Orally administered targeted nanoparticles have a large number of potential biomedical applications and display several putative advantages for oral drug delivery, such as the protection of fragile drugs or modification of drug pharmacokinetics. These advantages notwithstanding, oral drug delivery by nanoparticles remains challenging. The optimization of particle size and surface properties and targeting by ligand grafting have been shown to enhance nanoparticle transport across the intestinal epithelium. Here, different grafting strategies for non-peptidic ligands, e.g., peptidomimetics, lectin mimetics, sugars and vitamins, that are stable in the gastrointestinal tract are discussed. We demonstrate that the grafting of these non-peptidic ligands allows nanoparticles to be targeted to M cells, enterocytes, immune cells or L cells. We show that these grafted nanoparticles could be promising vehicles for oral vaccination by targeting M cells or for the delivery of therapeutic proteins. We suggest that targeting L cells could be useful for the treatment of type 2 diabetes or obesity.

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1. Introduction

1.1. Carriers and general properties

The intestinal epithelium allows the absorption of nutrients, electrolytes and fluids while acting as a defense system and an efficient barrier to macromolecules, toxins and microorganisms. When particulate drug delivery systems are administered orally, they are exposed to the harsh environment of the gastrointestinal tract. They must cross the mucus layer before they come into contact with intestinal cells. Moreover, they do not permeate easily across the intestinal barrier. However, fine-tuning the size, shape and surface properties of the delivery system allows the enhancement of drug-loaded nanoparticle (NP) absorption. A specific targeting effect can be achieved by conjugating the ligands of receptors that are expressed at the apical site of intestinal epithelial cells to the surfaces of the particulate delivery system. In other words, the targeted delivery of drugs through the oral administration of particles requires “smart” vehicles that are able to tolerate different conditions and cross various barriers to entry via specific interactions with the targeted cell surface.

The general physicochemical properties required for orally delivered particles to reach the attended sites of entry (e.g., gut-associated lymphoid tissue (GALT) or enterocytes) or the desired sites of action (e.g., cancer cells or a zone of inflammation) are beyond the scope of this paper and have been reviewed elsewhere [1–6]. This review will focus on cell–NP-specific interactions that could be induced by specific ligands that are present on the surfaces of the carriers. Nevertheless, we provide a short overview of the influence of NP surface properties on their uptake by intestinal cells because adequate surface chemistry promotes NP stability in gastrointestinal (GI) tract fluids, increases their transit time in the gut and finally enhances their ability to cross the mucosal barrier, including the mucus. This review will help to identify the potential benefits of receptor targeting.

The nanometric particle size is of prime importance to allow the particle to cross the mucus, avoid rapid clearance and finally enhance transmucosal transport. The external shell should have a good hydrophilic and hydrophobic balance. Hydrophilic molecules (i.e., PEGs, carbohydrates) are useful for the stabilization and diffusion of the particles in the fluids and mucus, whereas a hydrophobic coating enhances cellular and lymphatic uptake. The chemical functions of the materials play an important role in the surface charge, the overall stability of the system and the non-specific interactions with surrounding media. Neutral surfaces facilitate the crossing of the mucus barrier, whereas positively charged surfaces enhance interactions with the mucus and negatively charged cell surfaces. In contrast, thiols, amminons and lipophilic carbon chains reinforce adhesiveness and thus residency time by forming disulfide bonds, electrostatic interactions and hydrophobic interactions, respectively. Moreover, the presence of acidic or basic functional groups leads to pH-dependent behaviors that are useful for targeting different portions of the GI tract.

