CHAPTER SIX

GASTROINTESTINAL DELIVERY OF ANTI-INFLAMMATORY NANOPARTICLES

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Abstract
The concept of nanomedicine has risen to be the future of medicine. Advantages of using nanoobjects as vectors for drug delivery systems are numerous, such as fewer side effects due to a low drug dose, and high specificity between drug and target. Unlike systemic therapy, targeting a specific target is more efficient and less costly. In inflammatory bowel disease, including ulcerative colitis and Crohn disease, the colon represents the targeted organ. A large number of drugs are candidates for loading into nanoparticles (NPs). Small molecules, such as tripeptides and siRNA, or larger molecules, such as proteins (hormones,
antibodies (Ab), etc.), can be encapsulated alone or in a complex form inside the NPs. In our studies, once NPs are synthesized and loaded with anti-inflammatory compounds, they are delivered to the colon. An efficient technique has been developed for specific NP targeting to digestive tract regions, including the colon, using a hydrogel based on electrostatic interactions between positive ions and negative polysaccharides. An *in situ* double cross-linking process, mediated by Ca$^{2+}$ and SO$_4^{2-}$, of chitosan and alginate administered to the mouse gastrointestinal (GI) tract by double gavage, is used for gel formation. When the drug is given in NPs, NPs are targeted to the colon, and NP degradation by aggressive environmental conditions in the GI tract is significantly reduced. Using a biomaterial (hydrogel) associated with nanotechnology, lower doses of drug can be loaded efficiently and delivered to the colon to reduce colonic inflammation.

1. **Introduction**

Techniques to deliver drugs into the gastrointestinal (GI) tract can include the provision of drugs in solution. However, such drugs will be directly affected by the pH of the stomach and are likely to be degraded under acidic pH conditions. To circumvent degradation by stomach acidic pH or small intestine digestive enzymes, high drug doses or frequent administration are commonly used, and side effects may be problematic. Recently, complicated techniques, such as sprays of micro- or miniemulsions with high drug doses, have been used. These approaches used nasal and esophageal drug delivery systems, as opposed to lower GI tract-directed systems (Eslamian and Shekarriz, 2009; Li *et al.*, 2002; Sintov *et al.*, 2010). Enemas are often used to target drugs to the colon, but the procedure is cumbersome and is associated with high risk of local complications, including bleeding or perforation, especially in small animals. Thus, there is an unmet need for targeted drug delivery to specific areas in the GI tract, particularly the colon. The colon is important to target, as it is one location for colon cancer or inflammatory bowel disease (IBD), including ulcerative colitis (UC) and Crohn disease (CD). The enema strategy is limited to the distal part of the colon and is not able to reach the ascending (proximal) part of the colon. As the last step of the digestive tract, the colon is challenging to target with intact and quantitative amounts of drug. From oral uptake (saliva enzymes) to colon (pH 7, higher pressure), through the stomach (pH 1–3) and the small intestine (enzymatic release and pH 3–6), drugs face a deleterious environment.

Pharmaceutical nanotechnologies are a promising future for medicine. They can be defined as nanoscale technological innovations or “nanomedicine.” This high-interest field provides the opportunity to design and develop several modified or complexed molecules or vectors that can target, treat, and diagnose several diseases, including diseases of the colon. Several
drug delivery nanosystems have been developed to target the GI tract since the early 1960s, including micelles (Bromberg, 2005; Gou et al., 2011), liposomes (Garg and Kokkoli, 2011; Riviere et al., 2011), dendrimers (Navarro and Tros de Ilarduya, 2009; Wiwattanapatapee et al., 2003), and nanoparticles (NPs; Larouï et al., 2010a, 2011a,b; Lasic, 1992). Drug delivery systems have been engineered for: (1) specificity: to target only tissues or cells related to the disorder detected; (2) efficiency: to protect a drug against early biological environmental degradation (pH, enzymes, and oxidative agents); and (3) modulation: to modulate and control drug pharmacokinetics.

NPs loaded with drug require a specific vector to efficiently deliver them to a strategic area like the colon. Two strategies can be performed to locally deliver nonsteroidal anti-inflammatory drugs (NSAIDs) using NPs. First, NP matrices can be engineered to favorably collapse in the targeted area, using specific characteristics of the inflamed colon, such as formation of a prodrug, time, pH (Camma et al., 1997; Ewe et al., 1999; Lamprecht et al., 2005), release of oxidative species (Metz et al., 2009; Wilson et al., 2010), aberrant secretion of bacterial enzymes (Kabbaj and Phillips, 2001; Kinget et al., 1998; Levitt et al., 1987), local increase of heat by an external magnetic field (Sato et al., 2009), osmotic systems (Chaudhary et al., 2011), and pressure-controlled drug delivery systems (Jeong et al., 2001; Takaya et al., 1995). In addition, biomaterials such as hydrogels have biological, physical, and chemical characteristics that set them as major candidates for NP vectorization. Hydrogels are a network of polymer chains that are hydrophilic and can contain 99% of water. Hydrogels can protect the drug and/or the drug vector until the targeted organ is reached. They are then dissolved under particular conditions of pH, time, temperature, or enzyme activity. Again, the hydrogel has to be selected and/or engineered to be degraded at a specific GI location such as the colon.

