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Nanostructures in Therapeutic Medicine

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**Title : Nanostructures for oral delivery of therapeutic nucleic acids**

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**Abstract**

The promise of using nucleic acid as a drug has opened new therapeutics strategy for a large variety of diseases. Several types of therapeutic nucleic acid exist, ranging from short synthetic RNA- or DNA-based oligonucleotides to plasmid-based DNA expressing constructs. According to their functional activity, they can either induce the expression of a protein (gene therapy strategy) or trigger the silencing of a gene (RNA interference or antisense strategy). Current nucleic acid-based treatment strategies are largely injection-based, but effective oral delivery platforms for nucleic acids may result in improved patient comfort and compliance. In this chapter, we propose to discuss the barriers to oral delivery of nucleic acids as well as review the various technologies already tested in preclinical study and the experimental models available to assay oral delivery systems.

## Keywords

RNA interference, plasmid DNA, vaccination, non-viral delivery, cationic lipid, polymer, oral administration

## 1. Introduction

Since the demonstration that gene therapy can actually treat patients (Wang 2014) and the multiplication of clinical trials based on RNA interference (RNAi) (Ozcan 2015), the concept of therapeutic nucleic acid, i.e. the possibility to use a nucleic acid as a drug, has become a reality. Gene therapy involves local or systemic administration of a nucleic acid construct that will result in expression of the gene-encoded protein. The expected effect can be prevention, treatment and even curing of diseases. RNAi-based drugs harness a small RNAs-based endogenous process of gene silencing to treat disease. Both strategies rely on the efficient administration of nucleic acid molecules. **<Insert Figure 1 here>** Thus, therapeutic nucleic acid molecules can be distinguished (Figure 1), in terms of function on the one hand, and in terms of structure on the other hand. In terms of function, nucleic acid molecules can either switch on expression of a protein (gene therapy), or switch off expression of a protein (siRNA, shRNA or DNA-directed RNAi therapeutics, antisense oligonucleotide, decoy oligonucleotide). In terms of structure, gene and shRNA are based on DNA coding sequences that will trigger either the production of a protein via transcription of cDNA then translation of mRNA in the case of gene, or the production of interfering duplex shRNA. Antisense or decoy oligonucleotides and siRNA are small synthetic DNA- or RNA-based oligonucleotides. Nucleic acids are susceptible to degradation, particularly by nucleases, so efficacy may be enhanced by delivery system strategies that protect the drug. Moreover, in order to fulfill their function, the nucleic acids must penetrate the cells where the machineries required for

the implementation of their function are located. Cellular delivery of all types of nucleic acid molecules is challenging because these negatively charged, water soluble molecules are high molecular weight compounds with very low membrane permeability. Since they are unable to cross cell membrane on their own, they have to be vectorised. Viral and non viral vectorization strategies exist. We will focus here on non viral methods (Yin 2014). To date most non viral nucleic acids delivery strategies have concentrated on the parenteral route of delivery and oral administration has been less investigated. Yet, oral drug products account for half of the annual drug delivery market, so oral route for therapeutic nucleic acid has to be considered.

The two main obstacles for oral delivery strategies to achieving this goal are the harsh environment of the digestive tract, in particular the acidic milieu of the stomach, and subsequently the crossing of the intestinal epithelia to reach the circulation (O'Neill 2011). Nucleic acid modification and formulation into pH-responsive slow-release capsules can be used for stability purposes, while tight junction modulators such as caprate may be used to penetrate the epithelium. Several methods and delivery systems have been developed for parenteral delivery of nucleic acids (Yin 2014). Numerous *in vivo* studies have used cationic lipid-based vectors but they are not very popular for oral gene delivery. This is mainly due to their instability in the gut environment and could explain why polymer-based approaches have been used more commonly. Both condensing and non-condensing polymers have been utilized (Bahvsar 2007a). The cationic polysaccharide, chitosan, is among the most widely used condensing polymers for intestinal gene therapy research. It is a natural biodegradable mucoadhesive polysaccharide derived from crustacean shells, already available in pill form as an alternative therapy to reduce dietary fat and cholesterol absorption. This slowly degradable polymer has been shown to increase transcellular and paracellular transport of

macromolecules across intestinal epithelial monolayers. It forms nanoparticulate complexes with nucleic acids that are biodegradable, biocompatible and relatively non-toxic in nature. Chitosan also has mucoadhesive and tight junction destabilizing effects. PLGA has been one of the most commonly used non-condensing polymers in intestinal gene delivery. It has generally been formulated as microparticles to deliver DNA vaccines.

This chapter reviews the various delivery systems that have been developed for oral administration of therapeutic nucleic acids, with a focus on nanostructures. It also discusses the physiological and cellular barriers to this delivery, as well as the experimental models available to evaluate the stability of the delivery systems and the efficiency of the delivery.

## **2. Oral delivery of therapeutic nucleic acids**

### **2.1. Advantages of the oral route of administration**

Drugs can be administered by many routes but the most convenient route and then the most frequently used is the oral route. Several reasons may explain it. The first one is the patient's comfort. Compared to parenteral routes which consist in injecting the drug via a needle, this is a noninvasive and painless route for patients. This ease of administration results in a high patient compliance even for repeatable administration associated with a reduced healthcare cost (Raouf 2004; Bhavsar 2007a). Several therapeutic strategies are possible. It is first possible to cure local diseases such as for example food allergies, inflammatory bowel diseases (IBD), familial adenomatous polyposis (FAP), intestinal cancers or the intestinal symptoms of cystic fibrosis. Localized and targeted therapies make possible to reduce side effects. Secondly, considering that the gut epithelium is highly vascularized, the nucleic acid can access the systemic circulation and can be delivered to a distant site of

action after transport across the intestinal epithelium. This could be promising in the treatment of disease like hemophilia (Bhavsar 2008b, O'Neill 2011, Kriegel 2013).

The other advantages of the oral route are linked to the physiology of the gastro intestinal tract. The gut is characterized by a large surface of absorption composed of a high number of cells available for nucleic acid uptake. The crypts of Lieberkuhn consist in stem cells. The transfection of these cells could make possible the long-term expression of therapeutic genes. The gut is also an entry portal for pathogens. Gut-associated lymphoid tissues (GALT), antigen-sampling M-cells in the follicle-associated epithelium (FAE) of lymphoid follicles and Peyer's patches can be targeted in the perspective of orally delivered DNA vaccines (Bhavsar 2007a, O'Neill 2011). The last point to consider is the high turnover of intestinal epithelial cells (Kriegel 2013) since the enterocyte lifetime is estimated to be of five to seven days (O'Neill 2011). Considering this fast proliferation, repeated dosing can be considered avoiding an unreasonable cytotoxicity.

Nevertheless, over all the described advantages, the efficiency of gene delivery is compromised by the different barriers exhibited by the gastro intestinal tract. To better understand the obstacles encounter by nucleic acids after oral administration, the following part describes extracellular, intercellular and cellular barriers present in the gastro intestinal tract and the potential of targeting in therapeutic applications.

## **2.2. Obstacles to nucleic acid oral delivery and potential targets**

The nucleic acids are efficient only inside the cytosol of the cell. Nevertheless, it could be difficult to reach due to the several barriers that exist in the gastro intestinal tract. Figure 2 shows these barriers to oral gene delivery. As a consequence, nucleic acids must be

protected during transit to enable intestinal cellular uptake and then increase their transfection efficiency (Forbes 2012).

### **2.2.1. Physiological barriers**

The first barrier is the pH in the gastro-intestinal tract. The nucleic acids first reach the stomach known as a harsh acidic environment (pH 1.5 to 1.9). This low pH causes denaturation and depurination of nucleic acids leading to a decrease of their efficiency (Kriegel 2013). Moreover, stability over a wide range of pH is essential since the pH in the intestine can be variable and different from values observed in the stomach (pH 5 to 9 in the lower intestine).

Degradative enzymes are also found in gastro intestinal tract in order to degrade nucleic acid in food. We can cite pepsin in the stomach and amylase, trypsin or lipase in the gut lumen and on the brush border membrane. Obviously, the presence of these enzymes can lead to the rapid degradation of nucleic acid (Bhavsar 2007a, Akhtar 2009, O'Neill 2011).

Furthermore, the uptake of vectors containing nucleic acids can be reduced due to the fluid flow and peristaltic activity of the gastro intestinal tract since the contact time with the epithelial layer will be reduced.

Physical barriers also exist (O'Neill 2011, Kriegel 2013). The gastro intestinal tract is covered by a sticky layer of mucus under the epithelium. The role of the mucus is to catch foreign agents. Its thickness changes along the gastro intestinal tract, being thicker in the colon than in the ileum. Its turnover is quite short (50 to 270 minutes) and then can cause the excretion of the nucleic acids before reaching the epithelium. Another barrier is the glycocalyx. This is a glycoprotein and polysaccharide layer with a thickness of 400 to 500 nm present over the apical membrane of enterocytes. This layer acts as a size-selective diffusional barrier for

viruses, bacteria and also vectors that contain nucleic acids. Finally, the negatively charged cell surfaces can also be a barrier as nucleic acids are negatively charged and repelled by the cell surfaces.