In that context, degradable polymeric particles are particularly interesting [7]. Indeed, polymers have well-defined properties (i.e., chain length, chemical functions, shape and self-assembly) and can be derivatized using well-described chemistry. Therefore, they constitute an interesting tool to produce tailored delivery vehicles. The most widely described polymers for use in oral delivery are poly(glycolic acid) (PGA), poly(lactic acid) (PLA) and their copolymers (e.g., PLGA, PLAGA-PEG, and PLA-PEG) [3,8], polyanhydrides [9], chitosan and its derivatives [10–12] and other polysaccharides. As illustrated in Fig. 1, the surfaces of polymeric NPs can display ligands that will bind to cell-specific receptors. Consequently, this review focuses mainly on polymeric NPs.

1.2. Mechanisms of oral uptake of nanoparticles

The study of the mechanisms by which orally delivered drug-loaded NPs, whether targeted or untargeted, are absorbed have attracted less attention than their design. The design of new NPs for oral administration usually focuses on overcoming the different barriers in the GI tract. The NPs and/or cargo must resist the harsh GI environment, e.g., the low pH in the stomach and the degradative enzymes, but the major barrier to their absorption remains the intestinal mucosa.

The first barrier that must be crossed is the mucus bilayer, which covers and protects the epithelium. The NPs must adhere to and cross this highly viscoelastic layer, which is continuously secreted and cleared. Mucoadhesive NPs, particularly positively charged NPs, have been designed to promote strong interactions with the mucus and prolong the retention time of the NPs at the mucosal surface. However, the penetration of and diffusion through the mucus is also critical, as mucoadhesive NPs can be trapped in the loosely adherent mucus and thus rendered vulnerable to rapid clearance [13]. NPs that penetrate the mucus and release drugs and/or drug-loaded NPs closer to the epithelium have been engineered. Similar to viruses and bacteria, NPs that are capable of penetrating the mucus should i) be small to allow their diffusion in the mucin mesh; ii) have a non-mucoadhesive surface; and iii) be densely coated, with a net neutrally charged hydrophilic surface [13]. The group of J. Hanes has designed PEGylated muco-inert NPs that can penetrate the mucus. They found that mucus-penetrating particles could be designed by carefully modifying their surface properties, particularly by attaching a dense, low-molecular-weight PEG [6,14]. In conclusion, a balance between minimal mucoadhesion or interactions with the mucus and mucus penetration is required [6]. This also applies to targeted NPs, which must be PEGylated to allow their penetration into the mucus and subsequent access to the targeted receptor. Interestingly, the mucus layer is reduced at the surface of M cells.

The second barrier to overcome for drug-loaded NPs is their generally limited cellular uptake and translocation. In vitro models of the intestinal epithelium associated with specific inhibitors or markers of endocytosis have been used to better understand the fate of NPs in the intestine and optimize their design. It is generally accepted that NPs do not diffuse through the paracellular route, although NP components, such as chitosan, can affect tight junctions [1,6,12]. Rather, NPs can be taken up by phagocytosis, which is restricted to M cells, or by pinocytosis. The uptake and transport of particles by M cells is significantly higher than their transport by enterocytes. Pinocytosis can occur by macropinocytosis, clathrin- or caveolae-mediated endocytosis or clathrin- and caveolae-independent endocytosis [6,15,16]. The mechanism of uptake will affect subsequent intracellular trafficking and transcytosis. The physicochemical properties of the particles influence their uptake, e.g., the smaller the particle, the higher the uptake [1]. One strategy to enhance NP uptake by intestinal cells is to conjugate a ligand to the NP surface that will favor cell–NP interactions and enhance NP internalization, primarily through clathrin-mediated endocytosis.

1.3. Targeting cell receptors by grafting specific ligands to the nanoparticle surface

1.3.1. Importance of receptor-mediated targeting

Despite the promising results obtained for NPs with appropriate size and surface properties, the absorption and/or in vivo therapeutic efficiency of drugs following the oral administration of drug-loaded NPs typically remains low [1–6,17]. The mucus penetration, cellular uptake, NP trafficking inside the cells and biological fate of the delivered drugs are still not optimal. Some studies have shown that conjugating the NP surface with specific ligands for epithelial receptors or antibodies might enhance the specific cellular uptake and transepithelial transport of the NP [1,6,18]. Moreover, in the constantly moving environment of the gut, particles are rapidly cleared, and thus, strong associations (e.g., receptor–ligand interactions) would favor the accumulation of particles at their sites of action or absorption [2].