In this review, we present a protocol to specially target the colon with NPs. The schematic shown in Fig. 6.1 illustrates our targeting strategy. As the GI tract is very aggressive, we load the NPs into a hydrogel with the specific ability to collapse only in the colon.

2. Material According to Drug Application

NPs are made based on the double emulsion/evaporation of solvent technique (Fig. 6.2). The hydrophilic drug is first loaded with bovine serum albumin (BSA; 5% in water) in D,L polylactic acid (PLA; 25 g/L in dichloromethane). Each hydrophilic drug that is loaded has to be studied to optimize the loading rate, the kinetic release profile, and to ensure the molecular integrity. Sonication at 50% Pmax for 2 min of 50% active cycle is performed. Ultrasound provides energy to the system and allows emulsion formation. A water in oil (W/O) emulsion is obtained and dropped in a larger aqueous
phase containing polyvinyl alcohol (PVA; 3 g/L in distilled water). The same protocol is performed to generate a water in oil in water (W/O/W) emulsion. Then, a rotating evaporator removes dichloromethane from the solution entrapping the drug into the NPs. Centrifugation removes the PVA that is not adsorbed on the surface of the NPs (Laroui et al., 2007).

NPs allow for dramatically lowering the amount of the drug used by specifically and cellularly delivering the bioactive drug.

3. Anti-inflammatory Compounds as Encapsulated Drug

NSAIDs are important molecules to deliver to sites of inflammation. In this class of drugs, NSAIDs are widely used as a major treatment for IBD and colon cancer prevention and treatment. Studies from the 1980
and 1990s showed beneficial effects of NSAIDs on inflammation and tumor regression in animal models (mouse and rat), including indomethacin (Kudo et al., 1980; Narisawa et al., 1983; Pollard and Luckert, 1981) and piroxicam (Rao et al., 1991; Reddy et al., 1987, 1990). Those studies have since been continued in humans, and NSAIDs have shown positive effects on inflammation and colon cancer. Clinical trials tested sulindac (Koornstra et al., 2005; Waddell and Loughry, 1983), indomethacin (Itoh et al., 1988), and acetyl salicylic acid (Paganinihill et al., 1989; Rosenberg et al., 1991a,b). However, in the GI field, classic oral prescriptions of NSAIDs are limited. NSAIDs are never to be used in individuals with IBD, including CD or UC, due to their tendency to cause gastric bleeding and form ulceration in the gastric lining. Alternative molecules used as pain relievers, such as paracetamol (also known as acetaminophen) or drugs containing codeine (which slows down bowel activity), are safer medications for pain relief in IBD.

To prevent any deleterious effect of the NSAID, the active molecules can be loaded into NPs and be delivered efficiently to the inflamed location. NP encapsulation of the bioactive compound allows for lower doses and side effects related to NSAIDs. Depending on the context, the size, and the

![Figure 6.2](image_url)
charge of the drug loaded into NPs, an adjuvant can be used as a molecule of
complexation. Complexation of the drugs inside NPs has multiple advan-
tages, such as the protection of the drug from degradation (pH, enzyme,
oxidation, pressure, etc.). Also, the complexation improves the kinetics of
drug release from the NPs (mainly if the drug size is small and the release
could be potentially fast). Finally, it can increase the drug efficiency as some
positively charged adjuvants (polyethylenimine (PEI), chitosan, or lysine)
can boost the cytosolic uptake and release of the drug from the lysosomes.

Other classes of molecules have been used as anti-inflammatory com-
pounds with different associated beneficial effects. The main applications for
drug delivery systems use peptides, proteins, small interfering RNA (siRNA),
or plasmids.

3.1. Anti-inflammatory peptides

Peptides are short polymers of amino acids linked by peptide bonds. They
have the same peptide bonds as those in proteins, but are commonly shorter
in length.

Peptides with anti-inflammatory properties are of great interest as drug
delivery molecules. Several forms and origins of peptide make this drug class
attractive for drug delivery. For example, depsipeptides and biooligomers
found in microorganisms and marine invertebrates have been shown to
have anti-inflammatory and anticancer potentialities (Ballard et al., 2002).
Several key advantages are known such as (1) high specificity, (2) high
activity, (3) little unspecific binding with untargeted molecular structure,
(4) less accumulation in tissues, (5) lower toxicity, (6) minimization of peptide
intermolecular binding, and (7) unlimited potential for synthetic peptides.
Low oral bioavailability requires intravenous or local injection, as peptides
(used as a simple molecule alone) are difficult to deliver across biological
membranes, are rapidly cleared from the body and nonstable. Anti-inflam-
matory peptides such as the somatostatin analogs octreotide, lanreotide, and
vapreotide are now clinically available to treat GI tumors (Froidevaux and
Eberle, 2002).

