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miRNAs in Gastrointestinal diseases: Can we effectively deliver RNA-based therapeutics orally?

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Short running title: miRNAs in GI diseases and their oral delivery

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Abstract

Nucleic acid-based therapeutics are evaluated for their potential of treating a plethora of diseases, including cancer and inflammation. Short nucleic acids, such as miRNAs, have emerged as versatile regulators for gene expression and are studied for therapeutic purposes. However, their inherent instability *in vivo* following enteral and parenteral administration has prompted the development of novel methodologies for their delivery. Although research on the oral delivery of siRNAs is progressing, with the development and utilization of promising carrier-based methodologies for the treatment of a plethora of gastrointestinal diseases, research on miRNA-based oral therapeutics is lagging behind. In this review, we present the potential role of miRNAs in diseases of the Gastrointestinal tract, and analyze current research and the cardinal features of the novel carrier systems used for nucleic acid oral delivery that can be expanded for oral miRNA administration.

Keywords:

Oral delivery; non-viral vectors; miRNA therapeutics; delivery

1. Introduction

Small or short nucleic acids, such as small interfering RNAs (siRNAs) and microRNAs (miRNAs), have an increasing literature presence in the last decade, which is attributed to their relatively recent discovery and recognition as potential therapeutic agents [1]. Their versatility and multifaceted functions in multiple diseases, including inflammation, cancer and infections, potentiate an increasingly intensive and expanding research field [2-5].

The activity of non-coding siRNAs and miRNAs relies on the natural RNA interference (RNAi) mechanism of the cells to inhibit the translation of a single or multiple messenger RNAs (mRNA), inducing a silencing effect for their respective targeted genes [1, 6]. It is generally regarded that siRNA molecules utilize the RNAi cell mechanism to target a single, specific mRNA [7], granting off-target effects may take place [8], while miRNAs target multiple mRNAs [1]. This distinction stems from the fact that siRNAs present full complementarity to their targeted mRNA, leading to mRNA cleavage, while miRNAs require only partial complementarity to exert their translation repression [1]. Interestingly, a comparable activity of the miRNAs to the siRNAs can take place in plants, where miRNAs present full complementarity between them and targeted mRNAs, leading to the eventual mRNA cleavage [9].

Although significant structural similarities exist between siRNAs and miRNAs, their prominent difference relies on that siRNAs are regarded as synthetic, exogenous molecules delivered to the cells, while miRNAs are natural, endogenous molecules transcribed by non-coding genes of the cells, although they can be exogenously delivered as well [10]. miRNAs have attracted keen interest due to their natural origin, as the cell's natural transcription products, which frequently share the same introns with protein-expressing genes and they are transcribed along with them [11-13].

The ability to regulate a plethora of genes enables the miRNAs to function as crucial mediators of disease onset and progression, such as in cancer, where their action can be either oncogenic or tumor suppressive [14]. Similarly, in inflammation, miRNAs can have pro- and anti-inflammatory activity [15]. Due to these functions and to the observed dysregulation of miRNAs between healthy and diseased tissues, researchers are evaluating the use of miRNAs as predicting biomarkers of disease development, as well as potential therapeutic agents or targets [14, 16]. For example, miR-21 is commonly over-expressed in lung cancer samples, and the levels of this miRNA in sputum was used as a biomarker for lung cancer detection [17]. miR-34a is considered a master tumor suppressor, commonly downregulated in multiple cancers, and the exogenous delivery of miR-34a using cationic liposomes for the treatment of solid tumors was evaluated in Phase I clinical trials [17, 18]. Although the treatment showed evidence of antitumor activity in a subset of patients, the study was halted due to immune-related severe adverse events, which may have been induced by the cationic-lipid based liposomal delivery technology, i.e., Smarticles [18-20].

The delivery of nucleic acids presents significant challenges, such as instability in circulation, short half-life, and limited cell uptake, among others [1]. To overcome these challenges, novel viral or non-viral delivery carriers are developed for the *in vivo* therapeutic translation of nucleic acids. Due to the structural similarities between siRNAs and miRNAs, similar delivery approaches can be used for both types of nucleic acids, and we will be presenting delivery approaches for both cases, despite our interest being on miRNAs.

miRNAs are water-soluble molecules, and despite their short half-life in circulation [1], parenteral administration has been the primary approach evaluated for their delivery. In contrast, oral delivery of therapeutic molecules is ubiquitously used with a variety of pharmaceutical formulations. The intestinal epithelium provides a large surface area for the absorption of nutrients and active

molecules, and oral administration is generally regarded as easy and safe [21]. Unfortunately, oral delivery of nucleic acids is significantly lagging behind in development. Here, we review existing methodologies developed for the oral delivery of nucleic acids using non-viral delivery strategies, focusing on polymeric and lipid carriers. Interestingly, limited research has taken place on the oral delivery of miRNAs, due, in part, to the relatively recent discovery of the miRNAs, despite their apparent significance in multiple diseases. Thus, in the sections below, we summarize current methodologies based on the oral delivery of nucleic acids in general (i.e., DNAs or siRNAs), which can find similar applicability in miRNA oral delivery.

