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Polymeric nano- and microparticle technologies for oral gene delivery

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Gene therapy refers to local or systemic administration of a nucleic acid construct that can prevent, treat and even cure diseases by changing the expression of genes that are responsible for the pathological condition. Oral gene therapy has significant promise for treatment of local diseases such as inflammatory bowel disease and for systemic absorption of the expressed protein therapeutics. In addition, efficient oral delivery of DNA vaccines can have significant impact in disease prevention. The use of polymeric gene delivery vectors promises the translation of this experimental medical concept into clinical reality. This review addresses the challenges and opportunities in the development of polymer-based nano- and microparticle technologies for oral gene therapy. Specifically, the discussion is focused on different synthetic and natural polymers used for formulating nano- and microparticle technologies and the use of these delivery systems for oral DNA administration for therapeutic and vaccination purposes.

Keywords: gastrointestinal tract, gene therapy, microparticles, nanoparticles, plasmid DNA, polymeric vaccines

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1. Introduction

Gene therapy represents a new paradigm in the prevention and treatment of many different diseases. It is designed to either alleviate the genetic defects in cells or to provide additional, protective effect [1]. Gene therapy is an approach to prevent, treat and even cure diseases by changing the expression of genes that are responsible for the pathology. Given that genes regulate all the basic physiological processes in the body, there is tremendous potential for DNA-based molecules to be employed as therapeutic agents [2,3]. Over the last decade, the field of gene therapy research has seen significant growth in both academia and industry with ~ 900 clinical trials pursued over this period in many different countries [301]. A major challenge in systemic gene therapy is the development of a vector system that can allow for the safe and efficient delivery of plasmid DNA to the target tissue, followed by cellular internalization and processing, for continuous production of the gene product at therapeutic levels and for the necessary duration [4-7].

At present, viral and non-viral vectors are used for systemic gene delivery [8,9]. Although viruses are very efficient in gene delivery and transfection, their clinical utility is plagued with severe toxicity concerns that include immunogenic reactions and integration with the host chromosome [10,11]. Therefore, safe and effective non-viral gene delivery systems are being aggressively considered, in order to translate gene therapy strategies from an experimental approach into clinical reality for patients. Among the non-viral vectors, polymeric gene delivery systems have received a lot of attention [12]. Due to their versatility, ease of synthesis and fabrication into various geometries, and surface modification for targeted delivery to disease sites, polymer-based delivery systems offer tremendous promise for systemic

gene therapy. Polymers used for gene delivery include those with a positive charge that can condense with negatively charged DNA (i.e., polyplexes) or others that interact with DNA by physical incorporation or encapsulation (i.e., non-condensing systems) [13].

Low molecular weight pharmaceuticals are associated with a number of side effects due to their nonspecific actions in the body. Since DNA-based therapeutics are more selective in recognition of their molecular target and are preferentially meant to express the proteins at the target site by incorporating very specific promoters in nucleic acid constructs, there is potential that gene therapy will be associated with significantly less toxicity, compared with low molecular weight drugs [14]. Molecular therapies that include administration of plasmid DNA, antisense oligonucleotides, aptamers, small interfering RNAs (siRNAs), ribozymes and DNAzymes are all considered under the context of gene delivery. The delivery challenges ranging from optimization of the formulation to localization at the disease site, cellular uptake and transfection are common with all DNA- and RNA-based therapies. Without a delivery system, intracellular localization of nucleic acid molecules has been limited because of low biological half-life as a result of rapid degradation by endogenous nucleases, and natural cellular barriers such as the negative charge on the cell surface repelling the negatively charged DNA or RNA molecule upon systemic delivery [15].

Oral gene therapy has significant potential for local and systemic diseases, such as gastric and duodenal ulcers, inflammatory bowel disease (IBD), gastrointestinal infections and for the oral administration of DNA vaccines to provide mucosal and systemic immunity [11,16,17]. At present, gene therapy involving administration to the gastrointestinal tract as the target site has been limited to preclinical research. The gastrointestinal tract offers an interesting target for gene therapy as a patient-friendly, non-invasive route that also exhibits features such as a large surface area, presented by the gut epithelium for uptake and expression of DNA that encodes for therapeutic proteins. Long-lasting therapeutic gene expression can be achieved due to the presence of a large number of stem cells in the intestinal crypts. Oral adminstration also allows access to the luminal side of the intestine in the case of intestinal disorders [18]. Several investigators have researched the potential of DNA-based therapeutics for the gene therapy of local gastrointestinal disorders and allergies. The gastrointestinal tract is also a very interesting target because it serves as the entry portal for many pathogens. Using DNA-based vaccines, it is possible to establish an immunological barrier against pathogens entering via the mucosal membrane [17,19,20]. Proof-of-concept studies employing non-viral vectors for oral gene therapy and oral immunization using DNA vaccines have been provided [21,22]. However, the use of non-viral vectors in the past has resulted in a very low transfection efficiency. The reason for this low transfection efficiency of non-viral vectors, including polymeric delivery systems, is partly because of the formidable barriers exhibited by the gastrointestinal tract. Among the barriers to effective gene delivery are proteolytic enzymes in the gut lumen and on the brush border membrane, the mucus layer, gut flora, and epithelial cell lining, which prevent tissue-specific localization, cellular uptake and could prematurely degrade the translated protein. Nucleases are also present in the gastrointestinal tract, and can readily degrade the nucleic acid construct [23]. Figure 1 illustrates the biological barriers to oral gene delivery at the extracellular and intracellular level.

2. Polymers for gene delivery

Polymers have become an integral part of the development of any novel drug delivery system. Factors such as the chemical structure, composition, molecular weight and morphology of the polymer (amorphous/crystalline/residual stresses); the size and geometry of the delivery system; and the process of degradation (enzymatic or non-enzymatic); govern the behavior of polymer in the body when administered in a drug formulation [24-26]. Some examples of polymers used for gene delivery are shown in Table 1.

The polymer selection itself is a judicious process and the following issues should be considered:

- Non-reactivity: chemical inertness with respect to the active compound and the biological environment.
- Biocompatibility: the materials should be compatible with living cells and tissues that come in contact with the polymer. This also holds true for any degradation products.
- Non-pyrogenicity: should be free of any pyrogenic factors.
- Impurities: all the impurities should be well established and present in minimal amounts. The impurities, if present, also should be biocompatible or should not pose any toxicity at the amounts present.
- Regulatory issues: the polymer should be available in medical or pharmaceutical grade purity and must be approved by the regulatory authorities for human use.

In addition to the abovementioned characteristics, certain specific characteristics are required which include the following:

- Loading capacity: if complexation or chemical conjugation is the method used for preparation, then the polymer must have sufficient reactive groups to promote these interactions.
- Permeability: permeability to drug molecules and water will govern the diffusivity and release of the payload.
- Swellability: this is of relevance when designing a floating or a bioadhesive delivery system.
- Viscoelasticity: is an important controlling parameter for gel-forming and adhesive systems.
- Sensitivity to local environment: the triggering factor could be pH, specific enzymes, or even the microbial flora prevailing in the gastrointestinal tract.