Once coupled to drug delivery nanocarriers (NPs, liposomes, etc.), anti-
inflammatory peptides have tremendous results as the peptide has a high
activity and specificity. In one recent study (Laroui et al., 2010a), an anti-
inflammatory peptide (KPV, proline–lysine–valine) was successfully delivered
to the colon using PLA NPs transported to the colon in a hydrogel made of
alginate and chitosan. The authors successfully coupled KPV to BSA in the
inner phase of the NP to prevent the burst effect that leads to an early and
inefficient drug delivery from the NPs. Time and bacterial enzyme effects
specifically degraded the hydrogel in the colon and delivered KPV-loaded
NPs. Once delivered to the colon, NPs interact with cells like epithelial cells
and macrophages, and deliver KPV intracellularly (Laroui et al., 2010a).
3.2. Anti-inflammatory proteins

Proteins are biochemical compounds consisting of one or more polypeptides typically folded into a globular or fibrous form, facilitating a biological function. A polypeptide is a single linear polymer chain of amino acids bonded together by peptide bonds between the carboxyl and amino groups of adjacent amino acid residues.

Many proteins have an anti-inflammatory effect, and two main classes of protein intensively used in drug delivery are enzymes and antibodies. Challenges of protein drug delivery include fast elimination from the systemic circulation due to renal clearance and enzymatic degradation, danger of developing an immune response from the use of Ab, nonspecific uptake, and nonefficient translocation into the cell cytosol. Despite these limitations, several enzymes and antibodies have been used and approved by the FDA.

Many protein drugs, such as antibodies, exert their action extracellularly through receptor interactions. Recently, Theiss et al. (2010) have shown that encapsulation of prohibitin 1 (PHB) efficiently reduced DSS-induced colitis in mice. PHB is an evolutionarily conserved protein that has pleiotropic functions including mitochondrial protein folding, inhibition of cell-cycle progression, and regulation of transcription. Theiss et al. showed that levels of PHB are decreased in colonic biopsies from CD patients and in experimental models of UC (Theiss et al., 2007, 2009). Recently, the authors showed that villin–PHB transgenic mice, which exhibit intestinal epithelial cell-specific PHB overexpression, were protected from experimental colitis (Theiss et al., 2009).

PHB-loaded NPs produce significant anti-inflammatory effects as assessed by clinical and endoscopic scores, and significantly reduced myeloperoxidase (MPO) activity and proinflammatory cytokine levels.

Clinically available Ab molecules are applied to IBD. Infliximab, Adalimumab, and Certolizumab pegol are FDA-approved and TNFα antibodies are commercially available. TNFα Ab treatment showed reduction of the severity of IBD (Dignass et al., 2010; Kornbluth and Sachar, 2010; Lichtenstein et al., 2009; Travis et al., 2008). Despite many limitations as a problem with treating the immune response, TNFα Ab treatment remains an extensively used drug and the most efficient on the market.

3.3. Small interfering RNA

siRNA (also known as small interfering RNA or silencing RNA) is a class of double-stranded RNA molecules, 20–25 nucleotides in length, which play a variety of roles in biology. The most notable role of siRNA is its involvement in the RNA interference pathway, where it interferes with the expression of a specific gene (Ambros et al., 2003; Elbashir et al., 2001; Hammond et al., 2000; Zamore et al., 2000). siRNA duplexes are produced by processing of these longer double-stranded RNAs. Dicer ribonuclease
(Bernstein et al., 2001; Knight and Bass, 2001; Lau et al., 2001), and one strand of the duplex, is then incorporated into a ribonucleoprotein complex, the RNA-induced silencing complex (RISC) (Ambros et al., 2003; Martinez et al., 2002; Schwarz et al., 2002). The siRNA component guides RISC to mRNA molecules bearing a homologous antisense sequence, resulting in cleavage and degradation of that mRNA (Martinez et al., 2002; Schwarz et al., 2002). Figure 6.3 is an illustration of the mechanism required for the siRNA to be (1) chemically intact once interacting with the targeted cells and (2) to enter the cytosol of the cell and interact with the proinflammatory mRNA targeted. Due to these requirements, siRNA is a perfect candidate for therapeutic nanotechnology.