2. Advantage and limitations of oral delivery for nucleic acids

Nucleases in the circulation and in different tissues, such as RNase A-type nucleases in the blood, can rapidly degrade nucleic acids [22]. Specialized carriers are being developed to protect nucleic acids and deliver them to the targeted tissues [23]. miRNAs and siRNAs also need to be delivered to the cytoplasmic compartment of the cells, where they exert their RNAi functions [1]. Due to their negative charge, hydrophilicity and high molecular weight, miRNAs exhibit low membrane permeability and, consequently, their cellular uptake and cytoplasmic entry are challenging [1]. Currently, the main approaches for the *in vivo* delivery of nucleic acids rely on viral and non-viral carriers [24]. The viral vectors have demonstrated great potential on transfecting cells and delivering their load, with high transfection efficiency and with the potential to target specific subsets of cells, such as tumor cells. However, their immunogenic potential and high development cost have hindered their progression [25, 26]. Non-viral carrier systems were the apparent alternative for delivering nucleic acids, as they rely on chemical systems, such as cationic liposomes and polymers. Though not as effective in transfecting cells as the viral vectors, their

lower production cost, chemical versatility, ubiquitous availability, and lower immunogenicity attracted significant attention [26]. Below, we focus on non-viral methodologies for the local administration to the gastrointestinal (GI) tract, without considering viral methodologies nor aiming for systemic circulation and administration.

Intestines provide a large surface area (~250 m^2) specifically for the absorption of nutrients [27]. The oral route of administration of active compounds has significant advantages, with the most prominent being its simplicity. Although the oral administration accounts for a large portion of the current drug formulations, the digestive tract presents challenges for the integrity of nucleic acid-based products.

Nucleic acid-based therapeutics require the efficient local or systemic administration of the nucleic acids for disease prevention or treatment. Currently, the most frequently-studied administration of nucleic acids has focused on the intravenous delivery (parenteral route) with the molecules entrapped in carriers. This approach is invasive, and specific conditions are required for the successful execution, such as sterility and specialized personnel [28]. In contrast, oral administration is simple, does not cause patient discomfort (under normal conditions), does not require specific conditions (i.e., sterility), and can lead to increased patient compliance [29]. Additionally, the gut epithelium is highly vascularized and nucleic acids can potentially enter the circulation for systemic treatment applications [30].

Whether the ultimate target of the oral administration of nucleic acids is the systemic circulation or local action, the gastrointestinal tract presents several barriers that can hamper their successful delivery. Any oral administration is initially exposed to the acidic contents of the stomach, with pH values as low as 1.5, and with strong enzymatic activity, such as the presence of pepsin. Subsequently, any oral formulation will progress to the small intestine, where the pH transitions

to neutral, but significant enzymatic activity is present, such as the presence of trypsin, lipases, amylases, proteases and nucleases [31-34]. The resulting harsh environments can be detrimental to the stability of nucleic acids or any carrier that may be used [34]. Oral delivery systems must be able to withstand the changing environment and enzymatic conditions, to reach the small or large intestine areas, and deliver their payload. In addition, targeting specific portions of the GI tract constitutes a significant challenge by itself. For example, targeting the stomach requires the prolonged residence of any active compounds in the stomach area, while withstanding the harsh pH and enzymatic environment. Such application would be exceedingly challenging for the nucleic acids, even with the existing nucleic acid carriers. Not surprisingly, only limited research has taken place for targeting the stomach area, where alternative to carrier-based approaches were used [35, 36]. In contrast, the existing literature on carrier-based methodologies for nucleic acid delivery primarily target the intestinal area, i.e., small bowel and colon, and focus on the protection of the nucleotides from the stomach's environment. Chemical modifications, particle size, and composition define the residence time and intestinal targeting capacity of the carriers, and thus the nucleic acid activity (i.e., upper or lower intestinal portions), as we elaborate below.

The intestinal surface is covered by a mucus layer of varying thickness, which captures and removes hydrophilic molecules, and has turnover times between one [37] to five hours [38]. Mucus is a viscous layer, separating the intestinal bacteria from the epithelial cells, and its purpose is to protect tissues which may come into contact with the environment. Mucus consists of more than 90% of water and contains mucins, large glycoproteins that create the highly viscous mucus, produced by the goblet cells [39].

Delivery carriers may be trapped by the mucus and be eliminated. In fact, orally-delivered nanocarriers can potentially rapidly transit through the GI tract by association with the chyme or

be trapped in the mucus layer, eliminated through mucociliary clearance [40]. Mucoadhesive delivery carriers, such as carriers based on the cationic chitosan or lipids, have been used for enhancing the uptake from and penetration through the mucus layer [40]. Unfortunately, the nanoparticles need to transverse the mucus fast enough to reach the live cells, and the mucoadhesive carriers may not transverse the mucus layer fast enough and may be removed with the clearance of the mucus prior to reaching the underlying cells [38, 40].

In some cases, the mucus layer is thinner or ever absent in specific areas of the GI tract during diseases, such as in inflammatory bowel disease (IBD) and ulcerative colitis (UC), while is thicker under different conditions, such as in Crohn's disease [41], which may indicate a potential therapeutic path for the respective diseases. Additionally, the use of mucolytic agents can improve the penetration of carrier particles through the mucus. For example, N-acetyl-L-cysteine is a mucolytic compound and has been evaluated for its ability to disrupt the mucosal layer and facilitate the penetration of nanocarriers [40, 42]. Under the mucosal layer, there is a layer of cells, primarily consisting of a single layer of tightly packed epithelial cells, composed of goblet cells, M cells, enterocytes, lymphocytes and endocrine cells [43]. Moreover, in inflammatory scenarios, immune cells (i.e., lymphocytes, macrophages) may infiltrate the intestinal mucosa [44-46].

