid-in-liquid multi-compartmental systems—These systems consist of a solid hydrophilic internal phase (containing nucleic acids) and a liquid hydrophobic external protective phase. An example of this type of system is nanoparticles-in-emulsion (NiE) formulation, where IL-10 plasmid DNA was first encapsulated within gelatin nanoparticles, followed by encapsulation of gelatin nanoparticles within a safflower containing water-in-oil-in-water (w/o/w) multiple emulsion. This system when tested in vitro showed efficient plasmid DNA mediated IL-10 gene expression as well as anti-inflammatory effects resulting from IL-10 gene expression in murine adherent alveolar macrophage cell line (J774A.1)²³. This type of system has advantages such as ease of administration through parenteral or oral route, possibility of incorporating both hydrophilic (nucleic acids) and hydrophobic (small molecules) drugs within a single formulation for synergistic benefits along with capability of protecting encapsulated material within the innermost phase for efficient physiological transit and localization at desired site of action. Precise engineering of formulation with properties such as size, charge and surface chemistry of nanoparticles, along with choice material for external oil phase and size of oil droplets can be tailored for achieving stable transit and addressing target specific needs for preferential accumulation, uptake and effects at desired sites^{23–25}.

2.2.3 Liquid-in-liquid multi-compartmental systems—These systems consist of w/o/w multiple emulsion, where nucleic acids are incorporated within the innermost aqueous phase, surrounded by additional oil and aqueous phases. Both hydrophilic and hydrophobic drugs can be encapsulated within this system for synergistic effects in disease treatment. Choice of oil phase and size of oil droplets can be engineered depending upon desired therapeutic actions and physiological site for drug delivery. A systemic cancer DNA vaccine consisting of gp-100 plasmid DNA incorporated within squalane oil (having immunogenic effects) containing w/o/w multiple emulsion was developed for prophylaxis and active immunization against melanoma in mice^{26–28}.

Additionally, multi-compartmental systems consisting of solid microspheres or nanoparticles dispersed within semisolid gels such as chitosan or gelatin can also be formulated for payload protection and controlled release of drug substance over a prolonged duration²⁵.

Figure 2 shows a schematic representation of multi-compartmental systems.

3. BIOMEDICAL APPLICATIONS

3.1 IL-10 gene therapy

3.1.1 Functions of IL-10—IL-10 was first described as a cytokine synthesis inhibitory factor, secreted by CD4⁺ Th2-cells, that acts on antigen presenting cells and inhibits cytokine production²⁹. IL-10 can be considered as the cytokine of maximum importance in

the human anti-inflammatory response because of its complex and varied effects on different immune cell types³⁰. IL-10 is produced by a wide variety of immune cells including T- and B-lymphocytes, monocytes, dendritic cells, mast cells and macrophages^{31, 32}. IL-10 is considered to have a broad effect on host defense and immune regulation through its ability to affect both innate and adaptive immune systems by differentially acting upon varied subsets of immune cells³³. IL-10 can block inhibitor of kappaB kinase (IKK) activation and can directly inhibit nuclear localization of nuclear kappaB p65/p50 heterodimer^{34, 35}, thereby suppressing production of many pro-inflammatory cytokines regulated by nuclear factor kappa-light-chain enhancer of activated B cells (NF- κ B)^{36, 37}. Broadly speaking, IL-10 elicits an anti-inflammatory response by increasing production of anti-inflammatory mediators, and inhibiting production of pro-inflammatory mediators^{30, 37}.

3.1.2 IL-10 supplementation and IL-10 gene therapy for IBD—A number of animal studies have shown that IL-10 supplementation has a huge potential in blocking proinflammatory cytokines and inflammatory tissue damage in IBD^{37–39}. Based on compelling evidence rising from many animal models, IL-10 supplementation was evaluated in several human trials. The first human trial for recombinant IL-10 supplementation showed that IL-10 is safe and well tolerated in humans⁴⁰. Further, it was shown that IL-10 supplementation results in reduction of Crohn's disease activity index (CDAI) score, but that decrease is not significant⁴⁰. In another study, 5 μ g/kg of recombinant human IL-10 (Tenovil®) administered subcutaneously for 28 days to patients with mild to moderate Crohn's disease resulted in improved CDAI scores and improved endoscopic outcomes⁴¹. Schreiber et al⁴² showed that Tenovil® administered subcutaneously for 28 days at 8 μ g/kg to patients with mild to moderate Crohn's disease resulted in clinical improvements which were non-significant. In another study, Colombel et al⁴³ found that Tenovil® administered for 12 weeks did not result in preventing recurrence of Crohn's disease. Based on the human trials for recombinant IL-10 conducted so far, there is no compelling evidence that IL-10 supplementation can help prevent or improve symptoms of IBD. Among different potential explanations as to why recombinant IL-10 supplementation failed to achieve desired clinical outcomes in Crohn's disease³⁷, one major explanation is that systemically administered IL-10 results in low mucosal levels at the inflamed sites in the intestine, which are not enough to elicit a robust anti-inflammatory response.