### **2.2.2. Cellular barriers and potential cellular targets**

Three layers compose the intestinal mucosa. The first one is a single layer of epithelial cells. Under this first layer, the lamina propria is vascularized and composed of numerous immune cells. The last layer is called the muscularis mucosa. At the surface of intestinal mucosa is also found immune inductive tissue (GALT) including lymphoid follicles and Peyer's patches. These cells are important for mucosal and systemic immunity and are below the epithelial layer between villi.

When arriving in the gut after administration, nucleic acids first encounter epithelial layer which is composed of enterocytes, goblet cells, M-cells of the Peyer's patches, Paneth cells, intraepithelial lymphocytes and hormone-secreting endocrine cells (O'Neill 2011). The epithelial layer is mostly composed of enterocytes. Their apical membrane is covered by the glycocalyx. Beyond enterocytes, goblet cells are the next most common cells. These are the cells that produce mucus. M-cells are the specialized cells in the small intestine. These cells take up bacterial and viral antigens to make possible phagocytose by macrophages and finally initiate immune response in lamina propria. However, M-cells cover a small surface of the gut and when they are targeted, this can lead to patient-to-patient variability in bioavailability (Akhtar 2009).

Depending on the therapeutic strategy, the cells that compose epithelial layer can be targeted or they just represent a barrier to cross in order to access the vascularized lamina



propria. As shown on Figure 2, the crossing of epithelial layer can occur between the cells (it corresponds to paracellular route) or through the cells (it is called transcellular route).

The single way in the paracellular route is through the path tight junctions between cells. Since it represents less than 1 % of surface area in intestine, it limits the possibility of crossing. In addition, in human intestine the paracellular pores are characterized by a range diameter of 0.5 to 3 nm. This can restrain the paracellular way since the vectors used for the delivery of nucleic acids can be characterized by a higher size (O'Neill 2011).

**<Insert Figure 2 here>**

The transcellular route is more interesting than the previous one since the surface area for crossing is important. The first step consists in the endocytosis of the delivery system of nucleic acids at the apical pole of the epithelial cell. In the cytoplasm, the degradation of nucleic acids can occur due to the presence of nucleases and lysosomes (Kriegel 2013). The nucleic acids may also be captured by endosome and ejected to the lumen. Nuclear uptake is also possible. It is interesting if the goal is the transfection in epithelial cell. Nevertheless, lifetime of epithelial cells is short, about 5 to 7 days. As a consequence, repeated administration is essential for efficacy. Moreover, even if enterocytes are the most common cells of the epithelial layer, the access to enterocytes can be limited by the glycocalyx layer and by a lower endocytic activity of these cells. The glycocalyx layer on goblet cells is reduced compared to epithelial cells, but for the moment these cells are not identified as a target in gene therapies. Because of their role in immune response, the endocytic activity of M-cells is higher than the previous ones. Contrary to epithelial cells, they are covered with reduced glycocalyx and mucus layer. Furthermore, M-cells present other advantages. Lysosomes are lesser than in other cells leading to a reduced degradation of nucleic acids. Their basolateral membrane is like a pocket which limits the travel way for nucleic acids.

There are also closed to antigen-presenting cells that justify their potential in DNA vaccine delivery (Roy 1999). Nevertheless, as previously mentioned, the main restraint is their poor proportion in the gastro intestinal tract.

Lamina propria is richly vascularized and composed of immune cells with T and B cells, dendritic cells and macrophages. These cells could be targeted in therapy strategies. In inflammatory diseases such as inflammatory bowel diseases (IBD), macrophages produce proinflammatory cytokines that influence the disease state. It explains why these cells have been chosen as target in several studies (Bhavsar 2008a, Wilson 2010, Kriegel 2011a,b). In vaccine delivery and food allergy treatment (Jones 1997, Roy 1999), it could be interesting to target dendritic cells. In fact, these cells are the starting point of the immune response. They also could participate to the uptake of nucleic acids or their vectors across the epithelial layer thanks to their projection into the intestinal lumen. Since the endocytic activity of dendritic cells is equal to that of M-cells, they can contribute to the uptake of nucleic acid vectors (O'Neill 2011).

To conclude, Figure 2 summarizes the delivery of nucleic acids and/or their vectors in the gut after oral administration with the description of uptake and transport across the epithelium. The different barriers and targets can also be visualized. The delivery system of nucleic acids can make possible the transport from the intestinal lumen to lamina propria and bloodstream by paracellular route through tight junctions or by transcellular routes through enterocytes or M-cells. The nucleic acids can transfect three kinds of cells: the epithelial cells, the lamina propria cells or antigen-presenting cells. In the case of the transfection of epithelial cells, the expressed proteins can enter the lumen or the bloodstream. The transfection of antigen-presenting cells is desired in the case of vaccination. The transfection

results in stimulation of B cells and T lymphocytes. The produced antigen can be secreted in the intestinal lumen or in the bloodstream leading to systemic immunity.

### **2.3. Experimental models for studying the stability and efficiency of oral nucleic acid delivery systems**

<Insert Table 1 here>

The evaluation of the stability and efficiency of oral formulations of nucleic acids calls to several methods including *in vitro*, *in cellulo*, *ex vivo*, *in situ* and *in vivo* methods whose advantages and limitations are presented in Table 1. The first three types of experimental methods preceding the *in situ* and *in vivo* assays are implemented with the aim of predicting the efficiency of nucleic acid delivery systems in a whole living organism. Considering the successive obstacles that these systems have to overcome before delivering their load to its intracellular site of action, several complementary models are available to mimic each challenging step, from the *in vitro* ones to methods using biological supports like cells in culture or intestinal mucosa. *In situ* and *in vivo* models are the most elaborated ones with endoscopic/surgical intestinal administration and oral administration, respectively.

#### **2.3.1. *In vitro* models**

These are the simplest models employed. They allow assessing i) the stability of the formulations and their ability to protect the nucleic acids from degradation either by the gastrointestinal fluids (Ballarin-Gonzales 2013, O'Neill 2011) or by nucleases (Bhavsar 2007b, Han 2014); ii) the release of the nucleic acids from their delivery systems (Bhavsar 2007b, Bhavsar 2008b).

- Stability:

The stability of the formulations in extracellular conditions of the gastrointestinal tract is essential to their further activity. *In vitro* methods studying stability aim at mimicking the gastrointestinal environment in terms of pH, temperature, agitation, length of exposure, and biological composition. The integrity of the formulations and nucleic acids as well is determined after their exposure to the challenging medium. Structural stability of nanoparticulate formulations can be monitored in terms of particle size and zeta potential (Han 2014, He 2015). Polyacrylamide gel electrophoresis is also used to evaluate the biological stability (Ballarin-Gonzales 2013). The pH value may vary from 1.2-1.6 for gastric fluids, to 6.0-6.8 for intestinal fluids (O'Neill 2011). The temperature of 37°C is usually used as body temperature (Han 2014, He 2015) but room temperature is also employed (Ballarin-Gonzales 2013). Mechanical or magnetic agitation mimics peristalsis. The length of exposure shall reflect the transit time in each segment of the gastrointestinal tract. In practice, it varies from 1 hour (Ballarin-Gonzales 2013) to 2 hours (Han 2014, He 2015). As for composition, several media can be used including artificial ones like simulated gastric fluid and intestinal fluids (O'Neill 2011) prepared according to US Pharmacopoeia formulas, and natural ones such as intraluminal fluids collected in animals (He 2015). The composition of the medium is a critical parameter that may greatly influence the results of *in vitro* stability assays, especially depending on the presence or absence of bile salts, digestive enzymes (lipase, amylase, and trypsin), or mucus. In artificial media, the composition is well controlled since it reproduces formulas from the US Pharmacopoeia; while in natural intraluminal fluids, the composition may vary from one species to another, and within an animal species from one individual to another.

The ability of the formulations to protect their load from endogenous nucleases can also be determined through incubation with DNases or RNases. The principle is close to that of above-mentioned experiments in gastrointestinal conditions. The formulations are exposed to DNase-I (Bhavsar 2007b, Han 2014) or to RNase-A (Han 2014) at 37°C, for 20 minutes (Bhavsar 2007b) or 2 hours (Han 2014), and the biological stability of nucleic acids is determined by gel electrophoresis (Bhavsar 2007b, Han 2014).

- Release:

Beside stability studies, *in vitro* methods also comprise release studies. From a general point of view, the release of the therapeutic load from its pharmaceutical form is a necessary step for conventional drugs which activity depends on the absorption of their free soluble form. In such cases, drug release is classically evaluated *in vitro* by dissolution and dialysis assays. However, nucleic acid-based therapies are very different from conventional drugs since they cannot cross the cellular membrane unless carried by an adapted nanoscale delivery system. For that reason, nucleic acid release from their nanoparticulate carrier is not a key point in the development of such formulations. Nonetheless, some authors have implemented release studies to characterize oral formulations of nucleic acids because of the complexity of the oral system they have designed as a vehicle: gelatin nanoparticles, encapsulating the plasmid DNA, have been next included into poly( $\epsilon$ -caprolactone) microspheres (Bhavsar 2007b, Bhavsar 2008b). It was therefore necessary to determine whether these microspheres could release the nanoparticles carrying the nucleic acid. For that purpose, PBS containing protease was used as a release medium at 37°C, for 2 hours exposure, followed by 5 hours exposure in PBS with protease and lipase, the latter degrading the poly( $\epsilon$ -caprolactone) matrix and the former degrading the gelatin nanoparticles. The release was evaluated by fluorescence detection of the plasmid DNA whose stability was further studied

by agarose gel electrophoresis. The polymer degradation was also visualized by scanning electron microscopy (Bhavsar 2007b).