An additional consideration regarding the oral delivery of nanocarriers is its unavoidable interaction with the intestinal microbiota. During the last decade, there has been an increase in understanding of the role of the microbiome as a determinant of human health status [47]. The interaction between microbiota and nanocarriers can occur both ways. For example, given the well-characterized antimicrobial activities of numerous nanoparticles, one must understand how the nanocarrier will be affecting or modulating the host intestinal microbiota [48]. On the other hand, many investigators are exploring ways to use the host microbiota in order to enhance the delivery

of their load. For example, some polysaccharides can be selectively metabolized by the intestinal microbiota. Thus, nanocarriers prepared with these materials can deliver their load locally at the intestine site when the nanocarrier interacts with the select bacterial species that are able to metabolize these polysaccharides [49, 50]. This appears to be an important consideration when dealing with specific GI tract diseases, such as IBD, known to have an altered intestinal microbiota [51, 52].

Depending on the therapeutic objective, different parts or cell layers of the GI tract can be targeted with the nucleic acids (Figure 1). For example, if the oral delivery of nucleic acids is intended for the treatment of IBD, intestinal cancers or cystic fibrosis, targeting of the epithelial wall and superficial cells will be sufficient. Furthermore, GI-localized and GI-targeted oral therapies have the advantages of acting directly on the diseased tissue, minimizing the risks of side-effects to other organs and tissues.

3. miRNA dysregulation in diseases of the GI tract

miRNAs regulate a plethora of natural biological processes, such as cell proliferation [53], cell movement [54], cell cycle [55], apoptosis [56], cellular metabolic pathways [57], as well as immunity [54], and inflammation [15], among others. Not surprisingly, miRNAs are involved in the functional homeostasis regulation of the GI tract, and their dysregulation is associated with several diseases, spanning from IBD to cancer [58-60]. For example, regarding GI motility and maintaining smooth muscle functionality, the miR-143/145 and miR199a/214 clusters regulate differentiation and proliferation of smooth muscle cells, while gain- or loss-of-function studies indicated that these miRNAs switch smooth muscle cells between proliferating and differentiated

states [61]. In another study, Biton *et al*. showed the balance between the goblet cell-specific TH1 and TH2 response are regulated by the miR-375 [62].

In GI tract diseases, a frequently studied group of miRNAs is the miR-29 family. The miR-29a and -29c were significantly upregulated in diseased tissues from patients with Crohn's colitis, one of the two major types of IBD, when compared to healthy controls [63]. In ulcerative colitis, miR-29a was also upregulated in diseased tissues compared to non-diseased tissue samples [64]. Interestingly, interferon-gamma (IFN-γ) production is increased during Crohn's disease [65] and plays an essential role in UC [66], although the miR-29 is reported upregulated in these diseases and targets the IFN-γ mRNA.

Additionally, Crohn's disease has been associated with up-/down- regulation of miR-19a, miR-1273d, miR-886-5p, miR-3194, miR-192, and miR-200a [67]. Importantly, the Suppressor of cytokine signaling 3 (SOCS3) gene is critical for the inflammatory response in Crohn's disease. miR-19b directly targets and suppresses SOCS3 to prevent the pathogenesis of this disease [68]. Celiac disease (CD) is a lifelong autoimmune disease triggered by dietary gluten [69], and multiple miRNAs' dysregulations are correlated (e.g., miR-182, miR-196a, miR449a) with the disease progression [70]. Finally, a study on 60 different humans with 120 tissue samples from IBD patients identified that the Programmed Cell Death 4 (PDCD4) gene, which is the direct target of miR-21, is involved in the IBD-associated carcinogenesis [71]. Not surprisingly, miRNAs have important functions in tumorigenesis and tumor progression. Duan *et al.* [72] identified that miR-130 promotes cell proliferation and migration in gastric cancer (GC), the fourth most common cancer worldwide [72]. Similarly, Wu *et al*. [73] reported from the analysis of serum and peripheral blood mononuclear cells of 90 patients with GC and 90 healthy individuals that overexpression of miR-421 in serum can be a potential biomarker for identification of GC.

In Table 1, we present some of the most prominently identified and studied miRNAs, and their respective dysregulation depending on the type of disease. Table 1 is not an exhaustive review of the existing literature, as there are several specialized review papers on identifying possible miRNA mediators for various GI tract diseases [59, 60, 74-77]. Our analysis demonstrates how miRNA dysregulations are prominent in GI tract diseases. Furthermore, we highlight miRNAs that could potentially be therapeutically explored through oral administration, while at the same time we illustrate the limited presence of relevant literature on miRNA delivery for such a promising route of administration for localized therapeutic action.