Separate studies conducted by Steidler et al⁴⁴ and Bhavsar et al¹⁸ have utilized gene therapy to address the issue of low mucosal IL-10 bioavailability by engineering IL-10 plasmid DNA within a delivery vector, such that delivered IL-10 gene is expressed locally without resorting to high detrimental systemic levels of IL-10 protein. Steidler et al⁴⁴ embedded IL-10 plasmid DNA in *Lactococcus lactis* (*L. lactis*) to secrete IL-10 protein, which were successfully used to reduce inflammation by 50% or prevent onset of colitis in DSS mouse model with IL-10 knockout. Bhavsar et al¹⁸ used a multi-compartmental strategy for encapsulating IL-10 plasmid DNA within NiMOS, which resulted in a robust local expression of IL-10 protein, along with reversal of inflammatory damage in a mouse model of colitis. Two other studies^{45, 46} have demonstrated efficacy in mouse models, where local IL-10 expression was enabled by incorporating gene encoding IL-10 within replication-deficient adenoviral vectors. Given that all of the above studies utilizing gene therapy have

led to local production of IL-10 with favorable therapeutic outcomes, it is worth further exploring this potential therapeutic strategy in larger studies and in humans.

3.2 TNF- α gene silencing in IBD and other inflammatory disorders

Tumor necrosis factor- α (TNF- α) is a proinflammatory cytokine produced primarily by activated macrophages along with other cell types such as CD4⁺ T-cells, natural killer cells, neutrophils, mast cells, eosinophils and neurons⁴⁷. Dysregulation of TNF- α production is associated with onset and progression of a number of diseases including IBD, rheumatoid arthritis, Alzheimer's disease, cancer, Psoriasis, type 2 diabetes and major depression^{48–52}. Harmful effects of TNF- α are mainly thought to be mediated by the activation of NF- κ B, which regulates expression of over 200 genes⁵³. Over the last several years a number of approved therapies have emerged where blockage of TNF- α , either by monoclonal antibodies or circulating receptor fusion protein, is utilized for treating wide variety of autoimmune and inflammatory diseases including rheumatoid arthritis and IBD^{54, 55}. However, several side effects or contraindications such as risk of serious infections, malignancies, infusion reactions, reduced efficacy due to anti-drug antibody formation have hindered their unrestricted clinical use^{56, 57}. These side effects can be avoided if TNF- α inhibition occurs locally at the site of inflammation vs systemic inhibition. RNAi mediated suppression of TNF- α can be beneficial for overcoming several of these side effects, especially where local site specific suppression of TNF- α expression can be achieved. As discussed previously, Kriegel et al utilized NiMOS formulation for promoting local silencing of TNF- α in colon, which resulted in reversing inflammatory tissue damage in DSS-induced colitis bearing mice^{21, 22}. Wison et al⁵⁸ have reported development of TNF- α siRNA loaded thioketal nanoparticles for oral TNF- α silencing in the intestines, and have shown efficacy in protection against ulcerative colitis in mice. TNF- α silencing has also been utilized for treating systemic inflammations, for instance, Yin et al^{59, 60} have reported development of supramolecular self-assembled nanoparticles (SSNPs) for oral silencing of TNF- α . SSNPs were engineered via the hydrophobic and electrostatic self-assembly of a number of building blocks including oleyl trimethyl chitosan (OTMC), poly(γ -(4-(((2-(piperidin-1-yl)ethyl)amino)methyl)benzyl-L-glutamate) (PVBLG-8), oleyl-PEG-mannose (OPM), oleyl-PEG-cysteamine (OPC), sodium tripolyphosphate (TPP), and TNF- α siRNA. This system was shown to evade extracellular and intracellular barriers of GI tract and exhibit robust systemic oral gene silencing and systemic anti-inflammatory activity. Overall, results from these studies indicate that TNF- α silencing can be useful in treating number of local and systemic inflammatory diseases and that this therapeutic strategy needs to be evaluated for further confirmation in larger studies and in human trials.