In summary, the results of *in vitro* stability testing often allow to screen several formulations and to choose the most protective one. Advantages of *in vitro* methods are their easy implementation and low cost. Nonetheless, they present limitations since their results are highly dependent on the experimental conditions, in particular the composition of the tested media. In addition, they are relatively distant from *in vivo* conditions.

### **2.3.2. *In cellulo* models**

*In cellulo* methods for evaluating oral formulations mainly use intestinal cultured cells as a biological support. These techniques either study the uptake of nucleic acid into cultured cells by the direct mean of intracellular measurement, or explore the mechanisms involved in the uptake process by using endocytic inhibitors, or determine intracellular trafficking through examination with transmission electron microscopy, or assess permeation across cells, or evaluate transfection efficiency by detecting a biological effect within the cells.

Beside rapidly growing immortalized cells that are poor models of the intestinal epithelium, a variety of more physiologically relevant differentiated cell culture models are available, including Caco-2 cells, Ht29 cells, co-cultures of Caco-2 with Ht29 cells, or M-cells, or Raji-B cells, multilayer cell models of intestinal epithelial cells (Caco-2 or T84 cells) plus basolateral macrophages (O'Neill 2011), and other cell culture models.

- **Caco-2** cells established from a human colon adenocarcinoma represent a well characterized model for assessing oral delivery of drugs. Caco-2 cell monolayers consist in highly polarized cells possessing the characteristics of mature enterocytes, i.e. tight junctions, a glycocalyx and apical microvilli. They also express several membrane and

cytosolic enzymes common to enterocytes *in vivo*. The Caco-2 Transwell™ system that separates apical and basolateral compartments allows measuring the transcellular transport of nucleic acid delivery systems. However, Caco-2 cells present an increasing resistance to transfection in relation to their differentiation; this phenomenon may be partially due to decrease in particle uptake, as well as poor nuclear accessibility resulting from the non-dividing nature of these differentiated cells (O'Neill 2011).

It is also possible to quantify the level of expressed proteins in the basolateral compartment (Martien 2008); and to evaluate potential tight junction-destabilization or monolayer integrity disruption through the measurement of the transepithelial electrical resistance of Caco-2 monolayers (O'Neill 2011). However, Caco-2 cells are devoid of mucus layer and represent only enterocytes. For those reasons, other cell lines can be preferred.

- Among the clones of **Ht29** cell coming from a human colon carcinoma, the Ht29GlucH clone forms monolayers including mucin-secreting goblet cells able to produce a mucus layer (Wikman 1993). These cells as well as the co-culture of Caco-2 and Ht29GlucH cells present the advantages to allow assessing the effect of mucus on nucleic acid transport (Cryan 2003). However, Ht29GlucH present certain limitations, notably the multilayer growth of cells (Schipper 1999). This phenomenon is overcome by the sub-clone Ht29MTXE12 which could provide a more reliable model for gene delivery assessment (O'Neill 2011).
- Follicle-associated epithelium (**FAE**) models associate Caco-2 monolayers and M cells obtained by murine lymphocyte-stimulated conversion of enterocytes, or human Raji-B cells (O'Neill 2011). These models possess some of the characteristics of the FAE of Peyer's patches, i.e. fewer or no microvilli and reduced alkaline phosphatase activity. They permit to assess the contribution of M-cells to transfection and/or transepithelial transport, and may be of particular interest in evaluating M-cell targeted oral formulations (O'Neill 2011).

- Multilayer cell culture systems associating an epithelial monolayer of Caco-2 or T84 cells to a basolateral other cell type, are of special interest when the target cells are located beneath the intestinal epithelium. This is the case of **IEC (intestinal epithelial cells)-macrophage cocultures**. The stimulation of macrophages by LPS (lipopolysaccharide) can induce a barrier dysfunction characteristic of inflammatory bowel diseases, through the production of TNF $\alpha$  by macrophages and IL-8 by IECs (Clayburgh 2004).

- Other cell culture models

Beside intestinal cultured cells, other models are used to evaluate oral formulations of nucleic acids. For example, mouse peritoneal exsudate cell macrophages (PECs) were employed to test  $\beta$ 1,3-D-glucan-encapsulated siRNA (Aouadi 2009); and rat PECs were used to evaluate chitosan-siRNA nanoparticles (He 2015). Human hepatoma BEL-7402 cells allowed assessing uptake, intracellular distribution and gene silencing by shRNA and siRNA encapsulated in multifunctional polymeric nanoparticles (Han 2014). Mouse monocyte macrophages Raw 264.7 cells were used to study the cellular uptake and intracellular trafficking of polymeric siRNA nanoparticles (He 2013a).

The critical parameters of *in cellulo* methods include the cell line employed, the conditions of their culture and the composition of the culture medium in which the formulation will be tested, the concentrations at which the cells will be exposed and the duration of exposure. The extraction method of the nucleic acids from cells, and the detection limit of the measurement method are critical as well in uptake studies. Finally, the sensitivity and specificity of detection of a biological effect are also of major importance.

In summary, *in cellulo* methods are very useful to test nucleic acid formulations in terms of uptake and biological efficacy. Their advantages are a relatively easy implementation and a



moderate cost. They are also closer to biological conditions than *in vitro* methods. However, their results are highly dependent on the cell line and the testing conditions employed.

### **2.3.3. *Ex vivo* models**

*Ex vivo* methods consist in studying the permeation of nucleic acid delivery systems across the intestine. A part of the intestine is collected in euthanized animals, rinsed, tied at both ends to form a bag, and maintained at 37°C in an oxygenated buffer under smooth shaking. The formulation to be tested is placed either inside the bag in the non-everted intestine method or in the external buffer bath in the everted intestine method. Measurement of the transport across the gut membrane allows calculating the apparent permeability coefficient. A few studies have been implemented on either everted rat jejunum (Hughes 1995) or on non-everted rat ileum (Zhang 2013b, He 2015). In the first case, the study suggested that antisense oligonucleotide transport across the jejunum occurred through slow passive diffusion (Hughes 1995). In the second case, ternary polymeric nanoparticles enhanced siRNA transport across the ileum as compared to naked siRNA (Zhang 2013b). In the third case, the ileal segments were selected to contain either no Peyer's patch or 5-6 Peyer's patches in order to represent non-follicle associated epithelia and follicle associated epithelia, respectively. The apparent permeability of mannose-modified chitosan-siRNA nanoparticles was enhanced by targeting normal enterocytes and M cells that express mannose receptors (He 2015).

Although more physiological than *in vitro* and *in cellulo* methods, *ex vivo* methods present limitations linked to the difficulties of implementation and to limited survival of the isolated intestine. They are also costly.

#### **2.3.4. *In situ* models**

*In situ* methods take place at the limit between *ex vivo* and *in vivo* methods. They consist in endoscopically or surgically delivering the oral formulation directly into an isolated intestinal segment of anesthetized animals, and in further studying the nucleic acid effect. *In situ* methods have been rarely employed in the literature to test oral nucleic acid delivery systems. The colon epithelium could be transiently transfected by cationic liposomes of recombinant DNA (Schmid 1997, Liptay 1998). The model of ligated rat intestinal loop allowed assessing efficient intestinal transfection by various synthetic vectors of plasmid DNA (Cryan 2003).

Such methods may be of interest when the pharmaceutical form cannot be administered to the animal by the oral route. This is the case for solid dosage forms like tablets that could neither be swallowed by small animals like rodents, nor be crushed and administered by gavage as a suspension without losing a matrix effect or a coating protection. Nonetheless, these methods are difficult to implement and invasive since they necessitate an endoscopic or a surgical delivery in anesthetized animals; they are also costly.

#### **2.3.5. *In vivo* models**

Above-cited models are very useful but none of them fully reproduces the complex conditions of *in vivo* environment. *In vivo* models represent the most physiologically relevant approaches to study nucleic acid delivery via the oral route of administration. Most of these models are implemented in rodents (Roy 1999; Bhavsar 2007b, Bhavsar 2008a, Ballarin-Gonzales 2013, Zhang 2013a, Han 2014, He 2015) but dogs are also used (Raouf 2004). Oral gavage represents the unique way of administration of liquid suspensions in rodents while

tablets could be administered orally to dogs (Raof 2004). Experiments are carried out either in healthy animals or in diseased animals. Healthy animals are used for biodistribution studies (Raof 2004, Zhang 2013b, He 2013a) or translocation studies (Ballarin-Gonzales 2013), or *in vivo* protein expression (Bhavsar 2008b, or *in vivo* transgene expression (Bhavsar 2007b) after euthanasia.