Table 1

Disease	Upregulated miRNAs	Downregulated miRNAs	References
Crohn's disease	miR-31, miR-206, miR-146a, miR-424, miR-663, miR-29a, miR-29c	miR-194b, miR-216b, miR-548e, and miR-559, miR-200b, miR-19a-3p, miR-19b-3p	[63, 78-81]
Ulcerative colitis	miR-155, miR-31, miR-126, miR-7, miR-135b, miR-223, miR-29a, miR-29b, miR-127-3p, miR-324-3p, miR-150, miR-20b and miR-125b-1	miR-188-5p, miR-215, miR-320a, miR-346, miR-200b, let-7, miR-125, miR-101, miR-26, miR-124	[63, 78, 82-87]
Colorectal cancer	Let-7g, miR-18a, miR-21, miR-31, miR-17-3p, miR-92a, miR-29a, miR-135	miR-16, miR-22, let-7c, miR-93, miR-126, miR-143, miR-145, miR-320, miR-498,	[77, 88-101]
Gastric cancer	miR-17-5p/20a, miR-125b, miR-451, miR-486, miR-17-5p, miR-21, miR-106a, miR-106b, miR-195, miR-378	Let-7a/f/g, miR-100, miR-133b, miR-148a, miR-1182, miR-1207, miR-29a/b/c	[76, 102-113]
Celiac Disease	miR-503, miR-449a, miR-492, miR-644, miR-182, miR-196a,	miR-105, miR-409-5p, miR-631, miR-659, miR-379, miR-566, miR-512-3p, miR-614, miR-380-5p, miR-135a, miR-124a, miR-	[70, 75, 114]

	miR-504, miR-330, miR-500	600, miR-618, miR-616, miR-189, miR-576, miR-412, miR-202, miR-299-5p, miR-323, miR-219, miR-31-5p, miR-192-3p, miR-194-5p, miR-551a, miR-551b-5p, miR-638 and miR-1290	

4. Current strategies for the oral delivery of nucleic acids

Delivery of nucleic acids through oral administration provides the potential for the treatment of GI-specific disorders. miRNA-dysregulations have been observed between healthy and diseased tissues in the GI tract, and with the increased understanding of different diseases, it is evident that miRNAs are potential therapeutic tools or therapeutic targets. The harsh GI environment has limited the delivery of large or unstable molecules, such as plasmids and short RNAs, but the development of highly innovative, multifunctional, non-viral drug delivery carriers has overcome many of these limitations. Though not the focus of this review paper, it is worth mentioning that there have been significant efforts in chemically modifying naked oligonucleotides to enhance their stability and evade nuclease degradation. For example, GEM231, a clinical trial-studied antisense regulatory subunit α of type I protein kinase A mixed-backbone oligonucleotide with a hybrid DNA/RNA structure and 2′-*O*-methyl-ribonucleosides at the 5′ and 3′ ends [115], was evaluated by Tortora *et al.* for targeting the Protein kinase A type I subunit Ria, following oral administration [116]. The investigators reported a tumor growth inhibition using a subcutaneous xenograft model of colon cancer. Below, we summarize drug delivery carriers that have been used for oral delivery of nucleic acids, and by extension can potentially be utilized for delivering miRNAs.

a) Polymer-based vectors

Polymeric nano-/micro- carriers have attracted significant attention for the delivery of active compounds through different routes of administration. The versatility of the polymeric molecules, in terms of ease on altering their physicochemical properties, allowed for the development of novel structures and carriers (Figure 2).

There have been two major approaches on the utilization of polymers for transfection, associated with the mechanism that nucleic acids are incorporated into the carriers: a) polymeric carriers using electrostatic interactions between the polymer and nucleic acids (condensing systems), and; b) polymeric carriers that physically entrap nucleic acids (non-condensing systems).

In the first case, the negatively charged nucleic acids are electrostatically complexed with positively charged cationic polymers, developing structures called polyplexes [117]. One commonly used cationic polymer for nucleic acid condensation has been polyethyleneimine (PEI). PEI is composed by repeating ethyleneimine units, and can be either linear or branched (having primary, secondary, or tertiary amines) [1]. The PEI-based nanocarriers are up-taken by the cells through endocytosis, and due to their high proton buffering capacity, they can escape endosomes and release their cargo, based on the "proton-sponge effect" [117]. Additionally, the net charge of these polyplexes is positive, which facilitates their interactions with the cell membranes and their cellular uptake.

Transfection studies using PEI have used a variety of molecular weights and/or structures of the polymers. For example, Dai *et al.* [118] used branched-PEI (bPEI) polymers of molecular weight at approximately 25,000 Daltons to complex miR-193a-3p for colonic delivery. In their study, the authors did not utilize the oral route of administration. Instead, they directly delivered to the colon using a 100 ul rectal enema of the PEI-miR polyplexes in mice treated with dextran sodium sulfate

(DSS)-induced colitis. The investigators observed a significant amelioration of the induced colitis, following miR-193a-3p treatment, which was mediated by the PepT1 protein.

In a short communication, Klausner and Leong [119] reported the evaluation of bPEI and liner-PEI (lPEI) with a molecular weight of 25,000 for the oral delivery of a Secreted embryonic alkaline phosphatase (SEAP)-expressing plasmid. The polyplexes were fed to mice in the form of gelatin cubes over a period of 2 days. The investigators reported that although there was extremely overall low systemic SEAP activity following the oral administration of the different formulations, there was significantly increased activity compared to the baseline. Several studies have evaluated the use of PEI for oral delivery, while being incorporated inside another polymer, to enhance the PEI-nucleic acid complexes' stability. For example, Laroui *et al.* [120] reported the use of bPEI (MW: 1,800 D) for complexation with tumor necrosis alpha (TNF-α) siRNA and encapsulation inside polylactide nanoparticles for the oral delivery in a mouse model of IBD, induced by LPS-treatment. The researchers reported that the siRNA-nanoparticles, which had an approximate diameter of 380 nm, were taken up by macrophages *in vitro* and inhibited TNF-α expression, while oral administration of the nanoparticles reduced the TNF-α expression in the colonic tissue of the LPS-treated animals.