3.3 Potential of RNAi based therapies in iron overload disorders

Iron is an essential metal required for numerous physiological functions, including oxygen transport, growth, and neurotransmitter production. However, iron in excess is a well-defined risk factor for the pathogenesis of several diseases, including cardiovascular and neurodegenerative diseases^{61–68}. It is generally accepted that these toxic effects arise due to increased oxidative stress promoted by iron. Iron overload is best represented by hereditary hemochromatosis (HH), which is characterized by increased intestinal absorption and progressive tissue deposition of iron. Polymorphisms of the HFE (High Fe or hyperferremia)

gene are the major cause of HH, which is one of the most common autosomal recessive genetic defects of the Caucasian population^{69, 70}, affecting 7–32% with genetic variants^{70–72} (Figure 3). Iron overload also occurs in blood transfusion, which is required for several anemias (e.g. thalassemia, sickle cell anemia, myelodysplastic syndrome) due to defects in blood cell metabolism. Severe iron overload results in multiple organ failures, including cardiomyopathy, liver cirrhosis/cancer, arthritis, diabetes, hypertriglyceridemia and premature death^{69, 73}. For example, cardiomyopathy is the main cause of mortality in patients with HH and thalassemia^{73–75}. Notably, high iron stores in the brain are associated with several neurodegenerative diseases (e.g. Alzheimer's, Parkinson's and Huntington's diseases), as well as some pathological conditions, such as traumatic brain injury (TBI), Hallervorden-Spatz syndrome and neurodegeneration with brain iron accumulation (NBIA). In particular, elevated brain iron found in TBI, which affects 2.5 million people in the US, is associated with increased free radicals and neural dysfunction⁷⁶. Conversely, reduction of iron stores by phlebotomy, iron chelation therapy, or iron-restricted diet improves TBI, heart diseases, hypertriglyceridemia and diabetes^{77–84}.

Since there is no recognized active pathway of iron excretion^{85–87}, a disposal of extra iron from the body is the primary therapeutic goal of treating patients with iron overload. Although phlebotomy is effective for treating iron overload, patients prefer chelators because of phlebotomy-related side effects and substantial costs^{88, 89}. However, the use of chelators (Table 1) is limited due to non-specific distribution in non-target tissues, which results in a number of significant toxicities: hypotension, tachycardia, agranulocytosis, neutropenia, CNS and ocular/auditory toxicities, musculoskeletal-joint pains, gastrointestinal disturbances and even death^{90–100}. In addition, both phlebotomy and chelator therapy result in loss of essential metals and nutrients, which predisposes impaired body function. Considering hundreds of millions of people affected by various types of iron overload disorders, there are urgent needs for a new therapeutic strategy to control iron transport in the body.

The Divalent Metal Transporter 1 (DMT1) protein plays a well-established role in the absorption of iron as an essential nutrient^{101–103}. The primary site of DMT1 activity is the intestinal epithelium where the transporter provides sufficient iron to meet the body's daily demands in an iron-responsive manner (Figure 4); When iron stores are low (as in the anemic state), DMT1 expression is enhanced to enable greater absorption of iron from the diet. Conversely, when iron stores are high, the activity of DMT1 is reduced to avoid the toxic consequences of iron overload. These lines of evidence suggest that altered DMT1 activity can modulate the transport and toxicity of iron. Importantly, several investigations have revealed that intestinal DMT1 is up-regulated upon HH in both humans and mice^{104–106}. Since DMT1 also plays an important role in iron delivery during erythropoiesis^{107–109}, a “selective” suppression of intestinal DMT1 can be an excellent therapeutic target by direct delivery of DMT1 inhibitors to the site of absorption (i.e. oral administration) with no systemic effects.

Despite increased health concerns about high iron storages and increased susceptibilities to a number of major diseases, there are no studies about the modulation of iron transporters to mitigate iron-induced toxicity by *in vivo* gene silencing. Although a few small molecule-

based DMT1 inhibitors have been studied, overall enthusiasm is low because these inhibitors indirectly alter DMT1 function, for example, by modifying redox status^{110, 111}, only in *in vitro* systems. Also, poor pharmacokinetic properties of these compounds (high lipophilicity and rapid metabolism) make them unsuitable for effective therapy. Thus, it is plausible that oral DMT1 silencing by siRNA-encapsulated nanoparticles-in-microspheres (NiMOS) decreases intestinal uptake of dietary iron and improves iron overload and iron-mediated toxicity (Fig 6). The mechanism-based translational capability of oral gene silencing also offers several advantages, including:

1. Selective inhibition of DMT1 expression in the intestine, but not in the erythropoietic system, will ensure that red blood cell production is not compromised.
2. The model of oral gene silencing specific to DMT1 can be validated by using DMT1 mutant rats expressing non-functional DMT1^{112–115}.
3. The availability of HFE-deficient mice, a genetic model of iron overload hemochromatosis in humans^{116, 117}, will allow investigators to explore the therapeutic efficacy of DMT1 siRNA against iron overload.
4. Ability to simultaneously inhibit other iron transporters (multi-transporter inhibition) along with DMT1, as well as a potential combination with chelators (at low doses) can maximize the therapeutic efficacy both by inhibiting entry and by enhancing excretion of iron.

This approach can provide therapeutic benefits over numerous iron overload disorders by a combination of siRNA and nanotechnology.

3.4 Potential of RNAi based therapy for celiac disease

Celiac disease is caused by a T-cell mediated immune response in the small intestine against deamidated cereal gluten peptides modified by the enzyme transglutaminase 2 (TG2)^{118, 119}. Celiac disease is prevalent in approximately 1% of general population in the United States and Europe. Symptoms associated with celiac disease are abdominal pain, diarrhea, bloating, constipation, nutritional malabsorption and weight loss, along with other gastrointestinal abnormalities¹²⁰. The only way to prevent celiac symptoms is a strict adherence to a gluten-free diet. Minority of patients suffer from a condition called refractory celiac disease, where more severe disease symptoms are manifested and even consumption of extremely minor quantities of gluten can trigger a full blown inflammatory response^{121, 122}. Pathophysiology of celiac disease involves a combination of environmental, genetic and immunological factors^{123–126}. Among several immune mediators, enzyme tissue transglutaminase-2 (TG2) and the proinflammatory cytokine interleukin-15 (IL-15) have emerged as being very important in promoting inflammatory responses against dietary gluten^{123–126}. TG2 catalyzes deamidation of gluten peptides, which is required for their recognizing by antigen presenting expressing HLA-DQ2/8 major histocompatibility complex, whereas IL-15 promotes immune responses by altering homeostasis of intraepithelial lymphocytes resulting in innate and cellular immune responses^{127, 128}. Given their important role in the pathophysiology of celiac disease, siRNA mediated silencing of TG2 and IL-15 may result in neutralizing proinflammatory effects and alleviate disease

symptoms. In our further studies, we aim to utilize NiMOS for causing siRNA mediated silencing TG2 and IL-15 genes in the small intestine, and evaluate efficacy in a mouse model of celiac disease.

4. ILLUSTRATIVE EXAMPLES OF ORAL NUCLEIC ACID DELIVERY WITH MULTI-COMPARTMENTAL FORMULATIONS

4.1 Oral IL-10 plasmid DNA delivery for treatment of colitis

4.1.1 NiMOS Formulation—Bhavsar et al.^{18–20} developed a solid-in-solid multi-compartmental system consisting of nucleic acid containing gelatin nanoparticles encapsulated within poly(ϵ -caprolactone) (PCL) based microspheres. The major hypothesis underlying the engineering of NiMOS was that the encapsulated gelatin nanoparticles and nucleic acids would remain protected against low gastric pH, proteases, nucleases and physiological fluids by the outer hydrophobic PCL matrix, while allowing intestinal release through degradation by lipases and uptake by desired cells where pharmacologic action is desired²⁵. NiMOS formulation was optimized using a 3³ factorial design, where particle size of NiMOS was used as response. Three variables affecting particle size of NiMOS were evaluated. They were (1) concentration of PCL in the organic phase, (2) mass of gelatin particles encapsulated within PCL microspheres, and (3) speed of homogenization used for the formation of double emulsion. With this design, it was possible to tailor particle size of NiMOS by altering one or more of the above parameters. NiMOS with particle size ranging between 2 to 5 μm were formulated (Figure 5) as this size was thought to be favorable for uptake by intestinal immune cells such as macrophages, and hence suitable for oral anti-inflammatory gene therapy. Gelatin biopolymer was selected based on its proven gene encapsulation and transfection, along with its highly favorable oral safety profile efficiency^{23, 129}. Gelatin nanoparticles were embedded in PCL matrix, which can degrade over time in the small intestine in a controlled fashion under the influence of enzymes such as lipases and release gelatin nanoparticles at the inflamed site followed by endocytosis by enterocytes or other cells at the release site. Alternatively, NiMOS can be directly taken up by intestinal immune cells, such as macrophages, where intracellular degradation can cause release of gelatin particles along with nucleic acids²⁵.