Although the maneuverability around each of the above factors is very limited for a sterile dosage form, it becomes more flexible when developing an oral product. Parallel

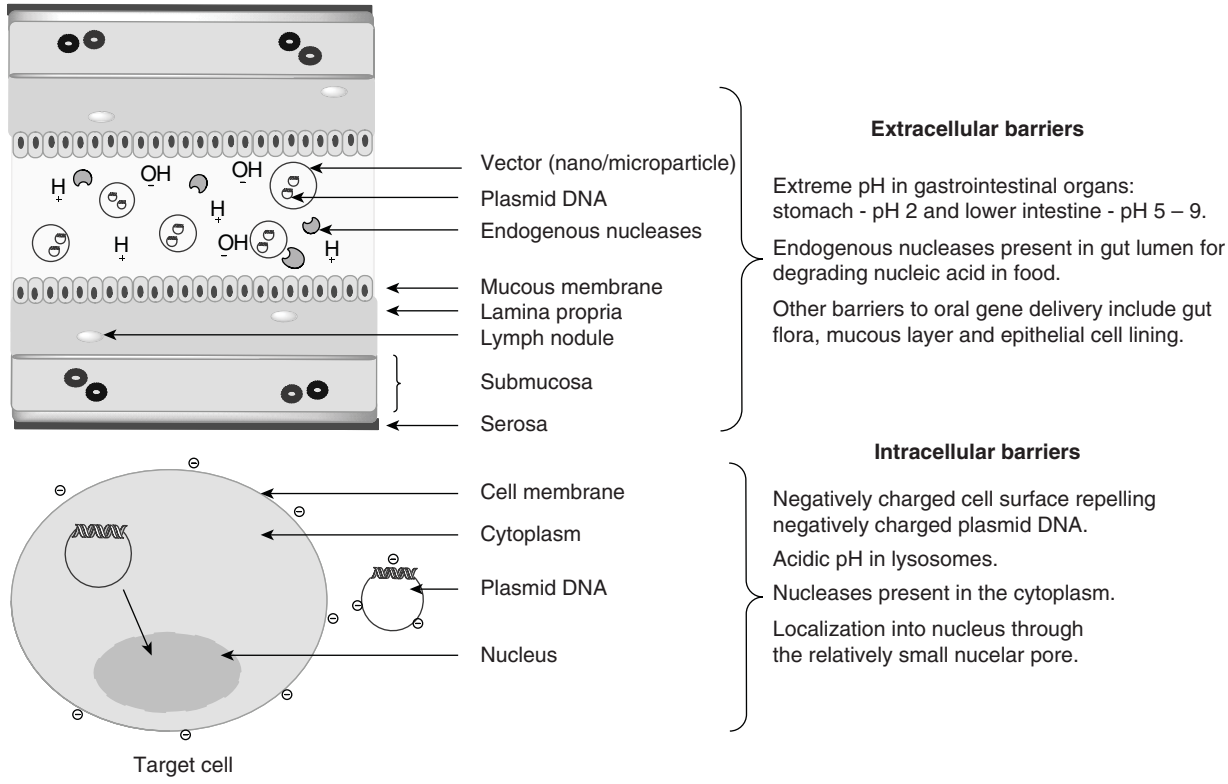


Figure 1. Anatomical and cellular barriers to oral gene delivery and transfection. Although there is significant potential for oral gene therapy, it faces several challenging, extracellular and intracellular hurdles to overcome.

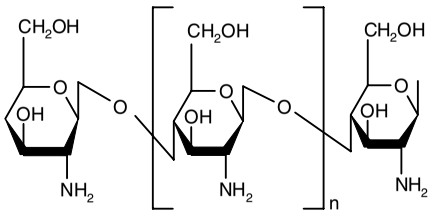
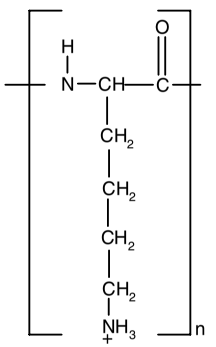
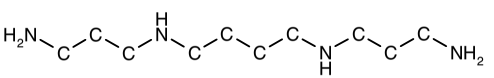
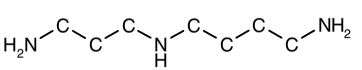
developments in the field of excipient science have contributed to a range of new high-performance polymers, making the choice of an approved polymer for oral administration relatively easier. Polymers that are employed for gene delivery purposes can be further classified into condensing and non-condensing systems based on their ability to form electrostatic interactions or physically encapsulate the DNA molecule.

2.1 Condensing polymers

An effective gene delivery system should be able to successfully mask the negative charge of DNA, resulting in efficient cellular internalization. Polycationic polymers have the ability to form electrostatic interactions between their positively charged functional groups and the negatively charged phosphate groups of DNA. This results in some degree of neutralization of the negative charge on the DNA molecule to form condensed particles, called polyplexes, which are capable of undergoing endocytosis [28-30]. The DNA condensing property of positively charged polymers can be exploited for formulating micro- and nanoparticles by easily manipulating the polymer molecular weight, salt concentration in buffer, pH of the buffer solution and the ratio of polymer to DNA. Condensed particles possess a net positive surface charge, which usually translates into reasonably efficient cellular uptake. Such systems are also very efficient in protecting their payload from degradation by endogenous nucleases [31,32]. Most condensing polymers, due to their cationic nature because of the presence of amino groups, can also be easily modified to include a targeting ligand and/or grafting polymer to make them specific and targeted to a specific cell type. The overall goal of any polymeric system is to enhance gene transfection at the target site. A range of natural and synthetic polycations have been used, including modified gelatin, chitosan, poly(L-lysine), poly(L-arginine), protamine, speramine, spermidine, poly(ethyleneimine) (PEI), poly(β-amino ester)s and several modified polysaccharides to prepare the complex coacervates with DNA and other polynucleotides to form discrete nanoparticles.

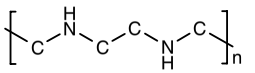
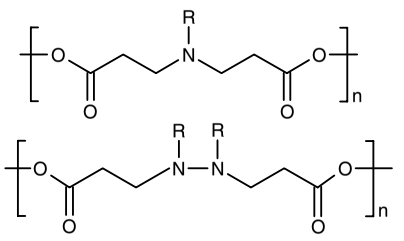
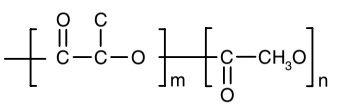
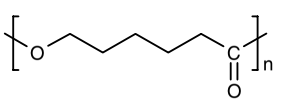
From the group of condensing polymers, chitosan has been extensively examined for oral gene delivery because of several reasons, ranging from its DNA condensing ability to its natural origin, biodegradability, low toxicity and muco-adhesive property [33]. Chitosan can also enhance the permeability of large, hydrophilic molecules across the mucosal

Table 1. Condensing and non-condensing polymers commonly employed for gene delivery purposes.