Animal disease models are necessary to assess the ability of nucleic acid delivery systems to induce a biological response, either protein expression, transgene expression, gene silencing, or therapeutic effect. Local as well as distant biological responses can be detected depending on the disease to be treated. Therapies to the intestine are intended for the treatment of local intestinal diseases. These pathologies include inflammatory bowel diseases, i.e. Crohn's disease and ulcerative colitis, colorectal cancer, and duodenal ulcers, which are good candidates for gene therapy intervention (O'Neill 2011). Local therapies also cover oral DNA administration for vaccination purposes because the intestinal mucosal surface offers an opportunity for generation of both mucosal and systemic immunity (Bhavsar 2007a). Therapies via the intestine are intended for the systemic treatment of distant pathologies like cancers, cystic fibrosis, hemophilia, diabetes (O'Neill 2011), hepatic tumor (Han 2014), and hepatic injury (Yin 2013, He 2013b, He 2015, Aouadi 2009).

- Models of local intestinal diseases

Crohn's disease is one of the two major forms of inflammatory bowel diseases. It mainly occurs in the distal ileum or the proximal colon while the second major form of inflammatory bowel diseases, ulcerative colitis, affects only the large intestine. Over forty models of intestinal inflammation are described in rodents. They can be chemically induced by oral administration of dextran sodium sulfate (Wilson 2010, Kriegel 2011a, b, Zhang 2013a), or by rectal administration of trinitrobenzene sulfonic acid (Bhavsar 2008a), or by

intraperitoneal injection of lipopolysaccharide (Laroui 2011). Intestinal inflammation can also result from genetic manipulation like IL-10 knockout (Kühn 1993), or depend on transfer of cells to immunodeficient animals (Wirtz 2007). These models are characterized by alterations close to human ones including epithelium disruption, immune cell infiltration and luminal bacterial translocation (O'Neill 2011). The severity indicators are body weight loss, stool consistency and rectal bleeding as clinical signs; colon macroscopic score, colon length and weight as macroscopic markers; proinflammatory cytokines, colon histology and myeloperoxidase activity as inflammation markers.

Colorectal cancer is one of the most common forms of intestinal cancer. It can be chemically induced in rodents by the carcinogen azoxymethane, or its precursor 1,2-dimethylhydrazine, reproducing histological and morphological features of human colon cancer (O'Neill 2011). Both above-cited chemicals used in combination can induce the development of colitis-associated colon tumor (Tanaka 2003). In addition, genetic mouse models spontaneously developing intestinal cancer can be obtained by mutations in the Apc gene, like the ApcMin mouse with multiple intestinal neoplasia, for example (Moser 1990).

Finally, experimental chronic duodenal ulceration can be chemically induced by cysteamine in rats (Szabo 2001).

- Models for vaccination study

These models study the induction of immunity in healthy mice against pathogen agents like rotavirus (Chen 1998, Herrmann 1999), bovine adenovirus type-3 (Mittal 2001), or pathologies like HIV (Kaneko 2000), and hepatitis B (He 2005).

- Models of pathologies distant from the digestive tract

Several models of non-intestinal diseases have been used to evaluate oral nucleic acid formulations intended for systemic effect. They include cystic fibrosis model (Alton 1993),

hemophilia model (Bowman 2008), diabetes model (Yoon 2002), hepatic tumor model (Han 2014), and lipopolysaccharide/D-galactosamine-induced hepatic injury (Yin 2013, He 2013b, He 2015, Aouadi 2009).

The critical parameters of *in vivo* models are the modalities of administration, mostly by gavage in rodents, the relevance of the experimental model compared to human diseases, and the choice of indicators of disease severity. *In vivo* methods offer the closest conditions to future utilization in medicine and allow the determination of the nucleic acid delivery system fate in a whole organism. Their limitations include difficulties of implementation and high cost. Of note, differences in anatomy and physiology between animal models and humans may hamper prediction of oral nucleic acid delivery performance in future clinical application. Hence, several parameters like for example gastrointestinal transit time, intraluminal pH under fasted or fed conditions, bile concentration, metabolic activity of phase I and phase II enzymes, membrane transporters, may notably vary in animals compared to human gastrointestinal characteristics (Sjögren 2014).

### **3. Oral delivery systems for nucleic acid administration (Table 2)**

<Insert Table 2 here>

#### **3.1. Naked molecules (essentially oligonucleotides)**

The base sequence of antisense oligonucleotide is complementary to its targeted gene's mRNA. Since unmodified DNA oligonucleotide structure is based on a phosphodiester linkage sensitive to naturally occurring nucleases, antisense drug candidates are generally modified to decrease their sensitivity to nuclease. They exhibit modified backbones, where the classical phosphodiester backbone is replaced by phosphorothioate backbone, or else sugar moieties of the nucleotides are altered. Modified oligodeoxynucleotides of a novel

class, defined as MBOs (novel hybrid DNA/RNA mixed-backbone oligonucleotides) have shown a significant improvement of pharmacokinetic properties *in vivo* compared with phosphorothioate-oligodeoxynucleotides. MBOs administered p.o. are absorbed in the gastrointestinal tract and distributed to major organs of mice and rats. GEM 231, a MBO with a hybrid DNA/RNA structure containing 2'-O-methyl-ribonucleosides at the 5' and 3' ends targets the protein kinase A type I (PKAI) subunit R1 $\alpha$ . This protein transduces mitogenic signals from different growth factors and oncogenes and is overexpressed in the majority of human cancers. When administered p.o. to nude mice bearing GEO human colon carcinoma xenografts, GEM 231 has anti-tumour activity (Tortora 2000). Similar results were obtained in SCID or nude mice with xenografts of human cancers of the colon (LS174T), breast (MDA-MD-468), and lung (A549) (Wang 1999). The mechanism by which modified oligonucleotide reaches its targeted cells is still unclear.

### **3.2. Cationic lipid-based nanostructures**

Cationic lipids are amphiphilic molecules able to interact with nucleic acid molecules. They form complex upon mixing via electrostatic interactions between the positive charges of the cationic lipid and the negative charges of the nucleic acid. Once complexed with cationic lipids, nucleic acid molecules are protected against nuclease activity and are readily taken up by cells by endocytosis or other mechanisms. Since the complexes formation lies on electrostatic interactions, cationic lipids can interact with both DNA and RNA and this irrespective of the size of the molecule, even if a collapse event is not expected when smaller molecules like siRNA or oligonucleotides are used.

#### **3.2.1. *Per se***

Only two publications show oral administration of particles formed with plasmid and cationic lipids. Liu et al (Liu 2014) used commercial cationic liposome Lipofectamine™ to prepare self-assembled complexes with plasmid DNA containing influenza M1 gene and administered intragastrically by gavage 100µg of plasmid to mice, three times at weekly intervals. One week after the final booster vaccination, mice were challenged with influenza virus to examine the efficiency of oral vaccination. Similarly, Lew et al (Lew 2002) associated plasmid DNA expressing human APC (adenomatous polyposis coli) gene with cationic liposome Lipofectin™ and administered transorally by gavage 20µg of plasmid to Min (Multiple Intestinal Neoplasia) mouse, that exhibit a mutation of the APC gene and consequently develop neoplastic intestinal polyps with elevated COX-2 levels similar to patients with familial adenomatous polyposis and sporadic colorectal cancer. The effects of APC gene replacement on intestinal neoplasia were observed 2 months after the oral administration. Although these publications show some efficacy following oral administration of these relatively simple formulations, most teams prefer to include the complex in a polymer matrix to provide better protection against the harsh conditions in the stomach.

### **3.2.2. Inside a polymeric matrix**

Wilson et al (Wilson 2010) prepared formulations of siRNA termed TKN (thioketal nanoparticles) generated by first preparing siRNA-DOTAP complexes then using a single-emulsion protocol to encapsulate these complexes into nanoparticles using a polymer, poly-(1,4-phenyleneacetone dimethylene thioketal) or PPADT, that degrades selectively in response to reactive oxygen species (ROS). DOTAP, or N-[1-(2,3-Dioleoyloxy)propyl]-N,N,N-trimethylammonium methyl, is a quaternary ammonium salt lipid capable of binding nucleic acid via electrostatic interactions and forming complexes or particles in which the nucleic

acid is protected from nucleases. Once siRNA/DOTAP complexes were formed, they were added to an organic solution containing PPADT to obtain nanoparticles. The authors showed that PPADT is composed of thioketal linkages that are stable to acid-, base- and protease-catalysed degradation and ROS-sensitive. Therefore, upon oral administration, the siRNA-particles remain stable in the gastrointestinal tract, thereby protecting siRNA and preventing its release and, at sites of intestinal inflammation, where high levels of ROS are produced, ROS-induced degradation of PPADT triggers the release of siRNA to inflamed intestinal tissues. Tahara et al described the preparation of nanospheres of chitosan-modified PLGA encapsulated complexes of oligonucleotides and DOTAP (Tahara 2011). Chitosan modified and unmodified PLGA provided 4h of protection for the DNA cargo from DNase I degradation and the chitosan modified provided protection from acidic pH for 2h. Once formulated, the efficiency of this decoy oligonucleotide targeting the nuclear factor  $\kappa$ B was tested upon oral administration in DSS-induced acute colitis in rats. Daily oral administration of high doses (2mg oligonucleotide / rat) of nanospheres significantly improved DSS-induced diarrhea, bloody feces, shortening of colon length, and myeloperoxidase activity. Nanospheres exhibited more adhesion and penetration in inflamed tissues than in normal tissues.