Chitosan, a natural polysaccharide derived by deacetylation of chitin [121], has attracted significant attention for oral delivery of nucleic acids. It is regarded as a biocompatible and biodegradable polymer, and due to its cationic nature, it can condense with nucleic acids [121]. Furthermore, chitosan demonstrates mucoadhesive properties, being capable of penetrating the mucosal layer and transfecting epithelial and immune cells, as well as can enhance transcellular and paracellular transport of active compounds across intestinal epithelial monolayers [122]. Roy *et al.* [122] demonstrated that chitosan nanoparticles of an approximate size of 100 – 200 nm,

complexed with plasmid DNA (pDNA; pCMVArah2), orally delivered to a murine model of peanut allergen-induced hypersensitivity, reduced the severity of anaphylactic responses following intraperitoneal challenge with Arah2 protein after sensitization with peanut butter. The researchers concluded that oral administration of the chitosan-pDNA nanoparticles can transfect and induce immune responses in mice, as increased levels of IgA were detected in fecal extracts, and increased IgG2a levels were detected in the serum.

To enhance chitosan's solubility, affinity with and protection of nucleic acids, as well as its bioadhesive properties, several derivatives have been developed. Chunbai *et al*. [123] developed mannose-modified trimethyl chitosan-cysteine nanoparticles for oral delivery of siRNA against TNF-a. The nanocarriers with the siRNA successfully inhibited TNF-α production in macrophages *in vivo*, protecting mice with acute hepatic injury from inflammation-induced liver damage. Subsequently, the same research team evaluated the modified-chitosan nanocarriers with the same siRNA in a rat model of the disease, and acquired similar results [124].

Bernkop-Schnurch and Krajicek [125] studied the mucoadhesive properties of chitosan, when complexed with EDTA and determined that the adhesive force of the conjugate was higher than that of chitosan-HCl [125]. In another study, Loretz *et al.* [34] assessed different methodologies for improving the stability of pDNA for oral delivery. They determined that EDTA had the strongest inhibitory activity against nucleases. They synthesized and evaluated an EDTA-chitosan conjugate and determined that it was efficient to protect pDNA and inhibit its degradation by nucleases.

Zhang *et al.* [126] developed galactosylated trimethyl chitosan (GTC)-cysteine nanoparticles for the oral delivery of a mitogen-activated protein kinase kinase kinase kinase 4 (Map4k4) siRNA for the treatment of DSS-induced ulcerative colitis. The nanoparticles were prepared using ionic

gelation of GTC with tripolyphosphate or hyaluronic acid. The researchers determined that daily administration of the nanoparticles with the siRNA significantly improved body weight loss and colon length shortening, due to DSS treatment. Additional examples of chitosan particles for the delivery of nucleic acids are presented in Table 2.

Gelatin is a mixture of water-soluble macromolecules (peptides or proteins) derived from the hydrolysis of collagen present in animal skin, connective tissue and bones of animals. Depending on its method of hydrolysis, the gelatin products can have a varying isoelectric point of neutral to basic (pH 7-9; Type A) or acidic (pH 4.5-6; Type B) [127]. Gelatin is commonly used for capsule preparation and has historically been used in food products, cosmetics and pharmaceuticals, being considered as "generally regarded as safe (GRAS)" material, according to the United States Food and Drug Administration [128]. Gelatin naturally carries the Arg-Gly-Asp (RGD) amino acid sequences, which results in improved cell adhesion [129]. Gelatin is a biodegradable and biocompatible natural product, and has multiple functional groups that can be modified to endow to the polymer desired properties, such as attachment of positively charged molecules. Representatively, Kaul and Amiji [128] utilized gelatin Type B to prepare nanoparticles of unmodified gelatin and pegylated gelatin containing pDNA expressing β-galactosidase. Initially, the researchers reported that the pegylated gelatin nanoparticles efficiently transfected Lewis Lung Carcinoma (LLC) cells using intravenous injections to LLC-bearing C57BL/6J mice, as indicated by significant expression of β-galactosidase in the tumors. Subsequently, the same research group [130] formulated a multicompartmental oral delivery system, consisting of gelatin nanoparticles entrapped inside poly-caprolactone (PCL) microparticles. The researchers initially optimized the conditions for the preparation of the microparticles using a factorial design and continued by using this formulation for the oral delivery of pDNA expressing β-galactosidase. The formulation relied

on the initial preparation of the Type B gelatin nanoparticles loaded with the pDNA, which were subsequently entrapped inside the PCL microparticles and administered orally to rats [131]. The microparticles demonstrated prolonged residence in the small and large intestine, while plain gelatin nanoparticles traversed quickly through the GI tract and accumulated in the large intestine within 1 h post oral administration [131]. In another study, the same team [132] utilized the microparticles loaded with gelatin nanoparticles to entrap TNF-α specific siRNA for oral delivery in a DSS-induced acute colitis mouse model. The administration resulted in decreased colonic levels of TNF-α, reduction of pro-inflammatory cytokine levels, such as IL-1β and IFN-γ, and an increase in body weight for treated animals vs. untreated.