Polymer type	Polymer name	Characteristics	Chemical structure
Condensing polymers	Chitosan	Biodegradable polysaccharide of natural origin. Efficiently condenses with plasmid DNA. Mucoadhesive, safe and increases permeability of macromolecules across gastrointestinal tract.	
	Poly(L-lysine)	Can condense with negatively charged plasmid DNA using its positively charged ε-amino group. Polyplexes are efficient in protecting the delicate payload from endogenous nucleases.	
	Spermine	Oligoamine used by sperm cells to tightly pack DNA into very small structures. This property can also be used for condensing plasmid DNA for delivery purposes.	
	Spermidine	Also an oligoamine. It is a precursor of spermine and can condense plasmid DNA as efficiently as spermine.	

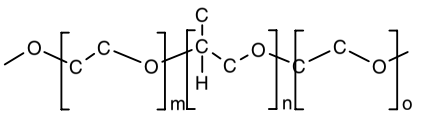
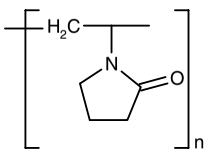
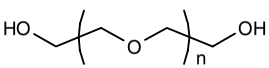
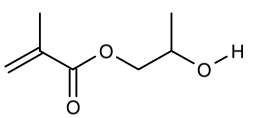
PEO: Poly(ethylene oxide); PPO: Poly(propylene oxide).

Table 1. Condensing and non-condensing polymers commonly employed for gene delivery purposes.

Polymer type	Polymer name	Characteristics	Chemical structure
	Poly(ethylene-imine)	Excellent plasmid DNA condensing polymer, forming polyplexes in nanometer range. These particles show excellent transfection efficiency. Higher molecular weight polymer, has very high cytotoxicity.	
	Gelatins	Protein from hydrolysis of collagen, which degrades in the presence of proteases, and hence is biodegradable. Cationic gelatin condenses plasmid DNA. Type A is a protein with a large fraction of basic amino acids such as arginine and lysine, which are protonated at physiological pH. Type B gelatin, with an isoelectric point of ~ 4.5 and a net negative charge at biological pH, can physically-encapsulate plasmid DNA very efficiently and protect it from degradation.	
	Poly(β-amino ester)s	Biodegradable polymer, can self assemble with plasmid DNA by forming electrostatic complexes under physiological conditions. Degradation occurs under physiological conditions via hydrolysis of backbone esters.	
Non-condensing polymers	Poly(D,L-lactide-co-glycolide)	Hydrophobic polymer, can efficiently protect plasmid DNA from degradation by physically encapsulating it. Usually formulated into microparticles. FDA approved.	
	Poly(ε-caprolactone)	Can efficiently protect plasmid DNA from degradation by physically encapsulating it. Degrades in the presence of lipases. This property can be utilized for oral gene delivery.	

PEO: Poly(ethylene oxide); PPO: Poly(propylene oxide).

Table 1. Condensing and non-condensing polymers commonly employed for gene delivery purposes.

Polymer type	Polymer name	Characteristics	Chemical structure
	Poloxamer (Pluronic®)	Amphiphilic triblock co-polymer made of PEO-PPO-PEO. Poloxamers can self assemble to form micelles. PEO chains can condense DNA by hydrogen bonding. Protects polyplexes from immune recognition.	
	Poly(*N*-vinyl pyrrolidone)	Synthetic homopolymer with mild adhesive property. Can bind to base pairs in the major groove of DNA through hydrogen bonding. This binding reduces the extracellular degradation of DNA.	
	Poly(ethylene glycol)	Used for polymeric gene delivery because of properties ranging from high water solubility to biocompatibility and low immunogenicity.	
	Hydroxypropyl-methacrylate copolymers	HPMA copolymers widely used for macromolecular therapeutics because of their biodegradability, hydrophilicity and biocompatibility. Provides adequate protection to payload, that is, plasmid DNA	 HPMA monomer

PEO: Poly(ethylene oxide); PPO: Poly(propylene oxide).

surface. Chitosan–DNA nanoparticles, with a mean diameter of < 200 nm, were first reported in 1995 [34]. Following this study, several other groups have reported on the utility of chitosan and its derivatives for delivery of DNA-based therapeutics [31,35-38]. Other polymers such as poly(L-lysine) also have excellent DNA-condensing ability because of the presence of positive charge on its ε-amine groups forming particles smaller than 100 nm, which are very efficient in transfecting many different cell lines. PEI, ranging from a low molecular weight (5 kDa) to higher ones (25 kDa), has been shown to be an efficient and versatile agent for *in vitro* and *in vivo* gene delivery via a number of routes [39-42]. PEIs can effectively condense plasmids into colloidal particles, which exhibit high transfection activity due to the polymer's endosomal and lysosomal buffering capacity [43-45]. However, in the case of poly(L-lysine) and PEI, poor degradability, high cytotoxicity, aggregation and a short circulation time in the bloodstream make them less desirable as gene delivery vehicles [45-47]. A cationic derivative of methacrylate polymers, poly(2-(dimethylamino)ethyl methacrylate) (pDMAEMA), has also been successfully studied for gene transfer [48]. Other examples of condensing (cationic) and non-condensing polymers used for gene delivery are shown in Table 1.

2.2. Non-condensing polymers

Although condensing polymers improve the transfection efficiency by neutralizing the negative charge on the DNA molecule and forming particles in the nanometer range, non-condensing polymers can act as efficient gene transfer agents by physically encapsulating the DNA molecule. The majority of non-condensing delivery systems are made from neutral or slightly negatively charged polymers. This results in shielding of the encapsulated DNA molecule from endogenous nucleases and also prevents charge repulsion between the cell membrane and the DNA molecule. Several of the non-condensing polymers are hydrophobic, aiding in their efficient cellular internalization upon systemic administration. Microparticles have been traditionally formulated from non-condensing polymers for gene delivery purposes. The logic behind the use of polymeric microspheres is that non-condensed and encapsulated DNA/RNA molecules are directly delivered to antigen-presenting phagocytic cells, which include macrophages and dendritic cells, at site of injection or draining lymph nodes, due to the particulate nature of the formulation [49,50]. Micro-particle-based delivery is considered an ideal platform for administration of DNA vaccines, which can be passively targeted to antigen-presenting cells (APCs) [51,52]. Poly(*N*-vinyl pyrrolidone), poly(lactide-co-glycolide) and poly(ε-caprolactone) are examples of non-condensing polymers, which have been formulated as micropsheres for gene delivery purposes. Other non-condensing polymers used for gene delivery include poly(ethylene glycol) (PEG) or poly(ethylene oxide) (PEO), poloxamer block copolymers, gelatin and several hydrophilic cellulose derivatives [12].