4.1.2 Oral biodistribution and gene transfection—In vivo mouse and rat studies^{17–20} were conducted to evaluate gene delivery and transfection potential of NiMOS after oral administration. Qualitative gene transfection studies were conducted in Balb/C mice and Wistar Rats with plasmid DNA encoding either enhanced green fluorescent protein (EGFP-N1) or β -galactosidase (CMV- β gal). Results obtained from these studies showed efficient transgene expression in the small and large intestines, but not in the stomach. Results from biodistribution studies (Figure 6) showed that gelatin nanoparticles delivered via NiMOS were predominantly distributed to the small and large intestines and had increased residence time when compared to gelatin nanoparticles administered without PCL encapsulation. At one hour post oral dose, 55% of administered dose was recovered from small intestine, while only 10% was recovered from large intestine. At four hours post dose, approximately 25% of administered dose was recovered from small intestine, indicating sustained release of gelatin nanoparticles from the microsphere matrix with stable transit in the GI tract. Gelatin

nanoparticle exposure was primarily restricted to intestinal local sites with minimal systemic exposure as evidenced by minimal gelatin exposure in liver and blood. Collectively, these results suggest that NiMOS have potential for being used as carrier vehicle for therapies where intestine specific gene delivery is desired.

4.1.3 Oral IL-10 gene delivery for local transfection and efficacy in an IBD

model—Inflammatory bowel disease (IBD) consists of (1) Crohn's disease and (2) ulcerative colitis. IBD is prevalent in genetically susceptible individuals where dysregulation of mucosal immune system results in pro-inflammatory tissue damage^{130, 131}. IL-10 has an important role in modulating mucosal immune responses through both innate and cell mediated inflammatory pathways. Mice lacking IL-10 receptor and IL-10 knockout mice have shown to develop enterocolitis by 2 to 3 months of age, along with inflammatory lesions in discrete regions of the GI tract^{132–135}. IL-10 plays a crucial role in regulating different pro- and anti-inflammatory cytokines and chemokines such as IL-1, IL-6, IL-8, TNF- α . IL-10 also plays a role in activating regulatory T-cells¹³⁵. However, systemic IL-10 protein therapy in a clinical trial of Crohn's disease did not help in alleviating disease symptoms or reducing disease severity [55, 56]. These disappointing results may be attributed to low exposures of recombinant IL-10 protein at the inflamed intestinal target sites. In such a case, delivery systems capable of producing local high levels of IL-10 protein expression at the local inflamed intestinal sites may prove beneficial¹³⁵.

Murine IL-10 plasmid DNA containing NiMOS formulation (mIL10-NiMOS) was evaluated in Balb/C mice for the treatment of 2,4,6-trinitrobenzenesulfonic acid (TNBS) induced acute colitis¹⁸. Female Balb/C mice of age 8–10 weeks were injected rectally with TNBS at the start of the study and after four days. Stool consistency, rectal bleeding, tissue histology and body weight were monitored to confirm development of colitis. Naked mIL-10 plasmid DNA, IL-10 plasmid DNA encapsulated within gelatin nanoparticles, or mIL10-NiMOS was administered orally after colitis was established. Mice receiving blank formulations (formulations not containing mIL-10 plasmid DNA) served as vehicle control. After 4 days post treatment (i.e., at the end of the study), the experimental animals from control and test were euthanized and blood as well as tissue samples were collected for further analysis. IL-10 gene expression was analyzed by determination of both mRNA and protein expression in mice treated with different formulations. Results obtained from these analyses showed that mice treated with mIL10-NiMOS exhibited large intestinal expression of IL-10 mRNA as well as IL-10 protein, which was greatest when compared to the vehicle control group, as well as groups receiving naked mIL-10 plasmid DNA or mIL-10 plasmid DNA delivered via gelatin nanoparticles. For instance, IL-10 protein levels in the mIL10-NiMOS treated group were approximately 7.2-fold higher than in the group treated with vehicle control. Further, cytokine profiling was performed to evaluate anti-inflammatory potential of local colonic IL-10 protein expression. Results from cytokine profiling showed an overall greater reduction in the expression profiles of proinflammatory mediators such as TNF- α , interferon gamma (IFN- γ), IL-12, IL-1 α , IL-1 β and chemokines such as monocyte chemoattractant protein (MCP)-1, and monocyte inflammatory protein (MIP)-1 in the mIL10-NiMOS treated group, when compared to other control groups. In addition, mIL10-NiMOS demonstrated reduced loss in body weights and increased colon lengths, when compared to control groups.