Figure 6.3  Schematic of the delivery of siRNA-loaded nanoparticles to a macrophage to stop the translation of mRNA and decrease the level of the corresponding protein.
Since the discovery of siRNA (Fire et al., 1998), knock-down of specific genes has been extensively used in drugs delivery (Leung and Whittaker, 2005; Novina and Sharp, 2004). The limitations of siRNA as a drug delivery molecule are size and enzymatic degradation. As siRNA is composed of 19–22 bases pairs, drug delivery systems usually associate it with a positive complex molecule (polylysine (Becker et al., 2011), PEI (Laroui et al., 2011a), or cationic lipid (Tseng et al., 2009)). The formation of complexes allows for slower drug release (Laroui et al., 2011a) and the prevention of siRNA degradation for RNAses. As Laroui et al. (2011a) demonstrated, PEI can also be used as a “proton sponge.” Once the NP is taken up by the cell in an intracellular vesicle, lysosomes fuse to it. The pH of the lysosomes, known to be around 3, can quickly degrade the drug and lead to an inefficient treatment. In this case, as siRNA binds to excess PEI, the basic function of PEI quickly neutralizes the protons and disrupts the lysosomal membrane. siRNA is released into the cell cytosol and specifically knocks down proinflammatory gene expression by destroying mRNA. Recently, complexation agents have been associated with oligonucleotides (siRNA, miRNA, or plasmids used as anti-inflammatory agents) to increase the cell transfection rate. Positive molecules, such as lysine (Harada-Shiba et al., 2002; Wagner, 1999; Wu and Wu, 1987), chitosan (Salva and Akbuga, 2010; Yu and Pishko, 2011), or PEI (Neu et al., 2006; Thankappan et al., 2011) are extensively used. PEI, alkaline and positively charged, can alone induce endocytosis of oligonucleotides in cells. An important notion is the N/P ratio that modifies transfection rate (N, nitrogen functional groups and P, phosphorous functional groups). Depending on the applications and the efficiency required, the N/P ratio can be calculated to fit expectations. The N/P ratio has to be set regarding the balance between positive charge, promoting the transfection efficiency, and negative charge, characterizing the importance of the DNA plasmid or siRNA in the complex. The N/P ratio is highly correlated to transfection results. Limitations to positive charges associated with molecules have to be observed. Cytotoxicity related to high molecular PEI and high N/P ratio is a concern. MTT and LDH tests are important as a proof of cytocompatibility of new biomaterials made of positive complexes known to be deleterious for cells in some cases.

In short, siRNAs are derived from long, double-stranded RNAs that are transcribed endogenously or introduced into cells by viral infection or transfection.

3.4. Plasmids

A plasmid is a DNA molecule that is separate from, and can replicate independently of, the chromosomal DNA. They are double-stranded and, in many cases, circular. Plasmids usually occur naturally in bacteria, but eukaryotic organisms can be transfected by plasmids and overexpress a molecule of interest.
Starting from the same approach used for siRNA and miRNA, plasmids are usually used in gene therapy for anti-inflammatory. They are also associated with positive molecules such as chitosan (CHI), PEI, or polyllysine. Plasmid targeting is a “hot topic” of research for nanotechnology. Theoretically, plasmids could be delivered in a specific cell in a specific state. NPs could be covered with a specific Ab or overexpressed during inflammation in a specific cell type. Unlike siRNA or miRNA transfection, plasmids have the advantage of being permanently expressed during a cell lifetime and expression can be modulated by promoters sensitive to cellular inflammation state. On the other hand, designing effective plasmids can be challenging for molecular biologists.

4. NSAIDs-Loaded NPs

According to the solubility and hydrophilic characteristic of the drug, several protocols of NP synthesis can be proposed. Most of the NSAIDs are hydrophilic; therefore the double emulsion/evaporation of solvent represents an important and suitable technique.

Several types of surface engineering are possible based on the expectation for the targeting, such as nonspecific targeting (biologically neutral surface like polyvinyl alcohol, PVA) or specific targeting like antibodies, ligands as peptides or proteins adsorption on NPs.

4.1. PVA-covered NPs

Once the inner aqueous phase (1 mL of the aqueous drug) is sonicated with 25 g/L of PLA in dichloromethane (4 mL), the W/O emulsion” is dropped into an aqueous phase containing 3 g/L of PVA (8 mL) and sonicated (for 2 min, 50% active cycle). The second emulsion is called W/O/W emulsion. The final emulsion contains the hydrophilic active compound in an organic emulsion covered with PVA.

4.2. Ab-covered NPs

As targeting is a major challenge for any drug delivery system, NPs covered with ligands have been extensively used. One Ab has a high affinity for an antigen (even exclusive affinity to one antigen) and that characteristic makes it a major tool for NPs surfaces. Strategies using organic chemistry have led to a huge panel of potentialities to anchor an Ab on the NPs surface. Skewis and Reinhard (2010) used anti-EGFR Ab to cover gold NP and tested it in A431 cells. Using a specific bifunctional polyethylene glycol (PEG) (short thiolated alkyl-PEG-acetates
(HSC\textsubscript{11}H\textsubscript{22}(OC\textsubscript{2}H\textsubscript{4})\textsubscript{6}OCH\textsubscript{2}COOH), Skewis and Reinhard were able to bind the Ab to the gold NP by activation of the carboxylic functional groups of the PEG. They used the carboxylic acid as the surface group for cross-linking to primary amines of the desired protein (e.g., antibody) by activating it with 1-ethyl-3-[3-dimethylaminopropyl] carbodiimide hydrochloride (EDC) and N-hydroxysulfosuccinimide (sulfo-NHS). Interestingly, this study leads to applications in advanced biosensing and biophotonics.