### **3.3. Condensing polymer-based nanostructures**

Besides cationic lipids, other cationic molecules of polymeric structure are frequently used to vectorize therapeutic nucleic acids. For oral administration the most popular is chitosan, a biodegradable polysaccharide modified from chitin, abundant in crustacean shells. Chitin is converted to chitosan by alkaline deacetylation. The major method for the formulation of chitosan-based nucleic acid delivery systems is complex coacervation in which the anionic nucleic acid complexes with cationic chitosan with the aid of either stirring or vortex force.



Chitosan behaves as a linear polyelectrolyte in solution with the N-deacetylated subunits conferring a highly positive charge density.

### **3.3.1. *Per se***

#### **3.3.1.1. Chitosan**

In 1999, Roy and colleagues published a proof-of-concept study using chitosan-plasmid DNA nanoparticles containing a dominant peanut allergen gene to demonstrate transduced gene expression in the epithelium of the gut upon oral administration of the DNA vaccine (Roy 1999). Nanoparticles, fairly spherical and 150-300nm in size were prepared by complex coacervation of high-molecular weight (about 390,000 Da) chitosan and plasmid, by simply mixing pre heated (55°C) solutions of plasmid in sodium sulfate and chitosan in sodium acetate-acetic acid buffer, pH 5.7. Mice were immunized orally with a dose of 50µg plasmid by oral gavage. Mice immunized with nanoparticles showed a substantial reduction in allergen-induced anaphylaxis, demonstrating that mucosal as well as systemic immunity was successfully achieved upon DNA vaccine delivery via oral mucosal surfaces. The same preparation protocol was used by Chen et al (Chen 2004) to produce chitosan-based nanoparticles with plasmids containing either LacZ gene or mEPO under inducible promoter (tetOn system). They obtained nanoparticles fairly spherical with a size of 70-150nm. They observed expression of β-galactosidase in stomach and small intestine 3 days after oral gavage of mice with 50µg LacZ gene-containing plasmid. They also administered once a week nanoparticles with plasmid expressing mEpo and observed an inducible modification of hematocrit during the first four days of treatment. A second administration, 6 days after the first one, was still able to promote a second hematocrit increase.

Following the same preparation protocol, Li et al (Li 2009) were able to produce chitosan-based nanoparticles with plasmid expressing house dust mite allergen Der p2. The obtained nanoparticles were found fairly spherical with a diameter size of 100-400nm. Mice were immunized with nanoparticles administered by oral gavage (100µg plasmid). It was shown that chitosan-DNA complexes can generate high expression of allergen in stomach and small intestine, three days post-administration, and prevent subsequent sensitization of Th2 cell-regulated specific IgE responses in a complete immunization study. Dhadwar et al (Dhadwar 2010) examined the influence of chitosan-DNA plasmid ratio on the size, morphology and transfection abilities of nanoparticles. They identified the best ratio yielding the smallest particle sizes (200-400nm) without producing large aggregates and exhibiting transfection efficiency on cells. Nanoparticles were prepared with plasmid containing canine FVIII cDNA. Hemophilia A mice (C57Bl/6<sup>FVIII<sup>-/-</sup></sup>) were administered by oral gavage with chitosan nanoparticles (150µg DNA plasmid expressing FVIII) and demonstrated transient canine FVIII expression one day after treatment together with partial phenotypic correction. The delivered plasmid was detected in the intestine and, to a lesser extent, in the liver. Repeated weekly administrations restored FVIII activity. In order to enhance resistance towards acids of chitosan-based plasmid nanoparticles, Kai et Ochiya proposed a preparation method in which nanoparticles were N-acetylated once formed and combined with mouse food (Kai 2004). They showed that the N-acetylated particles were more efficient to deliver gene to the intestines than particles prepared without N-acetylation; this result was most significant in the case of the duodenum. Chitosan was also used to prepare nanoparticles with siRNA (Ballarin-Gonzales 2013) at various chitosan to siRNA ratios. After oral gavage in mice, northern and quantitative PCR detection were used 1h and 5h post administration to study structural integrity and localization of siRNA. High levels of intact siRNA were detected in the

stomach, small intestine and colon 1 and 5 hours after oral gavage of nanoparticles. Intact siRNA was also detected in the liver, spleen, and kidney 1h after administration, with an organ distribution pattern influenced by chitosan to plasmid ratio used to prepared nanoparticles. These observations suggest a systemic translocation to peripheral organs after oral gavage in mice.

The above publications used unmodified chitosan, that produces, upon gentle mixture with nucleic acid solutions, chitosan / nucleic acid particulate complexes with broad size distributions (mean sizes from 100 to 600 nm), depending on the molecular weight of chitosan and chitosan to nucleic acid ratio. Because chitosan is soluble in an acidic pH range, the standard method of nanoparticle preparation comprises dissolving chitosan in acetic and/or hydrochloric acid.

#### **3.3.1.2. Modified chitosan**

Chitosan as a carrier for oral delivery of nucleic acids has many advantages including its strong nucleic acid affinity, bioadhesion and biodegradability. However, its insolubility in neutral and alkaline environments limits its application. To improve the solubility of chitosan over a wide pH range, trimethylation modification of chitosan has been proposed. Zhang et al have synthesized TMC (N-trimethyl chitosan) with 34% trimethylation degree (Zhang 2013b) and used it to prepare fluorescent nanoparticles encapsulating TAMRA-labeled siRNA to perform a biodistribution study upon oral administration to mice (4µg siRNA/mouse). Nanoparticles were shown to be able to promote the intestinal absorption of siRNA and subsequent entry into the systemic circulation and other reticuloendothelial tissues (liver and spleen). To achieve nanoparticles with compact and stable structures, a new preparation method has been proposed, based on ionic gelation in the presence of an

anionic component, tripolyphosphate (TPP). This method has been proposed since siRNA does not carry sufficient negative surface charge to form stable nanoparticles with cationic modified chitosan via simple complexation initiated by electrostatic interaction. TPP is a known food additive approved by FDA. Ionic gelation occurs spontaneously in aqueous solution through electrostatic interaction between cationic polymers and anionic TPP without sonication or heating, which can preserve biological activity of siRNA (Zhang 2013b). Furthermore, further modifications of methylated chitosan have been proposed to enhance its bioadhesion capacity through covalent binding with mucin glycoproteins and improve its permeation-enhancing potency. First, the synthesis of a partially quaternized derivative of chitosan, namely N-((2-hydroxy-3-trimethylammonium) propyl) chitosan chloride (HTCC) has been described. Wei et al have prepared HTCC-based nanoparticles of fluorescent siRNA and followed the biodistribution of siRNA thanks to *in vivo* fluorescence imaging after oral administration to mice bearing LLC (Lewis Lung cells) tumors (Wei 2013). The fluorescence intensity in gastrointestinal tract remained visible even 12h after administration, suggesting an efficient retention of nanoparticles in the intestinal tract. Meanwhile, the fluorescence signal was visualized and gradually increased in the LLC tumor after 12h administration, suggesting entry into the circulation and probably tumor localization through the enhanced permeability and retention (EPR) effect. Thiolated modification of chitosan with thiol-bearing compounds can also improve bioadhesion properties of nanoparticles. Thiolated trimethyl chitosan (TTMC) has been synthesized and used to prepare nanoparticles of siRNA through ionic gelation with sodium TPP (Zhang 2013b, He 2013b). Compared to unmodified chitosan-based nanoparticles, TAMRA-siRNA/TTMC particles showed the highest distribution percentages in most organs and plasma several hours after oral gavage administration in mice (Zhang 2013b). In addition, orally delivered TTMC particles with siRNA targeting TNF $\alpha$

at a siRNA dose of 200µg/kg significantly blocked LPS/D-GalN-triggered serum TNFα production in an acute hepatic injury model (He 2013b). The last modification of chitosan that has been suggested is the addition of targeting groups able to bind specific extracellular receptors in order to target nanoparticles to specific cellular types. Mannose- (He 2013a, Chu 2015, He 2015) or galactose- (Zhang 2013a, Han 2014) modified trimethyl chitosan-cystein conjugates have been synthesized and used to prepare nanoparticles with siRNA via ionic gelation with addition of TPP. Compared with nanoparticles without galactose, galactosylated nanoparticles were shown to more efficiently promote the distribution of fluorescent siRNA in ulcerative colon (in dextran-sulfate sodium (DSS)-induced ulcerative colitis mouse model) following oral administration. In addition, daily oral administration of galactosylated nanoparticles containing siRNA targeting Map4ka (mitogen-activated protein kinase kinase kinase kinase 4) significantly improved DSS-induced body weight loss, colon length shortening and increase of myeloperoxidase activity (Zhang 2013a). Nanoparticles of galactosylated chitosan with various degrees of modification have been used to encapsulate siRNA and shRNA within the same nanoparticles (Han 2014). Nanoparticles modified with a moderate degree (17%) outperformed the other particles, with no galactosylation or with higher (27%) or lesser (7%) degree of modification, in terms of siRNA accumulation in organs including tumors after oral administration to mice bearing hepatoma BEL-7402 tumors. Likewise, chitosan has also been modified with mannose and mannosylated nanoparticles of siRNA have been administered orally to mice to evaluate the biodistribution of fluorescent siRNA (He 2013a). Mannose is expected to be bound and taken up by macrophages. Mannosylated nanoparticles promote siRNA distribution in most organs (liver, spleen, lung and intestine), suggesting epithelial absorption of siRNA and subsequent trafficking to systemic circulation and other reticuloendothelial tissues. A single gavage of nanoparticles of