Further, histopathological analysis the colonic tissue obtained from mice treated with mIL10-NiMOS showed a healthy tissue architecture resembling naïve mice (non-colitis bearing mice), whereas control groups showed prominent signs of inflammatory damage such as villus atrophy, thickening of luminal wall, loss of mucosal architecture, infiltration of immune cells and loss of protective epithelial layer (Figure 7). Collectively, these results indicate that mIL10-NiMOS formulation is capable of not only protecting the encapsulated payload in the GI tract, but can also allow delivery of the encapsulated cargo at the inflamed site, enable transgene expression, and elicit desired pharmacological effect. These results also suggest that NiMOS formulation can be utilized for delivery of other similar nucleic acid based therapeutics such as siRNAs, mRNAs, micro RNAs (miRNAs) and DNA/RNA vaccines in the small and large intestines, thereby produce desired pharmacological effect which can be beneficial in treating various GI disorders ¹⁸.

4.2 Oral TNF- α and cyclin D1 siRNA delivery for treatment of colitis

4.2.1 TNF- α and cyclin D1 siRNA containing NiMOS for the treatment of IBD—

TNF- α plays an important role in the pathogenesis of numerous inflammatory disorders including disorders of the GI tract such as IBD. Inhibition of TNF- α through monoclonal antibodies is being utilized for treatment of IBD and other inflammatory disorders ^{56, 136, 137}. Although systemic treatment with monoclonal antibodies has been successful in treating and maintaining clinical remission of these disorders, a number of patients fail to respond to therapy which is most likely attributed to limited local concentrations of systemically administered drug at the site of action and/or development of anti-drug antibodies. Moreover, systemic exposure of monoclonal antibodies against TNF- α results in adverse effects such as risk of infections and cancers such as skin cancer and lymphoma ¹³⁰. RNAi has emerged as an exciting new approach for inhibiting expression of target genes by knocking down complementary mRNA. Although significant clinical progress has been achieved in successfully knocking down liver expressed genes by subcutaneous delivery ^{138–140}, not much progress has been made with knocking down intestinal genes through oral siRNA delivery. This is primarily due to siRNA instability at low gastric pH and rapid degradation in the presence of abundant nucleases in the GI tract. These barriers have necessitated development of delivery systems capable of protecting siRNA in the GI tract, along with allowing adequate release and uptake by target cells of interest for promoting knockdown of desired genes.

A NiMOS formulation encapsulating siRNA targeting TNF- α and cyclin D1 was developed and evaluated by Kriegel *et al.* ^{21, 22} for the treatment of IBD. TNF- α is a cytokine with a prominent pro-inflammatory role in the development of IBD. Inhibition of TNF- α has been shown to improve symptoms of IBD in various animal models and in humans ^{137, 141}. Cyclin D1 plays an important role in progressing cell cycle from G1 to S phase, and is overexpressed in a number of inflammatory diseases and cancers ^{142–146}.

Kriegel *et al.* have conducted studies to test the underlying hypotheses, that inhibiting intestinal expression of TNF- α and/or cyclin D1 in the colon locally, through oral siRNA administration, will result in restoration of balance between pro- and anti-inflammatory mediators, thereby alleviating signs and symptoms of IBD.

4.2.2 Oral administration of siRNA containing NiMOS in colitis bearing mice—

Efficacy of TNF- α and cyclin D1 silencing was evaluated in dextran sulfate sodium (DSS) induced mouse model of colitis (in Balb/c mice). DSS (3.5% w/w) was dissolved in drinking water throughout the course of the study for all of the study group, except one group which received regular tap water at the same time. siRNAs targeting TNF- α and Cyclin D1 were encapsulated within NiMOS either separately or in combination. Mice were orally administered with NiMOS containing TNF- α and/or cyclin D1 siRNA, NiMOS containing scramble siRNA, naked siRNA (TNF α and/or cyclin D1), or vehicle control every other day for three days. Other control groups included mice receiving either tap water, NiMOS with no siRNA or naked scramble siRNA. At 3 and 5-days post last dose, animals were euthanized, followed by blood and tissue collection for evaluation of gene knockdown and profiling of inflammatory markers^{21,22}.