5. BIOMATERIAL CHOICE

In our study, hydrogel formation is based on the association of two polysaccharides, alginate and chitosan (Fig. 6.4). Both are biocompatible and biodegradable, so they are widely used in the food industry, biology,

![Diagram of biomaterial encapsulation](image)

**Figure 6.4** Schematic representation of biomaterial encapsulation of KPV-loaded NPs. (A) An alginate and chitosan hydrogel was formed by double linking of Ca\textsuperscript{2+} and SO\textsubscript{4}\textsuperscript{2−} ions. Suspension of NPs in the polysaccharide solution loads the final formed hydrogel with NPs. (B) Optical microscopy image of KPV-loaded NPs encapsulated into a hydrogel bead of alginate–chitosan linked via Ca\textsuperscript{2+} and SO\textsubscript{4}\textsuperscript{2−} ions (from Laroui et al., 2010a).
and medicine (Hiorth et al., 2010; Ho et al., 2009; Laroui et al., 2010b; Venkatesan et al., 2011).

Alginate, extracted from brown algae, is a linear copolymer with homopolymeric blocks of (1-4)-linked β-D-mannuronate (M) and the C-5 epimer, α-L-guluronate (G). By gelation using divalent ions (such as calcium), alginate can be easily used as a biomaterial and is biocompatible. Alginate has been used to form beads carrying divalent ions for cell encapsulation (Mazumder et al., 2009), as a sponge for drug delivery, or as a wound-healing gel (Thomas et al., 2000).

Chitosan is a linear polysaccharide composed of randomly distributed β-(1-4)-linked D-glucosamine (deacetylated units) and N-acetyl-D-glucosamine (acetylated units). Chitin is susceptible to gelation when SO₄²⁻ ions are added. Chitosan is used in our work because: (1) it is specifically degraded in the colon by colonic bacteria, thus collapsing the whole hydrogel (Yamamoto, 2007) and (2) chitin has a well-known positive effect on inflammation because the material has antibacterial action (Qi et al., 2004).

### 6. Targeting NPs to the Colon: Hydrogel-Encapsulated NPs

Biomaterials must show biocompatibility, biodegradability, and bioactivity. The latter is the main criterion for a biomaterial used as a drug delivery system. Such systems are varied, ranging from metallic implants to polymers. The latter is widely used because polymers can be associated with copolymers and can be grafted, degraded, and acquire hybrid characteristics when associated with cells. Polymers such as poly(lactic-co-glycolic) acid, PLA, and PEG are usually modified and used to form films (Gerhardt et al., 2007) or for nano- or microparticle-mediated (Raynaud et al., 2008) drug delivery. The range of possible polysaccharide uses is as wide as polysaccharides are diverse (Table 6.1). Polysaccharides are biodegradable and biocompatible natural polymers (and are approved by the U.S. Food and Drug Administration), so such materials are widely used in applications such as the cosmetic industry (Bais et al., 2005; Gautier et al., 2008), tissue engineering, or other biological projects, with attention to polysaccharide charge and mass characteristics. Thus, NPs (Boddohi et al., 2009; Laroui et al., 2007; Lemarchand et al., 2006) can be used as drug delivery systems (de Guzman et al., 2008; Ladet et al., 2008) and, more specifically, as electrically charged molecules in electronic applications such as those requiring electroactive nanocomposites (Zampa et al., 2007). When polysaccharides are used as biomaterials, size and charge regulate the kinetics of drug delivery (Chuah et al., 2009; Sezer...
and Akbuga, 2006). Our technique is based on the formation of a hydrogel by ions (Ca$^{2+}$ and SO$_4^{2-}$) that mediate cross-linking between alginate and chitosan. Other polysaccharides can be used in this technique because polysaccharides generally share similar electronic properties (Crouzier and Picart, 2009).

The principal point of the double-gavage method for drug delivery is that it allows a “macrohydrogel” to form in the stomach (Fig. 6.5). The first gavage contains the polysaccharide material that contains the drug. As the polysaccharide biomaterial is still liquid at the time of gavage, this technique overcomes the limitations of the size of the animal’s oral cavity and allows an easier way to administer in addition to the ability to administer higher concentration of drug. A second gavage is performed with an ionic solution of calcium and sulfate. As soon as the ions and the polysaccharide solution are mixed, a hydrogel is formed within the stomach. The final volume of the biomaterial formed will be 150 µL.