PEG or PEO (when the molecular weight is > 20kDa) can be used to complex with DNA by hydrogen bonding. PEO-based copolymers, such as poloxamers (or Pluronics®; BASF, Inc.), can also interact with DNA to form non-condensing nano-structures [53]. In addition, PEG or PEO chains on the surface of the nanoparticle can prevent protein adsorption and cell adhesion by the steric repulsion. PEG or PEO surface-modified nanoparticles can remain in the systemic circulation for a prolonged period of time and can be passively targeted to solid tumours and other disease sites due to the leaky vasculature. Nanoparticles made from type B gelatin, which has an isoelectric point of ~ 4.5, is also very effective in the encapsulation of DNA. Over the past several years, the authors of the present review have developed and optimized the process of formulating gelatin nanoparticles, with and without PEG modification, for encapsulation and delivery of DNA for gene therapy [54-57]. Several studies have shown that gelatin-based particles can efficiently encapsulate DNA and protect against degradation in the systemic circulation and upon cellular internalization for efficient transfection [54-60].

3. Nano- and microparticle-based oral gene delivery systems

Nanoparticles and microparticles, fabricated mostly from natural and synthetic polymers, have been extensively researched into as delivery systems for low molecular weight pharmaceuticals. These systems can form ideal platforms for the delivery of genetic material because they can be tailored for specific formulation requirements. Developments in the field of polymer science have made the delivery of proteins, peptides and nucleic acids drugs via the oral route possible by protecting these molecules against pH- and enzyme-induced degradation, and by prolonging the time of delivery to the mucosal sites [61-65]. Several polymers have been used for formulating oral delivery systems, including microspheres and nano-particles, for proteins and DNA.

Factors that govern the uptake of polymeric particles from the gut include particle size, the physicochemical nature of particles, surface charge and attachment of cellular uptake promoters such as antibodies and lectins. After oral administration of the formulation, the nanoparticle and microparticles would: i) adhere to the cells (bioadhesion) and remain; ii) undergo oral absorption; and/or iii) be eliminated in the feces. Oral absorption of the particles results in passage across the gastrointestinal barriers and delivery of the payload into the blood, lymphatic system and specific tissues such as the liver. Before this translocation can occur, the microparticles have to adhere to the surface of the intestine. Translocation of particles across the gastrointestinal wall can occur due to intracellular uptake by either the absorptive cells of the intestine, paracellular uptake (i.e., between the cells of the intestinal

wall), phagocytic uptake by intestinal macrophages or uptake by the M cells of the Peyer's patches [66]. The fate of polymeric nano- and microparticles delivering DNA-based therapeutics upon oral administration is shown in Figure 2.

3.1 Preparation of nano- and microparticles

There are several methods for preparation of polymeric micro- and nanoparticles for the incorporation and delivery of DNA and other therapeutic molecules. In general, one of the two principles methods is utilized: controlled precipitation or controlled dispersion of the polymer. A few of the most common methods include solvent displacement, salting out, emulsion-solvent-evaporation, emulsion-solvent-diffusion, polymerization, complexation and supercritical fluid technology.

3.1.1 *Solvent displacement method*

In the case of the solvent displacement method, which is the simplest of all, the polymer is dissolved in a good solvent that may be partially polar and water miscible (e.g., ethanol or acetone) [67,68]. When the drug is to be incorporated into the particles, it can be dissolved in the same phase along with the polymer. This polymer phase is introduced into a non-solvent aqueous phase containing a stabilizer (generally a hydrophilic surfactant) at a controlled rate under continuous mixing. As the partially polar solvent diffuses rapidly into the aqueous phase (i.e., as the partially polar phase is displaced by the polar phase), the polymer starts precipitating due to change in its solubility, resulting in formation of nanoparticles. The surfactant present in the aqueous phase helps in preventing particle aggregation. The choice of a drug/polymer/solvent/non-solvent system is the major limitation of this method and hence its applicability is confined to hydrophobic drugs and polymers. This method can also be used for preparation of microparticles.

3.1.2 *Salting-out method*

This technique is generally used for the preparation of drug-loaded biodegradable nanoparticles and was first applied to pseudo-latexes [67]. It is based on the separation of water-miscible solvent from aqueous solutions by a salting-out effect. An oil/water emulsion is formed by adding a solution of the polymer and drug in a water miscible solvent into an aqueous gel containing a salting-out agent and a colloidal stabilizer. Water is added to dilute this mixture, as a result of which nanoparticles are formed. Solvent and salting-out agents are then removed by cross-flow filtration.

3.1.3 *Emulsification-solvent evaporation method*

This is based on the formation of a biphasic (oil/water or water/oil) or triphasic (water/oil/water or oil/water/oil) emulsion [67]. Generally, a preformed polymer is dissolved in an organic solvent, which is water immiscible along with the drug and is emulsified in an aqueous solution (oil/water emulsion). The formed emulsion is then exposed to high-energy mixers (e.g., high-speed or high-pressure homogenizers, colloidal mills or ultrasonic devices) to reduce globule size. The organic solvent is removed either by using heat or vacuum or at times, even both. Nanoparticles are obtained as fine aqueous dispersions that can be collected and purified. The process variables involved in this method are complex and manifold and the nanoparticles obtained are often polydisperse. However, this method is very popular for preparing polymeric microparticles, rather than nanoparticles, as it facilitates industrial applicability and scalability.

3.1.4 *Emulsion-solvent diffusion method*

This is another method which is used for nanoparticle preparation. It is a modified salting-out technique and differs mainly in the organic solvent, which is partially miscible with water in this case [69]. The organic solvent is presaturated with water to achieve an initial thermodynamic equilibrium between water and the organic phase. Solvent diffuses out upon addition of water and results in the formation of a nanoparticle suspension.

3.1.5 *Controlled complexation*

Controlled complexation induced by electrostatic interactions between oppositely charged polymers can yield stable colloidal dispersions. The interacting polymers can be therapeutically active (e.g., oligonucleotides and plasmid DNA) or may have tailored properties (e.g., pH sensitivity) [70,71]. A wide variety of charge-bearing polymers can be utilized to manufacture composite nanoparticles and varying physicochemical properties [72-77].

3.1.6 *Supercritical fluid technology*

This is an emerging science for the production of micro and nanoparticles [78,79]. In this method, an organic liquid solution of the polymer and the active moiety is sprayed through a nozzle into a chamber containing a gas that is miscible with the solvent, but in which the polymer and the active compound are not soluble. The gaseous phase in this case is a supercritical fluid (e.g., supercritical CO_2). The dispersion of the liquid solution in such a condition generates a high degree of super saturation, leading to the formation of fine, uniform colloidal particles. The particles can be recovered from the solution by depressurizing the chamber and allowing the gas to escape [78].