To improve the gelatin's properties, several modifications of the polymer have taken place [127], though not all of these formulations have been evaluated for oral delivery. For example, due to the highly hydrophilic nature of gelatin, its nanoparticles may be unstable and require chemical modification of the polymer so that its nanostructured form will remain stable in blood circulation [133]. Kommareddy and Amiji [133] prepared gelatin nanoparticles with the gelatin polymer being crosslinked using 2-iminothiolane, introducing disulfide bonds in the macromolecular structure of the nanocarriers to stabilize them. The higher intracellular glutathione concentration in comparison to the extracellular regions, such as in the blood [134], allows for the selective destabilization of disulfide-stabilized nanocarriers within the cells' cytoplasm. Within the cells and in the presence of glutathione or other redox enzymes, the disulfide bonds would break, and the gelatin molecules would unfold, releasing the load of the nanocarriers. The researchers showed that the thiolated gelatin nanoparticles strongly transfected NIH-3T3 murine fibroblast cells, with the transfection being detected stable for up to 96 h [133].

In the case of non-condensing polymeric materials, poly(lactide-co-glycolide) (PLGA) polymers have extensively been studied for oral delivery of nucleic acids. These polymers can be produced in various molecular weights, from a few thousand to a few hundreds of thousands, are biodegradable, biocompatible and FDA approved, and they form a solid polymeric core, capable of isolating their load from the environment for protection [28]. Not surprisingly, these polymers have been used for the oral delivery of sensitive molecules, such as insulin [135, 136]. Furthermore, PLGA polymers promote endosomal escape, through a selective reversal of the surface charge of the particles (from anionic to cationic) in the acidic endosomal/lysosomal compartments, which causes the particles to interact with the endosomal/lysosomal membrane and escape into the cytoplasm [137]. The oral delivery of nucleic acids using PLGA polymers was evaluated in several studies. Kaneko *et al*. [138] showed that oral delivery of PLGA microparticles containing pDNA encoding HIV gp160 induced cellular and humoral responses. In fact, the oral delivery demonstrated improved effect compared to intramuscular delivery of the pDNA loaded-particles in protecting against recombinant HIV challenge. The microparticles were prepared using a double emulsification method. Similarly, He *et al.* [139] encapsulated DNA encoding hepatitis B virus (HBV) HBsAg in PLGA microparticles to evaluate the induction of local and systemic HBsAg-specific immunity after a single dose of administration. Mice treated orally with the microparticles showed an antigen-specific IFN-γ production and cytotoxic T lymphocyte responses in spleen and gut-associated lymph tissue following *in vitro* re-stimulation with HBsAg. Nonetheless, the researchers mentioned that the observed activities were relatively low, and attributed this to the single-dose administration of the microparticles. More recently, Du *et al.* [140] developed a Porcine Reproductive and Respiratory Syndrome Virus (PRRSV) DNA vaccine encapsulated in PLGA nanoparticles modified with Ulex europaeous agglutinin 1 (UEA-1) for

preferentially binding to M cells in the GI tract. The researchers observed enhanced mucosal and systemic immune responses following oral administration of the nanoparticles to mice and piglets. Huang *et al.* [141] encapsulated a TNF-α siRNA in PLGA nanoparticles decorated on their surface with galactosylated chitosan to target galacto-type lectin on macrophages. Following oral administration, the nanocarriers crossed the physiological barrier in the colon, and alleviated DSS-induced colitis in mice, as presented by the histological evaluation of the colonic tissue and animals' body weight changes. Additionally, the grafting of the galactosylated chitosan improved the macrophage uptake and the kinetics of endocytosis [141]. Laroui *et al.* [142] encapsulated siRNA targeting TNF-α in PLA-PEG nanoparticles that were decorated with the Fab' portion of the F4/80 antibody for improving the targeting against macrophages as well. The researchers orally administered the nanoparticles inside a chitosan/alginate hydrogel in DSS-treated mice, and concluded that the nanocarriers attenuated colitis, with improved efficacy compared to the undecorated nanoparticles, and the animals treated with the formulation exhibited reduced weight loss and improved myeloperoxidase activity [142].

Table 2

Polymer name	**Condensing / Natural**	**Representative studies/literature**
Polyethyleneimine (PEI)	Yes / No	i) branched-PEI (bPEI) polymer of MW at ~25 kDa complexed with miR-193a-3p for colonic delivery [118] ii) bPEI and lPEI with MW ~25 kDa for oral delivery of a SEAP-expressing plasmid [119] iii) a combination of lPEI and lipids were used to entrap plasmid DNA (pDNA; gWiz-luciferase) into nanocarriers for oral delivery and feasibility evaluation [143]