An option technique is to perform the above protocol twice. The method can be called the “double-gavage” method. The purpose of this is to prevent early drug degradation during the digestion process. The first double gavage is made with a concentrated drug solution. The second double gavage is done with a drug = free polysaccharide solution that will recover the biomaterial (Fig. 6.5). This is an “onion-like” structure (Ladet et al., 2008) and has two advantages (Fig. 6.5). First, this original structure can prevent a quick release or “burst effect” of the drug from the hydrogel because the external layer (containing no drug) is the first to be degraded. Secondly, the kinetics of drug release (mainly in GI tract with pH gradient from acid (pH 2) to neutral pH, and digestive enzymes) is surface dependent. This structure allows minimal surface contact between the hydrogel and the external medium and allows the drug to be completely separated from the degradation interface. After oral gavage of the polysaccharide solution into a small animal, the ions in the solution form a hydrogel within the stomach of the maximum possible size. Use of a “macrosize” biomaterial allows prevention of a large contact surface between the loaded drug and the aggressive digestive medium (Fig. 6.5). This technique allows all types of encapsulation, including that of NPs, liposomes, or drug molecules alone.

Using the unique “double-gavage” method, we have shown that a combination of alginate and chitosan at a weight ratio of 7:3 is appropriate for delivery of an encapsulated product to the colon. As shown in Fig. 6.6, most gel-loaded NPs labeled with dextran-FITC were released in the colon after complete collapse of the hydrogel. The alginate and chitosan can be made at different concentrations, ratios, or types of polysaccharides to make the hydrogel collapse in a specific region of the digestive tract.
6.1. Biomaterials and hydrogel

6.1.1. Polysaccharide hydrogels based on electrostatic interactions

Table 6.1  Common polysaccharides used in biology, medicine, and biotechnology with their structural units and specific applications

<table>
<thead>
<tr>
<th>Polymer</th>
<th>Structural unit</th>
<th>Example of application</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dextran</td>
<td>Glucose unit (glc)</td>
<td>Nanoparticle core</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Reis et al., 2008)</td>
</tr>
<tr>
<td>Beta-glucan</td>
<td>Fructose unit (Fru)</td>
<td>Microparticles</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Aouadi et al., 2009)</td>
</tr>
<tr>
<td>Hyaluronan</td>
<td>d-glucuronic acid (GlcA) linked to d-N-acetylglucosamine (GlcNAc)</td>
<td>Wound healing</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Pardue et al., 2008)</td>
</tr>
<tr>
<td>Heparan sulfate</td>
<td>Glucuronic acid (GlcA) linked to N-acetylglucosamine (GlcNAc)</td>
<td>Regenerative tissue</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(DeCarlo and Whitelock, 2006)</td>
</tr>
<tr>
<td>Keratin sulfate</td>
<td>d-galactose (Gal) linked to N-acetylglucosamine (GlcNAc)</td>
<td>Synovial fluid supplementation</td>
</tr>
<tr>
<td>Heparin</td>
<td>2-O-sulfated iduronic acid [IdoA (2S)] and 6-O-sulfated, N-sulfated glucosamine GlcNS(6S) (variable)</td>
<td>Antithrombic agent</td>
</tr>
<tr>
<td>Chondroitin sulfate</td>
<td>d-gluconuronic acid (GlcA) and N-acetyl-d-galactosamine (GalNAc)</td>
<td>Pain relief in osteoarthritis</td>
</tr>
<tr>
<td></td>
<td></td>
<td>(Vangsness et al., 2009)</td>
</tr>
</tbody>
</table>

6.2. Preparation of the hydrogel

6.2.1. Protocol of gavages of the hydrogel

Preparation of the sodium chloride solution

1. A 0.15-M solution of sodium chloride is prepared.
2. The solution is filtered through a 5-μm filter placed on a syringe to sterilize the solution.
3. The solution is kept under a cell culture hood in a 50-mL tube.

Preparation of the chelation solution

4. Sodium sulfate (60 mM) and calcium chloride (140 mM) solutions are prepared.
Figure 6.5  Double-gavage procedure of encapsulated KPV-loaded NPs. (A) The first gavage delivered 100 μL of the polymer mix solution (alginate, 7 g/L; chitosan, 3 g/L) containing a homogenous suspension of NPs (2 mg/mL). Red Ponceau has been added for visualization. (B) The second gavage delivered 50 μL of a solution containing 70 mmol/L calcium chloride and 30 mmol/L sodium sulfate. (C) Visualization of the mixed hydrogel formed by chelation of the polymers in the stomach. (D) The hydrogel after extraction from a mouse stomach 5 min after the double-gavage method (from Laroui et al., 2010a).

Figure 6.6  Localization of encapsulated dextran-FITC NPs (μg dextran–FITC per μg tissue protein) in the digestive tract after 3 days of gavage. The digestive tract was divided into six sections as follows: stomach, proximal (1/3) small intestine, medial (2/3) small intestine, distal (3/3) small intestine, cecum, and colon (n = 12) (from Laroui et al., 2010a).
5. The sodium chloride solution is mixed with the same volume of each solution in (4) to produce a 30-m\(M\) solution of sodium sulfate and a 70-m\(M\) solution of calcium chloride.
6. The solutions are filtered through 5-\(\mu\)m filters placed on syringes to sterilize the solutions.
7. The solutions are kept under a cell culture hood in 50-mL tubes.