A large variety of epithelial cell receptors have been investigated as potential targets of delivery systems [1,6,18]. Based on this knowledge,
studies on ligand (or antibody)-modified oral drug delivery systems have emerged in the last decade, leading to promising in vitro results but unclear and often disappointing in vivo conclusions.

1.3.2. Limitations of peptidic and proteinic ligands

In addition to the challenges presented by interactions with mucus and cells and the poor uptake of NPs by cells, the chemical nature of ligands grafted onto the NP surface can also play a role in the particle fate. In the GI tract, ligands present on the NP surface are exposed to different harsh chemical conditions, such as the acidic pH of the stomach and enzymatic digestion, among others. Moreover, most of the ligands employed are biomacromolecules (e.g., antibodies and bacterial proteins) that can diminish the progression of NP in the mucus by increasing their size and the incidence of non-specific interactions, and they may also induce steric hindrance when they approach the receptor surface [2]. Some of these biomolecules are also immunogenic (Fc).

One critical consideration for ligands is the chemical method used for their introduction in the drug delivery system. While it seems obvious that covalent conjugation is mandatory, the chemistries that can be used and the chemical properties of both the carrier and the ligand are not necessarily easy to combine. Biomacromolecules often have a large number of hydroxyl, amino or thiol functional groups and are delicate compounds. In addition, the chemical characterization of the obtained bioconjugates is a demanding task that requires an entire development process in itself. It is thus difficult to determine whether a ligand is attached to the particles by the desired chemistry, how much is bound and whether it is still bioactive after conjugation [19,20].

Another limitation of targeted drug delivery systems is the accessibility and adequate bioactive conformation of ligands in the biological media. These are indeed difficult questions to address because during the formulation of the NP, it is challenging to assess where the ligands will be located (i.e., outside or inside the particles). Moreover, in the GI environment, the carrier can be reorganized, and the conformation of the ligands can change. Several recent studies have attempted to answer some of these questions. Most notably, the water solubility of the ligand influences its localization in the NP and, consequently, its targeting efficiency. For instance, in a self-assembling process, a hydrophilic ligand is more efficient than a hydrophobic ligand because of its greater representation in the outer shell [21]. Furthermore, the nature of the ligands and their surface densities, might also play a role in the level and nature of the immune response when antigen-loaded NPs are targeted to dendritic cells [22]. These are controversial questions, and it is not yet clear whether higher ligand density will lead to improved targeting because targeting also depends strongly on the aforementioned surface characteristics of the NP [23], the way ligands are tethered [24,25] and the affinity of the ligand for the targeted receptor [22,25,26].

1.3.3. Advantages of non-peptidic small molecule ligands

Considering the above-mentioned limitations, the use of small molecule ligands (i.e., molecules of less than ~1500 Da), such as sugar derivatives, peptidomimetics or metabolites, seems to have several advantages over peptidic conjugates: i) they are chemically resistant to GI conditions (or could be modified for this purpose); ii) they are usually conformationally stable; iii) they do not induce steric hindrance at the receptor surface; iv) they can be conjugated to the NP through simple chemical procedures, and the obtained conjugates can be more easily characterized; v) the are easier and less expensive to produce; and vi) they do not have immunogenic effects. The main small molecule ligands that have been investigated in oral delivery are mannose derivatives, RGD or LDV peptide sequence mimetics, lectin mimetics, fatty acids and vitamins (see Table 1).