		iv) oral Delivery of siRNA using a combination of PEI and PLGA polymers for the formulation of nanoparticles [144] v) PEGylated PEI/pDNA polyplexes entrapped in PLGA microparticles for oral delivery [145]
Chitosan	Yes / Yes	i) chitosan -EDTA conjugates for oral delivery of plasmid DNA [34] ii) orally administered AuNP–siRNA–glycol chitosan–taurocholic acid nanoparticles for delivery of Akt2 siRNA [146] iii) oral administration of chitosan/siRNA nanoparticles [147] iv) trimethyl chitosan-cystein nanoparticles for the oral delivery of TNF-α siRNA [148] v) imidazole-modified chitosan and trimethylchitosan nanoparticles with encapsulated siRNA against CDX2 for oral treatment against gastric lesion [149] vi) Codelivery of mTERT siRNA and paclitaxel by N-((2-hydroxy-3-trimethylammonium) propyl) chitosan chloride nanoparticles [150] vii) Galactose-modified trimethyl chitosan-cysteine nanoparticles for encapsulating shRNA against surviving pDNA and siRNA against VEGF for treatment of hepatoma [151]
Gelatin	Yes / Yes	i) Gelatin nanoparticles inside PCL microparticles for the delivery of siRNAs or plasmid for the treatment of IBD [132, 152-154]
PLGA	No / No	i) Surface charge optimization of PEG5k-b-PLGA10k for oral delivery of siRNA [155] ii) PLGA nanoparticles decorated with galactosylated chitosan for the oral delivery of siRNA against TNF-a for treatment of colitis [141]

		iii) PLA-PEG nanoparticles decorated with Fab' and encapsulated of siRNA against TNF-a for the treatment of colitis [142] iv) hyaluronic acid (HA)-functionalized PLGA nanoparticles with encapsulated CD98 siRNA and curcumin for the treatment of ulcerative colitis [156]

b. Lipid-based vectors

Cationic lipids are commonly used for complexation with nucleic acids and their *in vivo* parenteral delivery [1]. These complexes, also termed as lipoplexes, have found extensive applicability for the therapeutic delivery of plasmids or short nucleic acids, such as siRNAs or miRNAs. Most frequently used methodologies rely on the formulation of liposomal carriers with cationic lipids, which in turn are complexed with the negatively charged nucleic acids [157].

Unfortunately, lipid-based carriers have found limited applicability in the oral delivery of nucleic acids. This is a direct result of the inherent lack of stability of liposomes and other lipid-based materials in the gut [158]. Not surprisingly, cationic lipids are predominately used with other routes of administration. For example, Zhang *et al.* [159] delivered intrarectally administered siRNA targeting TNF-α complexed with the commercially available transfecting agent, Lipofectamine 2000, in a DSS-induced mouse model of inflammatory bowel disease. The administration led to a relative reduction of the TNF-α levels and a significant reduction in the inflammatory cell infiltration in the colonic tissue. In another study, Fichera *et al.* [160] utilized the Lipofectin transfecting agent for delivering intrarectally a plasmid to express the normal human adenomatous polyposis coli (APC) gene to C57BL/6J-Apc^{Min} mice. The researchers reported a low transfection efficiency for the approach, though the prolonged treatment indicated an improvement on the transfection. Sugimoto *et al.* [161] evaluated the expression of IL-22 gene on

alleviating the DSS-induced colitis in mice, by the delivery of an IL-22 expressing plasmid using (1,2-dioleoyl-3-trimethylammonium propane–cholesterol [DOTAP-cholesterol])/DNA–condensing agent–2 complexes. For their optimal delivery to the colonic tissue, the authors evaluated i.v., intrarectal and direct microinjection of the lipids/DNA complexes and determined that the optimal approach was to directly microinject the treatment into the colonic mucosa to efficiently deliver the gene. Furthermore, the authors reported that the gene delivery enhanced mucus production and attenuated the inflammation.

More recently, Ball *et al.* [162] evaluated the fate of orally-delivered siRNA lipid nanoparticles (LNPs). During prior work, the team developed nanoparticles using lipidoids [163], cholesterol, DSPC and PEG2000-DMG for the delivery of siRNA against GAPDH, which they evaluated *in vitro* against Caco-2 cells [164]. In a later study, they evaluated the LNPs in different conditions *in vitro*, such as varying pH solutions. The LNPs maintained their potency and siRNA against luciferase induced ~80% gene downregulation in HeLa cells. In contrast, pepsin and bile salts greatly diminished the activity of the encapsulated siRNA, indicating LNPs inability to protect the nucleic acids under certain conditions, though the authors declared that at "fasting"-state pepsin concentration, siRNA activity was partially retained. Furthermore, the authors mentioned that mucin prevented LNP gene silencing activity *in vitro*, though orally administered LNPs in mice stayed in the GI tract for at least 8 h post-administration, and entered the cells of the small intestine and colon [162]. These representative studies demonstrate the challenges associated with lipid nanocarriers and oral administration.

c. The size of particles and intestinal absorption

We have described above several formulations for oral administration that have varying particle sizes, spanning from nanometers to few micrometers in diameters. The particle size greatly affects

the absorption through the mucus and intestinal walls [165]. Indeed, there is a size dependence of the deposition of the particles to the intestinal walls [166]. Particles of 10 μm demonstrated deposition, but particles with smaller sizes, particularly at the nanometer sizes, demonstrated the most robust binding to the tissue. One significant barrier for the particles' cellular uptake is their transport through the mucus layer. Sufficient pegylation of the surface of the particles enhances the transverse of the nanoparticles through the mucus layer. It has been reported that 40% lower surface coverage of the particles with 2 kDa PEG, causes a 700-fold decrease in the transport rate, while an increase of the PEG's molecular weight to 10 kDa results in a 1000-fold increase in transport. Thus, lower molecular weight PEG chains densely covering the particle's surface promotes transportation through the mucus [167]. Furthermore, nanoparticles with a size between 200 and 500 nm efficiently transported through mucus, when PEG-modified, representing a potential desirable particle size [168].