4.2.3 Efficacy of TNF- α and cyclin D1 gene silencing in the treatment of IBD—

siRNA mediated silencing of TNF- α and cyclin D1 was evaluated by analyzing mRNA expression levels in colon samples from all study animals. Animals treated with siRNA delivered via NiMOS showed robust target gene (TNF- α or cyclin D1) knockdown efficacy after oral administration to all of the DSS-colitis bearing mice. Of note, a more pronounced silencing of TNF- α was observed with dual TNF- α /cyclin D1 NiMOS (NiMOS containing siRNAs against TNF- α and cyclin D1), when compared to individual silencing of TNF- α at early time points. At later time points both TNF- α /cyclin D1 and individual TNF- α silencing gave more or less similar results. This result correlated with reductions in protein expression, where dual TNF- α /cyclin D1 silencing showed greater knockdown of TNF- α expression when compared to individual silencing of TNF- α . Further, evaluations for cytokine and chemokine profiling was performed where expression levels of interferon gamma, IL-1 α , IL-2, IL-15, IL-17, granulocyte macrophage colony stimulating factor and monocyte chemotactic protein 1 was performed. Individual silencing of cyclin D1 expression through cyclin D1 NiMOS resulted in most pronounced downregulation of proinflammatory cytokines/chemokines, followed by dual silencing of TNF- α /cyclin D1. Individual silencing of TNF- α through TNF- α NiMOS also resulted in downregulation of proinflammatory markers, but the effect was not as pronounced as that observed in the individual cyclin D1 NiMOS treated group (Figure 8). Further, intestinal tissue histopathology showed that treatment with NiMOS containing therapeutic siRNA (TNF- α , TNF- α /cyclin D1 or cyclin D1 siRNA) resulted in reduced inflammatory damage with signs of tissue regeneration and tissue architecture resembling healthy controls, regardless of individual or dual therapy, when compared to groups treated with control formulations (Figure 9). Collectively these results indicate that NiMOS can efficiently cause oral gene silencing with beneficial downstream effects for effective disease treatment.

5. CONCLUSIONS

Oral delivery of nucleic acid-based therapeutics has tremendous potential in treating numerous human diseases, as long as safe and effective target site delivery is possible. Encapsulation of nucleic acids within multi-compartmental systems has been utilized for enabling local gene or siRNA delivery in the intestine after oral delivery. As reported in

several studies, nucleic acids delivered through NiMOS have proven to be effective in promoting local gene expression or silencing in the colon, which has in turn resulted in reversal of signs and symptoms of colitis in mouse models. These promising results warrant further development of this system and testing its safety and efficacy in larger studies. Further evaluations include characterizing pharmacokinetics, biodistribution as well as pharmacokinetic-pharmacodynamic relationships for nucleic acids delivered via NiMOS. NiMOS will also be evaluated for treating other potential GI disease such as celiac disease, chronic inflammatory diseases and diseases associated with iron overload.

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Figure 1. Barriers to oral nucleic acid delivery

Opportunities and challenges in oral delivery of nanoparticles using microscale carriers. A) Aggregated particles (i.e., with blood components or carrier matrix molecules) leading to restricted release; B) physical docking and/or accumulation on cell surface; C) microparticle internalization into the cell followed by endosomal release of; D) nanoparticles within the microscale device or E) the microparticle itself; F) particle uptake into the nucleus; G) crossing of the particles into the bloodstream; H) particles that are contaminated with the microscale carrier matrix, interfering with further delivery. Reproduced with permission from ²³

Figure 2. Schematic representation of Multi-compartmental gene delivery systems. Reproduced with permission from ²³.

Figure 3. Iron homeostasis and disorders

Normal: ① Dietary iron enters plasma. ② Most iron (75%) is transferred to the bone marrow ③ for red blood cell (RBC) production. ④ RBCs circulate for 120 days and are digested by macrophages. ⑤ Iron is released back to plasma and recycled for ② another round of RBC production. ⑥ The rest of iron (25%) is taken up by organs for physiological function, stored in the liver or excreted by urine or bile secretion.

Iron overload: ⑦ When excess iron enters due to genetic disorders (hemochromatosis) or ⑧ blood transfusion (thalassemia, sickle cell anemia), ⑨ iron is accumulated in the liver and heart and exerts toxic effects. ⑩ Brain iron levels also increase with age or by

prolonged exposures to high iron. Chelation therapy: ⑩ Chelators increase iron excretion, but display serious side effects.