2. Grafting of non-peptidic ligands to polymers composing nanoparticles

2.1. Strategies for introducing non-peptidic ligands onto nanoparticles

The conjugation of small ligands to a wide variety of biomaterials has been extensively studied for applications ranging from tissue
engineering to the conception of targeted imaging agents. In targeted nanoparticulate delivery systems, particularly those designed for oral administration, the conjugation methods are more limited. Careful attention should be paid to the localization and density of the ligands. Moreover, the biodegradable polymers employed can be degraded during the grafting procedure. The chemical bonds should resist hydrolysis by GI pH and enzymes. Consequently, the conjugation strategy should consider i) the chemical reaction used for the anchorage (i.e., how to conjugate the ligands); ii) the localization of the ligand along the polymer backbone (i.e., along the chain or at its end); and iii) the timing of the conjugation step with respect to the overall NP synthesis process (i.e., before or after NP formulation).

For instance, PLA, PLGA and their derivatives suffer from transamination and hydrolysis reactions, even under very mild conditions [27]. Consequently, gentle, selective and efficient chemistries must be used for their functionalization. Click chemistry with copper-catalyzed azide alkyne cycloaddition (CuAAC) is the method of choice for this type of polymer because it is a very tolerant reaction that occurs in organic solvents without requiring amines.

Otherwise, modular approaches must be considered, such as our “clip and click” strategy, in which PEG is first functionalized through “clip photochemistry” grafting and then attached to a terpolymer of poly(lactide-co-glycolide-co-ε-caprolactone) by CuAAC [28,29]. A common strategy for attaching ligands to PLGA-based NPs is to use poly(ε-caprolactone) (PCL) or PCL-PEG, which have the same properties as PLGA but are more chemically resistant. Ligand-PCL conjugates can subsequently be introduced to PLGA-based NPs. To ensure the protection of PLGA while using more conventional chemistry (e.g., carbodiimide activation), some authors have worked directly with the particles after their formulation. This allows the PLGA core to be protected against degradation and ensures that the ligands are present on the external surface, but the risk of particle leakage and loss of payload cannot be avoided. Other carriers used for conjugation with small ligands include reactive polymers (e.g., poly(azide alkyne), and stable compounds that can tolerate typical chemical treatments (i.e., PEGs, lipids, and polysaccharides).

The methods of conjugation will also influence the localization of the ligand along the polymer backbone. However, it has not been clearly established that ligand localization at the end of a polymer chain will generate better nanoparticle targeting than a ligand grafted along the carrier backbone [24]. Moreover, the final availability of the ligand may depend on the self-assembly process and the ligand characteristic [21].

2.2. Grafting of non-peptidic ligands to a polymer

The strategies for ligand grafting are schematized in Fig. 2 and will mainly be illustrated with PLGA and PCL, two biodegradable polymers that are commonly used for nanoparticle formulation. Most authors have chosen chain-end approaches with copolymers bearing reactive functions at the chain end of the hydrophilic block that can react with the ligand via usual coupling methods (e.g., reductive amination, amonium formation, carbodiimide coupling, and polymerization initiated by ligands). Recently however, several interesting methods have been developed to obtain ligands that are grafted along the polymer chain (i.e., click chemistry, clip chemistry and reactive polymers).

Reactive polymers can be grafted directly. Because poly(ethylene glycol) (i.e., poly(methyl vinyl ether-co-maleic anhydride)) is a reactive polymer, it forms naturally covalent conjugates through the reaction of the amine functional group of modified vitamin B12 with the anhydride functional groups of the polymer (Fig. 2A) [30].