Although larger particle sizes provide significant advantages in formulation preparation and drug delivery, such as improved drug loading and prolonged release kinetics, very large particles may not diffuse sufficiently through the mucus [40]. Furthermore, cellular uptake depends on the particle size, with larger particles being less uptaken by cells [169]. For example, *in vitro* analysis of polystyrene nanoparticles of varying diameters coated with and without d-α-tocopherol polyethylene glycol 1,000 succinate (TPGS) indicated a reduction on the cellular uptake *in vitro*, when the particle size increased above 200 nm in Caco-2 and Madin-Darby Canine Kidney (MDCK) cell lines [170].

5. Conclusions and future perspective

The discovery of RNA-based therapeutics is one of the most significant breakthroughs in recent years. siRNA- and miRNA-based therapeutics have demonstrated great promise for the treatment

of various GI tract diseases and the field is still progressing rapidly. However, the successful oral delivery of these RNAs is in its infancy and still evolving, particularly regarding miRNA applications.

Among various strategies for oral delivery of RNAs, non-viral carrier systems appear to be a better alternative for delivering nucleic acids. Although not as effective in transfecting cells as the viral vectors, the lower production cost, chemical versatility, ubiquitous availability, and lower immunogenicity represent significant advantages for the non-viral carrier systems. In particular, we consider that polymeric nanocarriers represent an ideal strategy for the oral delivery of active compounds, miRNAs and siRNAs. The versatility of the polymeric molecules, in terms of ease on altering their physicochemical properties, allows for the development of novel structures and nanocarriers that can be tailored for each therapeutic application.

However, some limitations for successful oral administration of RNA-based therapeutics must be overcome. These include: 1) determining how to accurately deliver the therapeutic agents into the targeted GI tract cells; 2) evaluate the potential of co-delivery approaches of RNA-based therapeutics with drugs currently used for GI tract diseases, and; 3) how it interacts with the intestinal microbiota from, both, a healthy individual as well as an individual suffering from a GI disease. In this point, more research is warranted on the mechanisms and effects of select nanocarriers on the GI, the microbiota and on the impact that microbiota may have in the outcome of therapies involving drug delivery nanosystems through the GI tract. In summary, we estimate that in the near future, RNA-based therapeutics will overcome the existing limitations, and therapeutic oral delivery of miRNAs and siRNAs will progress into the clinic, having the potential to contribute significantly to the treatment of GI tract diseases.

Executive Summary

Background

- Although there are a plethora of studies demonstrating the significance of miRNAs in different GI tract diseases, limited research exists on the therapeutic oral delivery and applications of miRNAs.
- Nucleic acid products, such as siRNAs and plasmid DNAs have been studied for the oral delivery. The structural similarities between siRNAs and miRNAs indicate that the limited research of oral delivery of miRNAs is the result of the relatively recent discovery of these molecules and the existing delivery technologies used for siRNA oral delivery have not yet been utilized with miRNAs.

Limitations of nucleic acid-based oral delivery

- Three aspects of the GI physiology primarily define design, development and success of orally administered carriers for nucleic acids: a) the extreme pH environments that vary from the very acidic environment in the stomach to the neutral to basic environment at the small and large intestine; b) the strong enzymatic activity present in the GI tract, including pepsin, trypsin, lipases, amylases, proteases and nucleases, which can destabilize or degrade carriers and nucleic acids, and; c) a tight epithelium surface covered with mucus, which limits the penetration of smaller or larger structures.

Carriers for oral delivery of nucleic acids

- Stability, capacity to adequately protect nucleic acid-loads and to deliver them in the cells' cytoplasm are critical in the design of effective carriers.

- Polymeric molecules allow for versatile preparation of carriers with specific properties, as well as permit subsequent carrier surface modification for further functionalization.
- Particle size significantly affects particle uptake by the GI tract.
- Lipid-based carriers have found limited application in the oral delivery of nucleic acids.

Figure 1: Uptake of delivery carriers by the intestinal epithelium and the routes of the carriers can utilize to be taken up by and penetrate the intestine. Carriers can enter the lamina propria by: a) the paracellular route; b) via transcytosis through enterocytes; c) transfection of epithelial cells; d) the transport through dendritic cells or e) M-cells. The carriers can: 1) gain access to the systemic circulation; 2) transfect lamina propria cells; 3) induce the expression of genes through transfection, which can access 4) the bloodstream; or 5) be processed by lamina propria cells; and 6), 7) induce immune responses, depending on the carrier's load. The figure is a reprint with permissions from O'Neil *et al.* [43]

Figure 2: Structure of polymeric molecules used for the development of nucleic acid delivery systems. Gelatin structure was reprinted with permissions from Sahoo *et al.* [127]

Table 1: Representative miRNAs up- and downregulated in different disease conditions of the GI tract

Table 2: Representative research for the different studied polymers for oral administration of nucleic acids

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