siRNA targeting TNF $\alpha$  blocked LPS/DGalN-triggered serum TNF $\alpha$  production in both time- and dose-dependent manners in mice (He 2013a) and in rats (He 2015). As for galactosylated nanoparticles, the degree of modification has to be controlled since the ligand grafting density affects the efficiency of the mannosylated nanoparticles with TNF $\alpha$  siRNA orally administered to mice bearing DSS-induced colitis (Chu 2015). Finally, modified chitosan has also been incorporated into supramolecular self-assembled nanoparticles, (SSNP) designed to deliver siRNA and able to overcome the absorption and transfection barriers posed by intestine. These nanoparticles were composed of (i) oleyl conjugated trimethyl chitosan (OTMC) expected to enhance permeation and transfection, (ii) poly( $\gamma$ -(4((2-(piperidin-1-yl)ethyl)-amino)methyl)benzyl-L-glutamate (PVBLG-8), a cationic  $\alpha$ -helical polypeptide designed to promote cellular internalization and endosomal escape, (iii) oleyl-PEG-mannose (OPM) designed to target the nanoparticles to enterocytes and macrophages that express mannose receptors, thus improving the intestinal absorption and macrophage uptake, (iv) oleyl-PEG-cysteamine expected to improve the mucoadhesion of the nanoparticles by forming disulfide bonds with mucin glycoproteins enriched in the intestinal mucosa. In contrast with previous nanoparticles (see above), in this strategy, each group (ligand, SH-, ...) is provided by separate components instead of being grafted on the same chitosan molecule. SSNP were used to encapsulate siRNA targeting TNF $\alpha$  and were given by gavage to mice suffering from LPS/D-GalN-induced acute hepatic injury. SSNP orally administered 24h before LPS/D-GalN treatment significantly improved the survival rate and inhibited the elevation of transaminase serum levels (Yin 2013).

### **3.3.2. Inside a micrometric matrix**

Nanoparticles formed by condensing polymers, like chitosan or PEI, have also been incorporated inside a micrometric matrix before oral administration. Bowman et al mixed unmodified chitosan-based nanoparticles of plasmid DNA expressing FVIII with strawberry Jell-O brand gelatin (Bowman 2008) to avoid gavage and get mice to eat nanoparticles contained inside gelatin. Functional FVIII protein was detected in plasma following oral administration of the chitosan nanoparticles to hemophilia A mice. More sophisticated multicomponent systems consisting of pre condensing of nucleic acid with cationic polymer and subsequent incorporation of the obtained nanoparticles into micrometric matrix have been described for oral delivery of plasmid DNA or siRNA. Laroui et al prepared complexes of PEI and siRNA, subsequently loaded into polylactide (PLA) in a single emulsion, followed by a double emulsion covered with polyvinylalcohol (PVA) (Laroui 2011). The final particles of 300 to 500nm in diameter were further encapsulated into a biomaterial composed of alginate and chitosan and given by gavage to mice before induction of acute inflammation by LPS injection. Nanoparticles loaded with siRNA targeting TNF $\alpha$  were shown to specifically reduce the TNF $\alpha$  expression in colonic tissue in LPS-treated mice. Aouadi et al also reported oral delivery of siRNA with the so-called GeRP ( $\beta$ 1,3-D-glucan-encapsulated siRNA particles). These particles are prepared following a layer by layer approach where 2-3  $\mu$ m large porous hollow outer  $\beta$ 1,3-D-glucan shells from baker's yeast were first filled with tRNA and PEI to build the cationic core that will encapsulate the siRNA, associated with a cationic peptide, Endoporter, and followed by another PEI layer (Aoudi 2009). Mice were given by oral gavage 10 to 20 $\mu$ g / kg of siRNA targeting Map4k4 and were injected with LPS to induce acute inflammation. Oral delivery of GeRP containing siRNA anti Map4k4 blocked the increase of serum TNF $\alpha$  and lethality. GeRPs were suggested to target the M cells of the Peyer's patches in the intestine in order to reach the gut-associated lymphatic tissue (GALT) located below.

Over time, the ingested GeRPs may migrate throughout the lymphatic tissue away from the gut. The last system called NiMOS (for nanoparticles in microspheres oral system) consists of pDNA (Bhavsar 2007a, b, 2008a, b) or siRNA (Kriegel 2011a, b) physically encapsulated by type B gelatin nanoparticles, which are further encapsulated within poly( $\epsilon$ -caprolactone) (PCL) microparticles. This system has been designed to facilitate protection of the nanoparticles from the harsh environment of the stomach, and then upon reaching the intestine, PCL is expected to be degraded by intestinal lipases, releasing the nanoparticles, which are then available for uptake by cells of the small and large intestine, where gelatin can be degraded by proteases. NiMOS were used to orally deliver plasmid DNA expressing reporter genes (Bhavsar 2007b) or IL-10 (Bhavsar 2008a) to mice or rats. A 100 $\mu$ g dose given by oral gavage lead to transgene expression 5 days post-administration in intestine and even therapeutic efficiency in TNBS- (2,4,6-trinitrobenzenesulfonic acid) induced acute colitis model in mice. Similarly, NiMOS encapsulated siRNA targeting TNF $\alpha$  (Kriegel 2011a) or Cyclin D1 (Kriegel 2011b) were orally administered to mice suffering from DSS-induced acute colitis and successful gene silencing led to decreased expression of pro inflammatory cytokines, increase of body weight and reduced tissue myeloperoxidase activity.

### **3.4. Other nanostructures**

Jazayeri et al have reported silver-based nanoparticles (AgNP) for oral DNA vaccine delivery (Jazayeri 2012). They used a green method to synthesize AgNP from AgNO<sub>3</sub>,  $\beta$ -D-glucose and PEG as reduction agent and stabilizer, respectively. Although some concerns about their toxicity have been raised, in this study AgNP were conjugated to plasmid DNA expressing the avian influenza virus H5 gene and orally administered to one day old chicks. No toxicity induced by AgNP delivery was observed and rapidly increasing antibodies against H5 were



detected in serum samples collected on days 14 and 35 post-immunization. Polymeric micelles have also been reported to orally deliver plasmid DNA. Non ionic PEO-PPO-PEO (poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide) triblock copolymer has been used to condense pDNA to form micelles with a diameter of 170nm and nearly neutral ( $\zeta$  potential -4mV). Six doses of micelles containing LacZ encoding plasmid were orally administered to mice (Chang 2004) and led to expression of the encoded protein in the villi, crypts, and goblets cells of the duodenum and in the crypt cells of the stomach. LacZ gene activity was also seen in the liver. The same polymeric micelles were also administered to parathyroidectomized rats to deliver plasmid DNA expressing parathyroid hormone (PTH) (Chou 2009) and led to expression of PTH in gastrointestinal tissues. Triblock copolymer micelles were prepared with copolymers poly(D,L-lactic acid)-co-poly(ethylene glycol)-co-poly(D,L-lactic acid) (PLA-PEG-PLA) and poly(D,L-lactic-co-glycolic acid)-b-poly(ethylene glycol)-b-poly(D,L-lactic-co-glycolic acid) (PLGA-PEG-PLGA) and used to entrap plasmid DNA. The hydrophilic PEG segment has been incorporated into polylactide and glycolic acid to improve the hydrophilicity of the polymers. Micelles of PLA-PEG-PLA / PLGA-PEG-PLGA with encapsulated plasmid expressing multiple-epitope antigen gene of hepatitis C virus were orally administered to mice with 100 $\mu$ g pDNA per vaccination. The results indicated that oral immunization achieved satisfying immune responses (Yang 2011) with scarcely any side effects.

### **3.5. Micrometric delivery systems and minitablets**

Besides nanostructures, larger particles of rather micrometric size and minitablets have also been reported for oral delivery of nucleic acids. Although they do not fall within the

nanostructure category in the strict sense, we chose to present them as they are much used in particular for genetic vaccination.

### **3.5.1. PLGA micrometric formulations**

Poly (lactide-co-glycolide) (PLGA), which has FDA approval for drug implants, is widely used for the production of biodegradable and biocompatible micro- and nanoparticles. PLGA has been one of the most commonly used non-condensing polymers in intestinal gene delivery. It has generally been formulated as microparticles to deliver DNA vaccines. Although PLGA-based microparticles cannot themselves transduce cells, they are phagocytosed by M cells in the Peyer's patches, making them a useful polymer system for oral vaccination.

The first report of systemic and mucosal antibody responses to encoded protein upon oral administration of the plasmid expressing the antigen has been published in 1997 (Jones 1997). PLGA-based microparticles of plasmid expressing photinus luciferase (50µg pDNA) were orally administered to mice by gavage. Luciferase specific IgG and IgM antibodies were detected in serum samples 3 and 6 weeks post-administration. In addition, significant levels of specific mucosal IgA antibodies were also detectable, suggesting that PLGA encapsulation has the ability to protect pDNA against degradation after administration, and to facilitate its uptake into appropriate cells for the subsequent expression and presentation of antigen, in such a way as to elicit both systemic and mucosal antibody responses. Afterwards similar studies were conducted to induce immune responses towards various antigens, rotavirus VP6 protein (Chen 1998), HIV envelope glycoprotein (Kaneko 2000), hepatitis B virus small envelope protein (He 2005), by oral immunization with PLGA-based microparticles containing antigen-coding DNA plasmid. In these three reports, one or several doses of

vaccine orally given to mice elicited both antigen-specific serum antibodies and induced resistance to mucosal viral transmission in one model (Kaneko 2000).