Although all of the abovementioned procedures employ preformed and well-characterized polymers, there are other techniques for obtaining fine nanoparticles from monomers via *in situ* polymerization pathways. The most popular example for this method of synthesis is the nanoparticles made from poly(alkylcyanoacrylates), poly(methylmethacrylates) and poly(methylidenemalonates) [62]. Generally a water-insoluble monomer is dispersed in an aqueous medium containing a colloidal stabilizer, and the polymerization is induced and controlled by gradual addition of a chemical initiator or by variations in physical parameters such as pH,

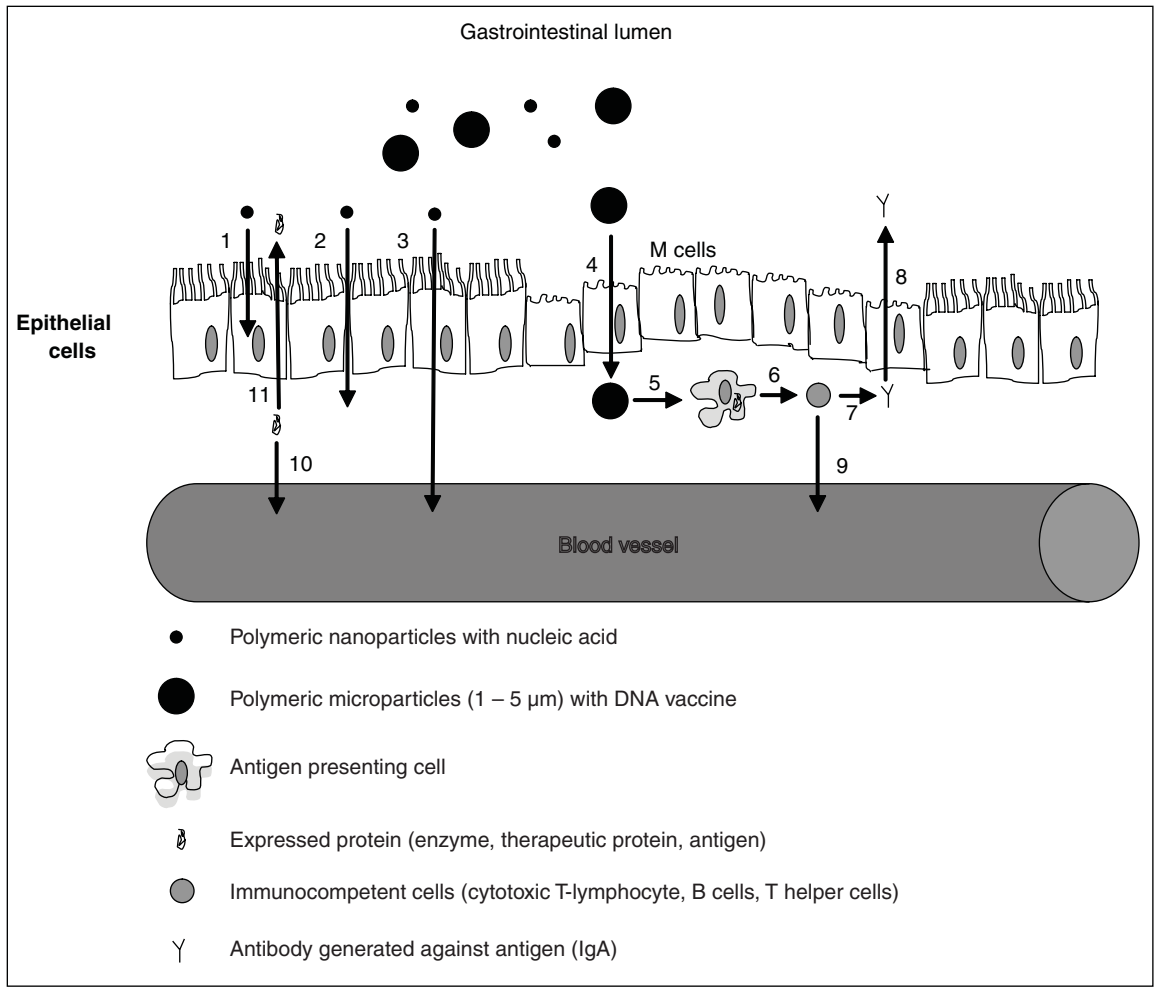


Figure 2. The fate of polymeric nano- and microparticles delivering DNA-based therapeutics upon oral administration. **1.** Uptake of nanoparticles by epithelial cells by endocytosis. **2.** Paracellular transport of nanoparticles to the underlying cell population. **3.** Transcytosis of nanoparticles from the upper epithelial cells to the lower underlying cells and also into the systemic circulation. **4.** Uptake of the microparticle formulation carrying DNA vaccine by M cells and passing them down to the underlying antigen-presenting cells. **5.** Uptake of the microparticle formulation by antigen-presenting cells by phagocytosis. **6.** Immunologic response upon transfection resulting in stimulation of B cells and/or cytotoxic T lymphocytes. **7.** The production of antibody (IgA) against transfection antigen by immunocompetent cells. **8.** The secretion of IgA into the intestinal lumen. **9.** The passing of activated lymphocytes into the systemic circulation leading to systemic immunity. **10.** The therapeutic protein expressed by cells of the gastrointestinal cells can either be secreted into systemic circulation or; **11.** secreted into the lumen.

temperature or light. Both hydrophilic and lipophilic drugs can be entrapped in the formed nanoparticles, based on the properties of the polymer and the solvent system used.

3.2 Characterization of DNA-containing delivery systems

To be successful in their goal of gene delivery to the cell nucleus, an ideal polymeric nano- and microparticle gene delivery system should exhibit the following attributes:

- The polymer used should exhibit a good loading capacity (i.e., the final formulation should have a high DNA loading efficiency for the maximum delivery of payload to the cells).
- The particle size should be in the nanometer range for optimal cellular internalization, unless used for gene delivery to phagocytic cells, in which case they can be in the micrometer range (1 – 5 µm).
- The particles should posses an optimum net positive surface charge, in the case of polyplexes, for interaction with the negatively charged cell membrane.
- The formed particles should be able to protect the payload from pH- and enzymatic degradation.

After formulating micro- and nanoparticles containing DNA- or RNA-based therapeutics, it becomes absolutely important to characterize the abovementioned parameters.