Preparation of the hydrogel solution

8. 140 mg of alginic acid sodium salt and 60 mg of chitosan are weighed for 7 and 3 g/L solutions, respectively.
9. Both powders are placed in a glass tube of 50-mL capacity and a magnetic bar is added to the tube.
10. The top of the tube is taped with autoclave tape and surrounded with aluminum foil.
11. The tube containing the polysaccharides is sterilized in an autoclave (120 °C under pressure) for 40 min.
12. The aluminum foil and the tape are removed under the cell culture hood and 20 mL of the sterile sodium chloride solution is added. At this stage, the bioactive compound (drug powder, particles) can be added. The tube is covered with Parafilm and placed on a stirring plate until total solubilization of the polysaccharides is evident.
13. The solution is stored at 4 °C overnight to remove all potential air bubbles formed during stirring or centrifuged at 3440 \(\times g\) at room temperature for 15 min.

Synthesis of NPs

Preparation of the matrix of NPs (Solution 1)

14. 4 mL of dichloromethane containing 25 g/L of D,L PLA is prepared. PLA crystals are dissolved in dichloromethane kept at 4 °C in a closed beaker to prevent evaporation.

Preparation of the inside of the NPs (Solution 2)

15. Solution 2 is 800 \(\mu\)L of aqueous solution containing the water soluble molecule of interest (protein, peptide, or oligonucleotides, etc) to load inside the NPs.

In a previous study (Laroui et al., 2011a), we determined N/P = 30 (N/P is the ratio of the number of positive charges of PEI (N as the ammonium charge) and the negative charges of siRNA (TNF\(\alpha\) siRNA or FITC-TNF\(\alpha\) siRNA) (P as the phosphorous charge)). We complex 29 \(\mu\)L of siRNA (5 \(\mu\)M) with 18 \(\mu\)L of PEI (5 \(\mu\)M) for 10 min at room temperature. To obtain the final solution 2, we add 750 \(\mu\)L of 5% BSA.

Preparation of the outside part of the NPs

16. Polyvinyl alcohol is dissolved in water to get 10 mL 0.3 g/L solution.
Synthesis of the NP

17. Solution 1 + solution 2: solution 1, containing the drug, is mixed with solution 2 to generate a W/O emulsion after 2 min of vortexing (Maxi Mix II, Thermodyne, Dubuque, Iowa, USA) and 1 min of sonication with 50% active cycle at 70% power ($P_{\text{max}} = 400$ W) (Digital Sonifier 450, Branson, Danbury, CT, USA).

This first emulsion is dropped in solution 3 containing 0.3 g/L of PVA to generate a W/O/W emulsion (same process as above).

The W/O/W emulsion is dropped in a dispersing phase of 0.1 g/L PVA and stirred at 45 °C under a vacuum to remove dichloromethane. As each synthesis makes around 50 mg of dry NPs, each group of NPs is the accumulation of three independent syntheses.

NPs are then centrifuged at 9953 g and freeze-dried overnight at 50 °C under 0.1 mbar pressure.

Delivery of the biomaterial encapsulated with NPs into the mouse stomach

18. The chelation and the polysaccharide solution tubes are placed into a 37 °C water bath. The chelation solution is collected in a 1-mL syringe without creating any bubbles. This step is repeated using a second syringe to collect the polysaccharide solution.

19. The mouse is left for 3 h without food but with water to allow the animal to receive a biomaterial of a final volume of 150-μL.

20. The gavage of the mouse starts with 100 μL polysaccharide solution containing the NPs and is followed by a second gavage of 50 μL chelation solution.

21. The hydrogel is formed as soon as the chelation solution reaches the polysaccharide solution and a hard gel forms in the stomach of the mouse.

22. A study is necessary to measure whether the biomaterial will reach the target, which depends on which part of the GI tract is targeted and what polysaccharide proportions are chosen.

For colon targeting, Laroui et al. have shown that the biomaterial must be made of 3 g/L of chitosan and 7 g/L of alginate (Laroui et al., 2010a).

6.3. Study of the hydrogel

6.3.1. Rate of release in colon versus other organs of NPs encapsulated in the hydrogel made of alginate and chitosan

Successful delivery of NPs to the colon is calculated using dextran-tagged FITC-loaded NPs (Laroui et al., 2010a). Once the experiments are performed, all the organs of the digestive tract (from the stomach to the colon) are collected. Then, tissues are homogenized with a tissue homogenizer (Power Gen 125, Fisher Scientific, Pittsburgh, PA, USA).
Using dextran–FITC-loaded NPs, we measure the NP distribution throughout the GI tract. Five days after gavage of the NPs loaded with dextran–FITC encapsulated into the hydrogel, the stomach, jejunum, duodenum, ileum, and the colon are collected and sonicated. After centrifugation, supernatants are collected and the fluorescent signal is measured. We found that the biomaterial composition (mix of alginate and chitosan, respectively 7 and 3 g/L) was optimal for colonic release of the NPs. As shown in Fig. 6.6, dextran–FITC-loaded NPs were mainly released in the colon versus all other areas of the digestive tract.