### **3.5.2. Alginate micrometric formulations**

Sodium alginate is a naturally occurring polysaccharide which can be easily cross-linked into a solid matrix with the addition of divalent cations, cross-linking in a water-in-oil emulsion resulting in the formation of microspheres. Alginate microparticles are safe to use in animals and have been used to encapsulate purified proteins, bacterial outer membrane proteins and virus. Mittal et al encapsulated plasmid encoding the bacterial  $\beta$ -galactosidase gene inside alginate microspheres and compared immune responses following various routes of administration, oral, intranasal, intramuscular, subcutaneous or intraperitoneal (Mittal 2001). They reported that oral immunization of mice induced slightly higher titers of LacZ specific IgA antibodies at mucosal sites compared to those obtained by systemic routes, namely intramuscular, subcutaneous and intraperitoneal. The ability of orally administered alginate microspheres to deliver plasmid DNA into intestinal cells of mice and induce the expression of the encoded protein was reported by Nograles et al (Nograles 2012) and Rosli et al (Rosli 2013). Using expression plasmid carrying gene-coding sequences for the cholera enterotoxin B subunit (ctxB) immunostimulatory antigen, the latter have observed IgA antibody responses at local mucosal sites following oral administration of alginate microspheres containing plasmid DNA (Rosli 2013).

### **3.5.3. Tablets**

ISIS 104838, a 2'-O-methoxy ethyl partially modified phosphorothioate antisense oligonucleotide targeting human TNF $\alpha$  mRNA was incorporated into minitables containing

sodium caprate permeation enhancer (Raouf 2004). Sodium caprate enhances drug absorption, causes dilatation of tight junctions, enhances paracellular transport and is FDA approved as an emulsifier or binder food additive. An Opadry™ subcoat was applied to the minitablets that were then further coated with hydroxy-propyl methylcellulose (HPMC-50) and orally administered to beagle dogs to investigate tolerability and tissue distribution of ISIS 104838. The tissue distribution profile showed that the highest concentrations of ISIS 104838 were observed in the kidney, followed by the liver, lymph nodes and spleen. In addition the study showed that multidose administration was well tolerated during the in-life phase of the study as well as through clinical pathology (standard clinical chemistry and haematology tests) and post-mortem examination at the end of the study. Oral absorption of ISIS 104838 was also investigated in human healthy volunteers (Tillman 2007). Four different capsule formulations were prepared, one containing a single population of minitablets designed to perform an immediate releasing of oligonucleotide, the others containing two populations of minitablets including the one permitting immediate release of ISIS 104838 and another minitablet population. The other minitablet populations were either a short or long delayed release of sodium caprate and were coated with different levels of methacrylate polymer Eudragit™ RS 30D (poly(ethyl acrylate-co-methyl methacrylate-co-trimethylammonioethyl methacrylate chloride). Minitablets were then encapsulated in gelatin capsules, and then an Eudragit™ L30D55-based (poly(methacrylic acid-co-ethyl acrylate)) enteric outer coating of the capsules was added. These various formulations were well tolerated in both fasted and fed volunteers. Oral absorption of ISIS 104838 was demonstrated with an average 9.5% plasma bioavailability.

#### **4. Conclusion**

Increasing nucleic acid delivery systems are developed for the oral route. Although therapeutic nucleic acids molecules exhibit unique properties in terms of stability and characteristics like global charge and size, an effective delivery through oral administration has been demonstrated in various preclinical models. However, at present, no system has reached clinical trials in humans, apart from the delivery of naked antisense oligonucleotides. It is therefore still necessary to improve the efficiency of these vectors as well as to characterize their toxicity, in particular for the most recent discovered molecules such as RNAi-trigger molecules.

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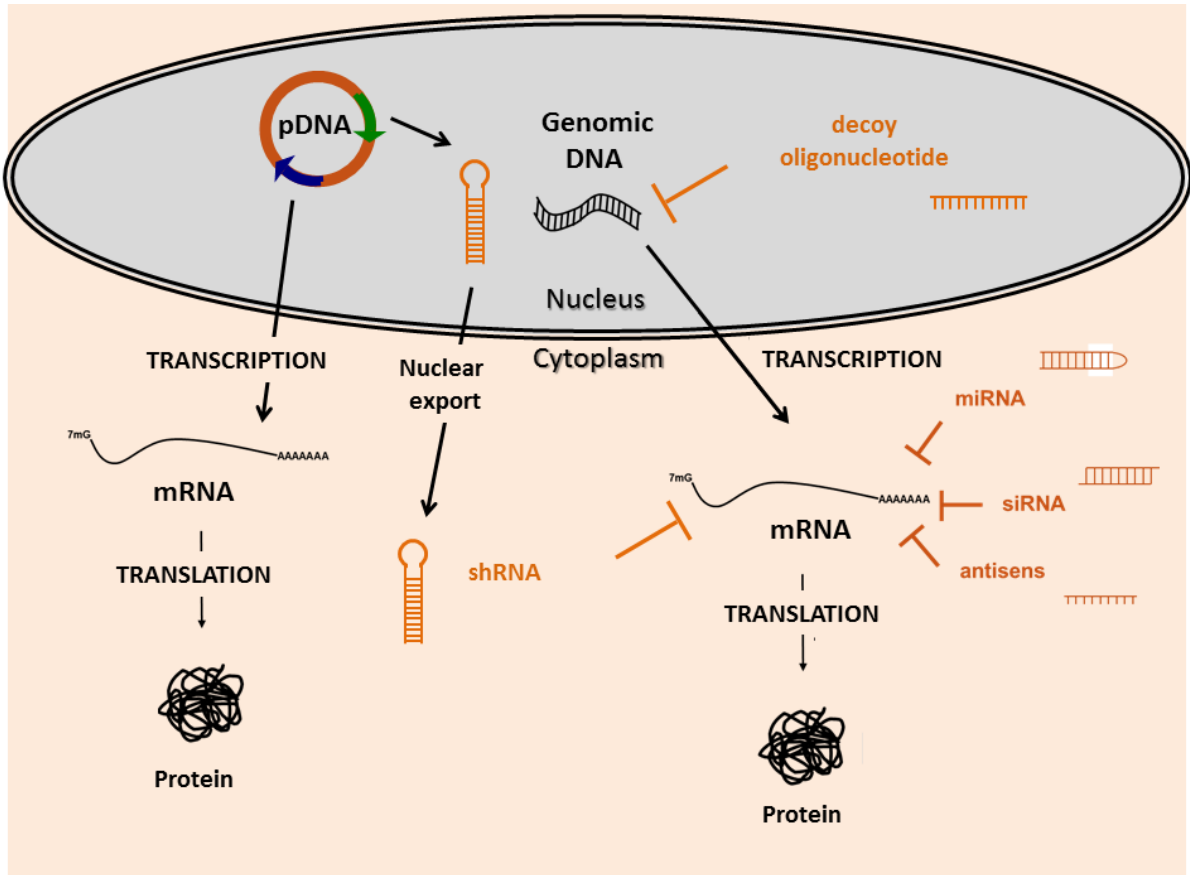
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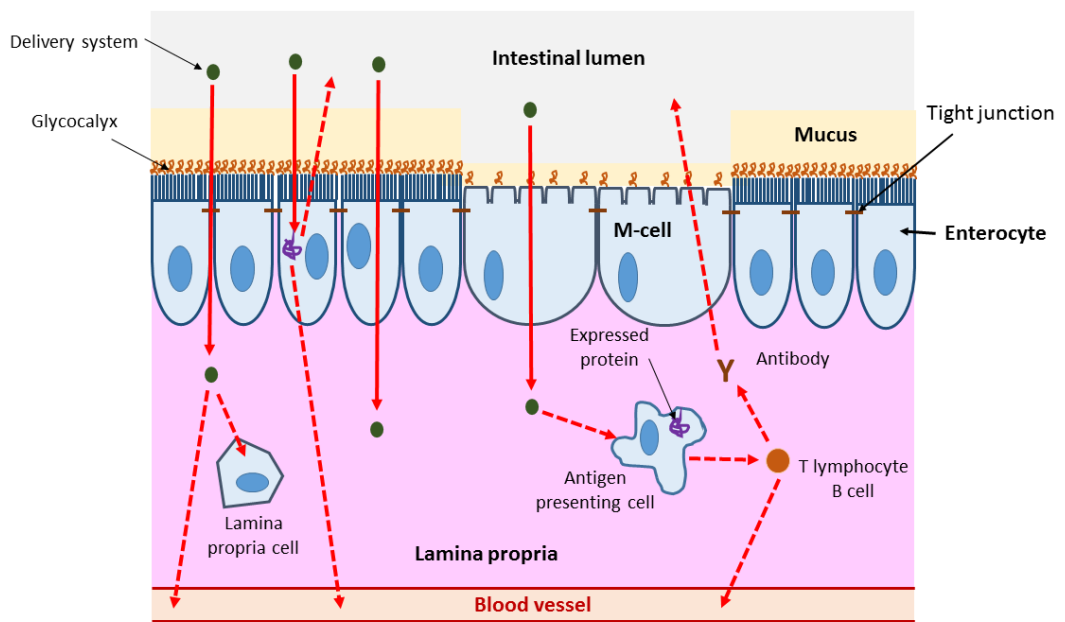
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## Figure legends