These parameters largely determine the gene transfer ability of the formulated system, although not completely. Either a direct or an indirect method can be used for determination of the DNA-loading efficiencies of formulations. In the case of systems that physically encapsulate DNA, a direct approach can be used that involves extracting and quantifying the loaded DNA to obtain the amount incorporated per mass of delivery system. The indirect method, mostly used in the case of polyplexes, determines the amount of DNA material that remains free and can then be subtracted from the total amount of DNA that was initially added. Often a quick test to determine the DNA-loading efficiency is to determine the absorbance of the sample in the ultraviolet region of the spectrum at 260 nm. However, the sample has to be relatively pure of interfering components [80]. Because of this limitation, fluorochromes such as ethidium bromide, 4′,6-diamidino-2-phenylindole, Hoechst 33258 and other dyes, which bind strongly with double stranded DNA and fluoresce, can also be used for quantitative analysis [81-83]. The stability of plasmid DNA can be determined by agarose gel electrophoresis, by running an appropriate standard along with the sample in the same gel and then comparing the bands of the sample and standards.

In the case of particulate formulations, particle size becomes the most important parameter to be assessed. Particle size is often measured using instruments that work on the light scattering principle. These can measure particles that are as small as 20 nm [302]. Other instruments are also available that employ a laser beam and work on the principle of laser diffraction. These can measure particles sizes from 0.6 nm to 6.0 µm. The Zetasizer Nano series particle sizers from Malvern Instruments employ laser beams for particle size measurement and can measure particles in the nanometer range very accurately. In addition to particle size, the surface charge of formulation also has to be determined. It is advantageous for a particulate formulation to have a net positive surface charge for maximizing their interaction with the negatively charged cell membrane. The surface charge on particles can be assessed with commercially available instruments that measure the electrophoretic mobility of particles under the influence of an electric field. Often, the particle sizing and zeta potential measurements can be done on the same instrument (e.g., Zetasizer Nano series [ZS and Z90], Malvern Instruments; and 90Plus® nanoparticle size analyser, Brookhaven Instruments Corp.) and can measure both particle size and zeta potential [302,84]. Most of the methods for preparation of micro- and nanoparticles mentioned above do not involve steps that would put significant physical strain on the DNA molecule, but the assessment of the physical integrity of the encapsulated payload becomes necessary, as it can affect the final outcome of gene therapy. In addition, the ability of the formulation to protect the payload from endogenous nucleases has to be determined. This can be done by incubating the formulation with DNase I. In both of the abovementioned cases, the stability of the DNA molecule can be very efficiently and positively determined by agarose gel electrophoresis. For a more precise result, the DNA sample can be sequenced to determine its physical integrity, but this is a more expensive and time consuming process.

4. Oral gene therapy using polymeric nano- and microparticles

Although there are a number of local gastrointestinal tract and systemic diseases that could benefit from protein-based therapies, oral or rectally administered protein drugs are rapidly degraded by the proteolytic enzymes. In addition, in certain cases, such as the administration of non-humanized antibodies, there is development of an immune response that limits chronic use. Local production of protein drugs using a gene therapy strategy could provide an efficient alternative to oral and rectal administration. Efficient oral gene therapy provides an opportunity for the sustained local production of therapeutic protein at the disease site in the gastrointestinal tract or at a site where maximum systemic absorption can occur due to low proteolytic activity [6].

The oral route is ideal for gene therapy of gastrointestinal disorders, and oral vaccination purposes, as it allows easy and rapid access to the site of action upon administration (see Table 2). Although very appealing from a patient compliance perspective, oral formulations have to overcome anatomical (mucus and epithelial layer) and physiological barriers (varying pH, degradative enzymes) of the gastrointestinal tract to become successful in drug delivery. These difficulties become even more pronounced when attempting gene delivery, as the DNA molecule has to reach the nucleus of cells for efficient transfection. Polymeric nano- and microparticle technologies have been used for oral drugs, including a number of protein therapeutics [85-87]. For gene therapy, there are few reported cases of success.

The pioneering study reporting oral plasmid DNA administration using polymeric nanoparticles was put forth by Roy *et al.* in 1999 [22,301]. In this study, chitosan nanoparticles in the size range of 100 – 200 nm were prepared by the salting-out technique with the plasmid DNA (pArah2), which encodes for the peanut allergen Arah2. The nanoparticles were orally administered to mice, and the serum and fecal levels of IgG or IgA were measured periodically. High levels of anti-arah2 IgG were observed in the titer of the group which was fed with low molecular weight chitosan nanoparticles complexed with the plasmid DNA, compared with other groups, which were dosed with high molecular weight chitosan nanoparticles. The mice from all groups were challenged with crude peanut extracts 4 weeks after the booster dose and positive antibody response were detected in groups immunized with DNA-containing nanoparticles. These results suggest that chitosan-plasmid DNA nanoparticles delivered through the oral route can modify the immune system in mice and protect against food allergen-induced hypersensitivity. This study indicates that with

Table 2. Summary of oral gene delivery for therapeutic and vaccination purposes.

Purpose	Polymer	Nanoparticle/microparticle (size) and plasmid DNA used	Ref.
Gene delivery formulation	Chitosan	Nanoparticles (70 – 150 nm) – pAAV-ET coding for erythropoietin gene.	[91]
	Chitosan	Nanoparticles (150 – 300 nm) – p43LacZ coding for β-galactosidase and pCMVArah2 coding for peanut allergen.	[21]
	Chitosan-EDTA conjugate	Nanoparticles (< 100 nm) – pSV-β-galactosidase coding for enzyme β-galactosidase.	[5]
	N-acetyl chitosan derivative	Polyplexes in mouse feed – pDNA coding for β-galactosidase and murine IL-10 both.	[27]
	PEG-DSPE	Folate linked cationic nanoparticle (230 – 340 nm) – pAAV-CMV-Luc coding for enzyme luciferase.	[103]
DNA vaccine formulation	Poly(lactide-co-glycolide)	Microparticles (2 µm) - pCMVLuc coding for firefly luciferase.	[22]
	Poly(lactide-co-glycolide)	Microparticles (2 µm) – pDNA coding for outer caspid surface protein of rotavirus.	[100]
	Poly(lactide-co-glycolide)	pDNA/PEI complexes encapsulated in PLGA microparticle – pGL3-control coding for enzyme luciferase.	[104]
	Poly(lactide-co-glycolide)	Microparticle (2 – 5 µm) – pDNA coding for envelop glycoprotein of HIV.	[101]
	Poly(lactide-co-glycolide)	Microparticles (2 – 5 µm) – pVAX(S) coding for hepatitis small envelop protein.	[104]
	Alginate	Microparticles (< 100 nm) – pDNA coding for bacterial β-galactosidase.	[102]

EDTA: Ethylenediaminetetraacetic acid; pDNA: Plasmid DNA; PEG-DSPE: Poly(ethylene glycol) conjugated 1,2-distearoyl-sn-glycero-3-phosphatidylethanolamine; PEI: Poly(ethyleneimine); PLGA: Poly(lactide-co-glycolide).

a well-designed polymeric nanoparticle system, it is possible to achieve successful oral gene delivery and transfection.