6.3.2. Effect of NP release in the colon on local inflammation

Laroui et al. (2010a) encapsulated a tripeptide made of lysine–proline–valine (KPV, derived from alpha-melanocyte-stimulating hormone, α-MSH) in NPs made of PLA and covered with PVA. Once the biomaterial (mix of alginate and chitosan) specifically collapsed in the colon, NPs directly interact with colonic cells. Interestingly, they showed that a low dose of KPV loaded in NPs stimulates anti-inflammatory activity in the colon, reducing the intake amount of KPV and thus potential side effects of overdosing (Fig. 6.7). They showed that the dose contained in NPs was 12,000-fold lower than the efficient concentration of KPV used in a free drinkable solution. These results showed the principal advantages of nanotechnology applied as a drug delivery system.

6.3.3. Analysis of cells involved in NP uptake

In a recent paper, Laroui et al. (2011a) delivered TNFα siRNA-loaded NPs to the colon. The authors showed that TNFα protein expression was dramatically downregulated in the colon, which was the targeted organ, but also the liver by an indirect effect (Fig. 6.8). We recently performed an analysis by flow cytometry (FACS) to determine which cells in the colon were preferentially took up the NPs. We found that mainly the CD11b+F4/80+CD11c− cells (macrophages) and epithelial cells phagocytosed the NPs (data not shown). CD11b+F4/80−CD11+ positive cells (dendritic cells) were also involved but in a lower range.

Our FACS experiment is coherent with the literature as TNFα is mainly secreted in the mucosa by macrophages. The significant decrease of this proinflammatory cytokine was mainly due to the siRNA-loaded NPs taken up by the macrophages.

7. Conclusion

Our technique using an association of polymer NPs and biomaterials (hydrogel) allowed us to efficiently target the colon in a mouse model. Using the characteristic of the hydrogel to specifically collapse in the colon,
we were able to treat colonic cells with bioactive compounds such as peptide, protein, or siRNA. Polysaccharides have many advantages that make them essential for colon targeting strategies, including pH and bacterial enzyme sensitivity and biocompatibility. One idea will be to develop and optimize NPs made of polysaccharides that are resistant to the digestive tract and only degradable in the colon. Chemical engineering can modify the polymer without modifying the main physical and chemical characteristics and allow the polymer to form NPs. Emerging applications for NP therapy in the GI tract are being discovered. In this review, we have described an example of a nanotechnology application for IBD. We specifically designed the NPs, the biomaterial and the technique of gavage to be suitable and efficient against IBD. However,
NPs are not limited to drug delivery and can also be an efficient tool to be used in diverse applications, such as bioimaging, diagnostics that can compete favorably with conventional molecular approaches, such as colon cancer therapy or diagnosis. NPs are not restricted to the digestive tract and applications in brain, liver, blood, bone, or cartilage disorders are being investigated. Recent evidence suggests that live oral Salmonella–HIV vaccine vectors have the potential to elicit HIV-specific T cell-mediated immunity in both the mucosal and systemic compartments. Oral vaccines using microfold cells are under investigation, and results could make several determinant advances in sciences as HIV vaccine. M cells, cells found in the follicle-associated epithelium of the Peyer’s patch. They transport organisms and particles from the gut lumen to immune cells across the epithelial barrier, and thus are important in stimulating mucosal immunity.

**Figure 6.8** TNFα siRNA/polyethylenimine (PEI)-loaded nanoparticles (NPs) inhibit LPS-induced TNFα secretion in macrophages and in mice: (A) RAW 264-7 macrophages were pretreated with TNFα siRNA-loaded NPs suspension (800 μg/mL) for 24 h. As a control, RAW 264-7 macrophages were pretreated with empty NPs, scrambled siRNA-loaded NPs, and lipofectamine with the same amount of TNFα siRNA compared to TNFα siRNA-loaded NPs suspension. Cells were then treated with LPS (10 mg/mL) for 1 h (positive control cells were not pretreated with NPs). Cells were lysed and TNFα protein measured using ELISA test. Data is SEM ± S.E, n = 6 (significantly different from ANOVA test **P < 0.005, ***P < 0.001). (B) C57BL/6 female wild-type mice were gavaged with TNFα siRNA-loaded NPs (5 mg/mL), scrambled siRNA NPs (5 mg/mL), and empty NPs (5 mg/mL) contained in a hydrogel (alginate and chitosan) daily for four consecutive days. Positive control mice were gavaged only with the hydrogel without NPs. Mice were then administered LPS (100 μg Kg⁻¹ i.p.) and euthanized after 1 h. The colon, spleen, and blood were collected and TNFα expression was measured using ELISA test. Data is represented as SEM ± S.E, n = 8 (significantly different from ANOVA test *P < 0.05, ***P < 0.001, ANOVA test) (from Laroui et al., 2011a).
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