### Figure 1 – Overview of therapeutic nucleic acids pathway and sites of action



**Figure 2 - Fate of nucleic acids delivery system in the gut following an oral administration with the description of the different routes, barriers and targets.**



**Table 1. Advantages and limitations of experimental methods for evaluating stability and efficiency of oral formulations of nucleic acids**

Experimental methods	Advantages	Limitations
<b><i>In vitro</i> methods</b>		
- Stability assessment	Easy implementation	Results highly dependent on the medium composition
- Release of nucleic acids	Low cost	Distant from <i>in vivo</i> conditions
<b><i>In cellulo</i> methods</b>		
- Cellular uptake	Implementation relatively easy	Results highly dependent on the cell line and on the testing conditions
- Intracellular trafficking	Closer to biological conditions than <i>in vitro</i> methods	Necessity to extract the nucleic acids from cells in uptake and intracellular distribution studies
- Permeation across cells	Methods of choice for testing nucleic acid biological effects	
- Transfection efficiency	Moderate cost	
- Biological effect detection		
<b><i>Ex vivo</i> methods</b>		
- Methods of transport across gastrointestinal membranes	Closer to <i>in vivo</i> conditions than <i>in vitro</i> and <i>in cellulo</i> methods	Difficulty of implementation Limited survival of the isolated intestine Higher cost than above-cited methods
<b><i>In situ</i> methods</b>		
- Intestinal transfection efficiency in anesthetized animals	Intermediate between <i>ex vivo</i> and <i>in vivo</i> methods Possibility to test conventional oral forms like tablets that can neither be crushed before administration nor be swallowed by rodents	Difficulty of implementation High cost
<b><i>In vivo</i> methods</b>		
- Biodistribution in healthy or diseased animals	Closest conditions to future utilization in medicine Determination of the nucleic acid delivery system fate in a whole organism	Difficulty of implementation High cost
- Translocation in target tissues		Anatomical and physiological differences between animal and human gastrointestinal tracts
- Therapeutic efficacy in diseased animals		



Table 2 – Delivery systems for oral delivery of nucleic acids

Molecule	Vector	Particle size	Gene	Specie	Dose	Model	Reference
siRNA	GeRP	2-4 µm	Map4k4	mouse	20µg/µg	LPS/DGalN-ALI	Aoudi 2009
	TKN	600 nm	TNFα	mouse	0.23 and 2.3 mg/kg	DSS-UC	Wilson 2010
	NiMOS	2-3 µm	Cyclin D1/ TNFα	mouse	1.2 mg/kg	DSS-UC	Kriegel 2011a, b
	(PEI/PLA) Cs modified - alginate	380 nm	TNFα	mouse	not given	LPS/DGalN-ALI	Laroui 2011
	Cs NP	150 nm	no target	mouse	28 and 78 µg / mouse	biodistribution	Ballarin-Gonzales 2013
	T – Cs NP	150 nm	TNFα	mouse		LPS/DGalN-ALI	He 2013a,b
	HTP- Cs NP	130 nm	TERT	mouse	0.2 and 6.5µg/kg	tumor	Wei 2013
	SSNP	130 nm	TNFα	mouse	200 µg/kg	LPS/DGalN-ALI	Yin 2013
	TTG- Cs NP	150 nm	Map4k4	mouse	250 µg/kg	DSS-UC	Zhang 2013a,b
	TTG- Cs NP	130 nm	VEGF (+ shRNA Survivin)	mouse	200 µg/kg (1mg pDNA/kg)	Hepatoma	Han 2014
	TTM – Cs NP	150 nm	TNFα	mouse	30 µg/kg	LPS/DGalN-ALI and DSS-UC	Chu 2015
	TTM – Cs NP	150 nm	TNFα	rat	20 and 50 µg/kg	LPS/DGalN-ALI	He 2015
Oligonucleotide							
	Naked		PKA I	mouse	0.5 to 10 mg/kg	tumor	Wang 1999
	Tablet		TNFα	dog	80 mg / dog	biodistribution	Raooof 2004
	Tablet / Capsules		TNFα	man	100 and 140 mg	toxicity / pharmacokinetics	Tillman 2007
	(PLA/PLGA)	370 nm	NF κB	rat	2 mg / rat	DSS-UC	Tahara 2011
	Chitosan						
	Naked		PKA I	mouse	1 to 10mg/kg	tumor	Tortora 2000

Cs NP : Chitosan Nanoparticle, DGalN-ALI : D-galactosamine-induced acute liver injury, DSS-UC : Dextran sulfate sodium-induced ulcerative colitis, GeRP : β1,3-D-Glucan-encapsulated siRNA Particles, HTP : N-((2-hydroxy-3-trimethylammonium) propyl), LPS : lipopolysaccharide, Map4k4 : Mitogen-activated protein kinase kinase kinase 4, NF κB : Nuclear factor κB, NiMOS : Nanoparticles in Microspheres Oral System, pDNA : plasmid DNA, PEI : polyethylenimine, PKAI : protein kinase A type I, PLA : poly(D,L-lactic acid), shRNA : small hairpin RNA, siRNA : small interfering RNA, SSNP : supramolecular self-assembled nanoparticles, T-Cs NP : Trimethyl chitosan nanoparticle, TERT :

Telomerase Reverse Transcriptase, TKN : thioketal nanoparticles, TNF $\alpha$  : Tumor Necrosis Factor  $\alpha$ , TTG : thiolated trimethyl galactose, TTM : thiolated trimethyl mannose, VEGF : vascular endothelial growth factor.

Molecule	Vector	Particle size	Gene	Specie	Dose	Model	Reference
Plasmid DNA							
	PLGA	2 µm	Luciferase	mouse	50 µg /mouse	vaccination	Jones 1997
	Cs NP	150 – 300 nm	Peanut arah2 allergen	mouse	50 µg / mouse	allergy	Roy 1999
	Cs NP	70 – 150 nm	LacZ /mEPO	mouse	50 µg / mouse	hemophilia	Chen 2004
	NiMOS	5 µm	LacZ / GFP	rat	100 µg / rat	gene expression	Bhavsar 2007
	NiMOS	2 – 5µm	IL-10	mouse	100 µg / mouse	TNBS-induced colitis	Bhavsar 2008
	Cs NP	200 – 400 nm	FVIII	mouse	50 µg / mouse	hemophilia	Dhadwar 2010
	PLGA	0.5 µm	rotavirus VP6	mouse	50 µg / mouse	vaccination	Chen 1998
	N-acetylated Cs NP	not given	LacZ / IL-10	mouse	6 mg / mouse	gene expression	Kai 2004
	Alginate	5-10 µm	LacZ	mouse	not given	vaccination	Mittal 2001
	PLGA	2-9 µm	env HBV	mouse	20 – 200 µg / mouse	vaccination	He 2005
	PLGA	1-10 µm	env HIV	mouse	10 µg / mouse	vaccination	Kaneko 2000
	Alginate	45 µm	CtxB	mouse	50 – 150 µg / mouse	vaccination	Rosli 2013
	Lipofecamine	not given	Influenza M1 gene	mouse	100 µg / mouse	vaccination	Liu 2014
	Cs NP	100-400 nm	Dust mite allergen Der p2	mouse	100 µg / mouse	vaccination	Li 2009
	PLA-PEG-PLA / PLGA – PEG–PLGA micelles	not given	HCV MA gene	mouse	100 µg / mouse	vaccination	Yang 2011
	Alginate	50 µm	GFP	mouse	50 to 150 µg / mouse	gene expression	Nogralas 2012
	Silver nanoparticle	25 nm	Avian virus H5 gene	chicken	not given	vaccination	Jazayeri 2012
	PEO-PPO-PEO micelles	170 nm	parathyroid hormone	rat	720 µg / rat	parathyroidectomised rat	Chou 2009
	Cs NP in Jello	300 nm	FVIII	mouse	50 to 600 µg / mouse	hemophilia	Bowman 2008
	Lipofectin	not given	APC	mouse	20 µg / mouse	tumor	Lew 2002

APC : adenomatous polyposis coli, Cs NP : chitosan nanoparticle, CtxB : cholera enterotoxin B subunit, FVIII : coagulation factor VIII, GFP : green fluorescent protein, HBV : hepatitis B virus, HCV : hepatitis C virus, HIV : human immunodeficiency virus, IL-10 : interleukin-10, MA : multiple-epitope antigen, NiMOS : Nanoparticles in Microspheres Oral System, PEG : polyethyleneglycol, PEO-PPO-PEO : poly(ethylene oxide)-poly(propylene oxide)-poly(ethylene oxide), PLA : poly(D,L-lactic acid), PLGA : poly(D,L-lactic-co-glycolic acid).