Such therapy systems can be potentially used for other incurable disorders such as IBD. IBD is a group of chronic clinical disorders involving inflammation of the mucosal layer of the gastrointestinal tract. The fundamental patho-physiological processes involved in pathogenesis of IBD are still unknown, but a great deal of clinical and non-clinical research focused in this direction has suggested multifactorial interactions of genetics, environmental factors and more importantly, inappropriate mucosal immune response to antigens arising due to the presence of normal gut flora [88]. Historically, IBD has been classified into two different diseases: Crohn's disease and ulcerative colitis. Both Crohn's disease and ulcerative colitis exhibit a common stem in their phenotypes, but branch out with different types of $CD4^+$ lymphocytes, which are activated and mediate the progression of the two diseases. Crohn's disease has been recognized to be mediated by a T helper 1 (T_H1) response, and ulcerative colitis shows activation of T helper 2 (T_H2) lymphocytes [89-91]. Both of these conditions predominantly affect the colon, but can also cause inflammation of the distal part of small intestine, leading finally to the destruction of the protective mucosal layer. It has been hypothesized that upon administration and transfection of plasmid DNA encoding for one or more of the cytokines, the balance can be restored, thus, leading to the possible treatment of IBD.

Kai and Ochiya [27] successfully used an *N*-acetyl derivative of chitosan to form polyplexes with plasmid DNA containing two genes – one encoding for β-galactosidase and the other for murine cytokine the IL-10. These polyplexes were delivered via the oral route by incorporating them in rodent feed. Five days after administration, qualitative and quantitative transfection efficiency with the DNA-containing formulations was confirmed by testing for β-galactosidase expression with X-gal staining and also quantifying the production of IL-10 by an ELISA, respectively. Both the upper and lower intestines were transfected with the formulated particles, with the colon showing the highest level of IL-10 expression among all of the test organs.

In another study with the use of chitosan nanoparticles for oral gene delivery by Chen *et al.* [92], an erythropoietin gene (*mEpo*) was transfected to the intestinal epithelium of mice. Erythropoietin is a glycoprotein that stimulates the production of red blood cells. It is used in patients with anemia associated with chronic renal failure and in cancer patients for simulation of erythropoieisis. Chitosan nano-particles containing plasmid DNA encoding for erythropoietin have been orally administered to mice along with other appropriate control formulations; erythropoietin gene expression was evaluated every 2 days by measuring the hematocrit of the mice. Mice that received chitosan nano-particles encapsulated with *mEpo* showed a 15% increase in hematocrit over the control formulations, indicating

successful transfection of the *mEpo* gene across the intestinal epithelium and systemic absorption of the erythropoietin for pharmacological activity. These results suggest that chitosan nanoparticles were able to protect *mEpo* from degradation by DNAses and, hence, there is a possibility of using them as gene delivery vehicles via the oral route, where the protein drug is absorbed for systemic therapeutic activity. In another study showing oral absorption of locally produced protein drug, nanoparticles prepared from cationic biopolymers, including chitin, chitosan and their derivatives, were used as carriers for the oral administration of a therapeutic gene [202]. In this case, the nanoparticles were encapsulated with plasmid DNA encoding for human coagulation Factor IX, which is absent in hemophilia. Human Factor IX was detected in the systemic circulation of the mice within 3 days, following oral delivery of the plasmid DNA-containing nanoparticles and declined after 14 days. The investigators also demonstrated the bioactivity of the Factor IX transgene product in Factor IX knockout mice. Hemophilia B is an X-linked bleeding disorder caused by a mutation in the Factor IX gene. After orally feeding Factor IX transgene-loaded nanoparticles to the knockout mice, the blood clotting time was reduced from 3.5 min to 1.3 min, which was comparable with a clotting time of 1 min observed with normal mice. This study elegantly shows that locally produced protein drug in the gastrointestinal tract can be systemically absorbed and shows therapeutic activity. This research has significant implications in non-invasive administration of protein-based therapies.

There are many additional examples of oral gene delivery using polymeric nano- and microparticle systems which have used reporter plasmids such as pEGFP-N1 and pCMV-β-gal [93-95]. Very few studies have actually been done where therapeutic efficacy of oral gene delivery methods has been evaluated with *in vivo* disease models. To further understand the difficulties and needs of successful therapeutic oral gene delivery system, it is absolutely important to research them on disease models. In the case of conventional drug delivery systems delivering small molecules, characteristics such as bioavailability and pharmacokinetic parameters, along with the therapeutic outcome, have been used as indicators of successful delivery systems. An effort has to be made to outline such pharmacokinetic parameters for gene delivery systems. These would be indicators of a successful formulation, having the ability to efficiently transfect at the desired site in the gastrointestinal tract

5. Nano- and microparticles for oral DNA vaccination

The various mucosal surfaces in the body provide an alternative route for vaccine administration, where there is an opportunity for generation of both mucosal and systemic immunity. The gastrointestinal mucosal surface is particularly attractive due to the ease of self-administration, reduced adverse effects, and the opportunity for frequent boosting for optimum vaccine effects [97]. The gastrointestinal mucosal surface exhibits an abundance of APCs, such as M cells in the Peyer's patch region of the small intestine that provide a very exciting and feasible target for immunization against infecting pathogens [66].

The first use of DNA vaccines was reported by Ulmer and colleagues in a series of experiments where they demonstrated that immunization of mice by intramuscular injection of naked plasmid DNA encoding for an influenza viral protein generated specific cytolytic T cells and protected the mice against subsequent challenge with live Influenza virus [97-99]. Cytolytic T cells have the ability to recognize epitopes derived from proteins, including conserved (often internal) proteins and this response is critically important for clearance of infections caused by viruses and bacteria [96].

Non-viral vectors, especially polymeric microparticles of < 5 μm in diameter, encapsulating plasmid DNA, have established themselves as efficient delivery platforms for DNA vaccination based on their ability to be taken up by a variety of phagocytic APCs [96]. The mucosal surface of the gastrointestinal tract provides an excellent opportunity for use of polymeric microparticles as delivery agent due to the presence of M cells and APCs. The translocation of particles across the gastrointestinal wall can occur due to phagocytic uptake by intestinal macrophages or uptake by the M cells of the Peyer's patches and their presentation to immunological cells [18]. By combining the attributes of DNA vaccines and polymeric microparticulate delivery systems, it might be possible to generate immunity against a wide range of local and systemic infectious diseases.