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Figure 4. RNA interference therapy to silence the DMT1 expression

A. Non-heme iron from diet is reduced to ferrous iron (Fe^{2+}) by the duodenal cytochrome B (Dcytb) and absorbed by the divalent metal transporter 1 (DMT1). Iron can either be stored in the cell by binding to ferritin, or released into the blood through ferroportin (FPN). Fe^{2+} is then oxidized by hephaestin and binds to transferrin (Tf). DMT1 siRNA in NiMOS given orally will promote degradation of DMT1 mRNA in the enterocyte and down-regulate DMT1 expression. As a result, intestinal uptake of non-heme iron from diet will be attenuated. **B.** Tf-bound iron in blood is first transported into the endosome of the erythropoietic tissues by the transferrin receptor 1 (TfR) and then reduced by Steap3. The resultant Fe^{2+} is transported by endosomal DMT1 to mitochondria for heme synthesis. DMT1 siRNA in NiMOS will not affect the transcription of DMT1 in the reticulocyte since the complex is not absorbed and unavailable in the circulation.

Figure 5. Schematic illustration showing the cross-sectional view of nanoparticles-in-microsphere oral system (NiMOS)

On the left is the scanning electron microscopy (SEM) image of gelatin nanoparticles, which are less than 200 nm in diameter, and can physically encapsulate plasmid DNA at a loading efficiency of >93%. On the right is the SEM image of 2–5 μm NiMOS with the overall DNA encapsulation efficiency of ~46%. Reproduced with permission from ¹⁸.

Figure 6. Distribution of NiMOS particles after oral administration

Gastrointestinal distribution following oral administration of ^{111}In -labeled gelatin nanoparticles and ^{111}In -labeled gelatin nanoparticles encapsulated in the NiMOS in 24-h fasted female Balb/C mice (A & B) or Wistar rats (C). Reproduced with permission from ²³.

Figure 7. IL-10 expression and therapeutic effects in IBD

Murine interleukin (IL)-10 transgene expression (A) and Reduction in the levels of proinflammatory cytokines TNF α (B) and IL-1 β (C) upon delivery of murine interleukin (IL)-10-expressing plasmid DNA administered in nanoparticles-in-microsphere oral system (NiMOS). (D) The therapeutic benefits of oral interleukin (IL)-10 gene therapy as determined by tissue histology upon oral administration of murine IL-10-expressing plasmid DNA in nanoparticles-in-microsphere oral system (NiMOS). Reproduced with permission from ¹⁸.

Figure 8. TNF- α and Cyclin-D1 silencing with NiMOS in IBD

mRNA and protein expression profiles in control and short interfering RNA (siRNA)-treated mice. Reproduced with permission from: Kriegel C, Amiji MM: Dual TNF-alpha/Cyclin D1 Gene Silencing With an Oral Polymeric Microparticle System as a Novel Strategy for the Treatment of Inflammatory Bowel Disease. *Clinical and Translational Gastroenterology* 2, e2 (2011). Reproduced with permission from ²¹.

Figure 9. Intestinal tissue histology before and after treatment

Microscopic evaluation of colonic tissue histopathology. Bright-field images of haematoxylin and eosin stained sections of the colon harvested from each control and test group. Images are shown at magnifications of $\times 10$ and $\times 40$ from tissue cryosections obtained on day 10 and day 12 of the study. Sections from the first control group show normal and healthy colon tissue. Intestinal tissues from the dextran sulfate sodium (DSS) control group, the group treated with blank and scrambled short interfering RNA (siRNA) nanoparticles-in-microsphere oral system (NiMOS) showed a severe infiltration of white blood cells, abnormal mucosal structure, and a certain degree of goblet cell depletion. Tissue

from the group receiving tumor necrosis factor- α (TNF- α), Cyclin D1 (CyD1), or combined TNF/CyD1 silencing NiMOS showed signs of regeneration and exhibited a tissue architecture more closely resembling that of healthy tissue in the normal control group. Occurrence of goblet cells is indicated by red arrows; cell infiltration and abnormal tissue histology is indicated by black arrows. Reproduced with permission from ²¹.

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Table 1

Iron chelators approved by the US Food and Drug Administration

	Deferoxamine (Desferal)	Deferasirox (Jajunu; Exjade)	Defepirone (Ferriprox)
Dose (mg/kg/day)	20–80	20–40	75
Dose route	s.c., i.v.	Oral	Oral
Oral bioavailability	Negligible	70%	40%
Half-life	6 h	8–16 h	2 h
Elimination route	Urine, Fecal	Urine, Fecal	Urine