In 1997, Jones *et al.* first reported on the use of polymeric microparticle formulations made from Poly(lactide-co-glycolide) (PLGA) for delivery of DNA vaccine [21]. They used plasmid DNA expressing the insect protein luciferase under the transcriptional control of the human cytomegalovirus immediate-early promoter. The DNA-containing formulation was administered orally and by intraperitoneal injection. Upon comparison of serum Ig levels after both routes of administration, they found that intraperitoneal route elicited good serum IgG and IgM responses and a modest IgA response; the oral route was able to elicit a good serum response of all three immunoglobulins. The oral route was also able to generate a good mucosal IgA response. With this study, the authors were able to show that PLGA microparticles were able to facilitate DNA vaccine delivery into appropriate cells for the subsequent expression and presentation of antigen in such a way as to elicit both systemic and mucosal antibody responses.

The same group also reported on the *in vivo* therapeutic potential of oral DNA vaccine using PLGA microparticle technology [100,101]. They used plasmid DNA encoding for the rotavirus coat protein called VP6. After administration of just a single dose of DNA vaccine formulation in Balb/c

mice, a protective immune response was observed in terms of serum antibodies, as well as intestinal IgA production, resulting in stimulation of both systemic and humoral immunity. Animals showed adequate protection from challenge with homologous rotavirus after 12 weeks postimmunization. This finding was significant in the mucosal DNA vaccine field, as the first evidence of successful outcome from an orally administered DNA vaccine using microparticle technology in an *in vivo* disease model. Similar type of PLGA microparticles were used subsequently by Kaneko *et al.* [102] for oral delivery of plasmid DNA encoding for HIV envelope glycoprotein. This study reported the induction of systemic and humoral immune responses to HIV specific antigens and protection upon oral vaccination in Balb/C mice.

In addition to the abovementioned reports, Mittal *et al.*, [103] investigated the effect of the route of administration and co-administration of bovine adenovirus type-3 (BAdV3) DNA vaccine, along with biodegradable alginate microspheres, on systemic and mucosal immune responses in mice. Three different microsphere formulations (i.e., plasmid DNA [pDNA], BAdV3 and pDNA plus BAdV3) were administered to mice via the oral, intranasal, intramuscular, subcutaneous and intraperitoneal routes. Antibody titer results showed that systemic routes were able to induce a better response, but low levels were observed at the mucosal sites. In contrast, the mucosal routes showed high levels of induction at the mucosal sites and low levels of systemic induction. In 2005, He *et al.* [104] reported on the use of PLGA microsphere encapsulating DNA vaccine for induction of immunity against hepatitis B after a single oral dose. They obtained the gene for hepatitis B virus antigen from the serum of a patient positive for hepatitis B virus infection. Oral administration of PLGA-pDNA microparticles in Balb/c mice induced a long-lasting and stable, antigen-specific antibody response in the systemic circulation and at the mucosal surface.

The abovementioned are few examples of successful oral DNA vaccination using polymeric microparticle technologies. Although there have been some advances, tremendous work is needed to optimize polymeric nano- and microparticle technologies for successful oral DNA vaccination.

6. Conclusions

Oral gene therapy offers tremendous promise for the production of protein therapeutics and for mucosal vaccination. Improved understanding of the field of molecular biology, polymer characteristics and the use of innovative techniques for the development and production of particles in the nanometer and micrometer length scales has led to the development of many novel strategies for oral gene therapy. Polymeric nano- and microparticle technologies have been used successfully for the development of delivery systems for therapeutic gene delivery in the gastrointestinal tract, for the local production of proteins or for systemic absorption and therapeutic effect. In addition, oral DNA vaccination using microparticle technologies have shown to elicit both systemic and mucosal immune protection.

7. Expert opinion

With increased understanding of disease pathology, the next generation of therapeutics is expected to be based on large hydrophilic and charged molecules (e.g., plasmid DNA, siRNA, oligonucleotides and proteins) that have poor stability and diffusion properties in the biological environment. Convergence of expertise in molecular biology, disease pathology, polymer chemistry and pharmaceutical sciences, among others, has led to the development of gene delivery systems for local and systemic therapeutics and vaccine administration. Oral gene therapy can be extremely beneficial for local treatment of diseases, systemic absorption of protein drugs and for DNA vaccination. However, the challenges of oral gene delivery, including poor stability in the gastrointestinal fluid, poor availability at the target site and lack of appreciable uptake by the cells, lead to poor transfection efficiency. Nano- and microparticle technologies made with biodegradable and biocompatible polymers may pave the way to enhancing the transfection efficiency of orally administered plasmid DNA.

Nano- and microparticles, made from the synthetic and natural polymers, are likely to dominate the novel drug delivery systems in the oral market because of the cost-to-benefit ratio, excellent stability and flexibility for industrial production. These nano- and micro-technologies are also adaptable for surface modification to enhance target-specific delivery, improvement in the stability of the encapsulate payload, sustained or stimuli-responsive release mechanisms, and promoting cellular stability of the plasmid for nuclear uptake in order to optimize transfection efficiency. Although present nano- and microparticle technologies are focused on the efficient delivery of a therapeutic or vaccine to the target site, a future goal may be to even further enhance the functionality by introducing multiple types of payload (e.g., drug and gene combination), the ability to externally actuate the release at a desired time point, or combine delivery with a polymeric material that responds to environmental stimuli (e.g., a pH-sensitive polymer)

The prospect of using targeted gene delivery to a specific site in the gastrointestinal tract where there is less proteolytic activity, such that the expressed protein can be systemically absorbed, offers tremendous potential for noninvasive administration of protein therapeutics with reduced frequency. The future success of polymeric technologies for oral DNA administration will depend on their ability to efficiently bypass the barriers presented by the gastrointestinal tract, selectively targeting a specific cell type *in vivo* and expressing therapeutic genes in a regulated

fashion. These goals can be achieved by improving the formulation characteristics using polymers that are able to effectively protect the delicate payload, surface modification with targeting moieties, and by perfecting the gene vector construct.

Furthermore, oral formulations for siRNA and gene administration have tremendous potential. Although siRNA delivery has so far been limited to systemic diseases, it has tremendous therapeutic potential for oral administration to modulate protein expression by RNA interference in the intestinal cells. For instance, short-term silencing of P-glycoprotein in the intestinal lumen could potentially improve the bioavailability of a large number of drug candidates that are substrates for P-glycoprotein-mediated drug efflux. In addition, RNA interference can also be employed for the treatment of local diseases, such as gastrointestinal carcinomas and IBD, where genes encoding for specific proteins, such as angiogenic factors, apoptosis inhibitors or immune stimulation can be silenced with siRNA administration. Polymeric nano- or microparticulate systems can form an ideal platform for oral siRNA delivery.

It is clear that the success of oral gene therapy, either for gastrointestinal disorders or for vaccination purposes, needs to be evaluated in higher organism disease models before they can be moved up to human trials. However, despite many obstacles, the development of oral gene therapy has great clinical promise and must continue to move forward simultaneously with developments in innovative delivery strategies.

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