Grafting of the RGD sequence to NPs has been investigated as a strategy to target drug-loaded NP to integrins that are overexpressed by specific cells, particularly αvβ3 in the angiogenic tumor endothelium [31] and β1 integrin in intestinal M cells of the follicle-associated epithelium [32]. However, because RGD might be degraded in the GI tract, non-peptidic analogs have also been investigated. RGD peptidomimetic (RGDp) sequences have been developed for potential applications in antiangiogenesis treatment [33] or tissue engineering, but few have been used in targeted drug delivery systems [34–38]. The works conducted by our group [32,39] provide clear evidence that RGDp-targeted PLGA NPs are superior to peptidic RGD-targeted NPs in inducing an immune response against a model antigen (ovalbumin) in mice. These RGDps are built on a tyrosine template [40] and mimic the bioactive conformation of cRGD(NMe)3 inside the binding pocket of αvβ3 integrin. These ligands were aimed at biomaterial applications [41] and have a short oligo ethylene glycol (OEG) spacer arm. They were conjugated via a “clip photochemistry” process to the PEG parts of a PCL-PEG polymer before their incorporation with PLGA during the formulation of the NPs [32,39,42]. The presence of the ligands in the shell of the NPs was confirmed by surface chemistry analysis using X-ray photoelectron spectroscopy (XPS) [43]. Other targeting ligands, such as a Leu-Asp-Val (LDV) modified sequence and a LDV peptidomimetic (LDVp) with an OEG spacer arm, initially designed to bind αvβ3 integrins, have also been grafted onto PCL-PEG and incorporated into PLGA-based NPs [39,44].

C-type lectins and mannosse receptors are the most explored molecules for the targeting of antigen-presenting cell (APC) receptors. Consequently, mannosse-grafted particles, intended for dendritic cell or macrophage targeting, have been investigated [45], including their activity when administered via the oral route. Recent reviews [46–48] have more precisely detailed such chemistry. Thus, they will not be described here, but some examples of the most relevant strategies should be mentioned. For example, Riger et al. produced

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<th>Cells targeted</th>
<th>Active targeting ligand</th>
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<td>Enterocytes</td>
<td>Lectins</td>
<td>Glycoproteins and glycolipids of enterocyte membranes</td>
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<td>– Wheat Germ Agglutinin (WGA)</td>
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<td>Lectins</td>
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<td>Immune cells</td>
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PCL-PEG with a mannose residue on the chain end of the PEG block [49,50]. They synthesized PCL-PEG terminating with a secondary amine function that reacted with a mannose derivative equipped with a small ethyl spacer ended by bromine to form an ammonium link. The obtained copolymer easily formed positively charged micelles bearing mannose residues on their surfaces (Fig. 2B). Similarly, Freichels et al. produced aldehyde-terminated PCL-PEG that, through a reductive amination protocol with 2-aminoethyl-α-D-mannopyroside, allowed for the production of end-capped mannosylated PCL-PEG [51]. PCL-PEG micelles prepared with these modified polymers displayed a neutral surface charge. Click chemistry can also be used to produce mannosylated PCL bearing sugar residues along the entire polymer chain. For that purpose, Xu et al. produced PCL diblock polymers in which one block had azide functional groups that could be engaged in a CuAAC reaction with propargylic sugar derivatives [52] (Fig. 2C). The large number of carbohydrates fixed on one block of the polymer confers amphiphilic properties to this PCL-based polymer. We also successfully applied our “clip photochemistry” process to the preparation of PCL-PEG grafted with mannose (Fig. 2C) [42]. This mannosylated PCL-PEG was subsequently introduced into the formulation of PLGA-based NPs [39].

Careful and demanding strategies are required to functionalize PLGA or PLA polymers with mannose derivatives. For instance, Nagasaki used protected sugars to induce the sequential ring-opening polymerization of ethylene oxide and DL-lactide [53]. Deprotection yielded PLA-PEG chains that were end capped with carbohydrates and demonstrated interesting micellization properties. Hamdy et al. synthesized mannan-conjugated PLGA NPs by direct functionalization of the carbohydrate functions on the surface of preformed OVA-loaded NPs via a classical carbodiimide protocol [54] (Fig. 2B). Similarly, Brandhonneur et al. produced PLGA particles bearing different ligands (including lectin, RGD, and mannose) and demonstrated their enhanced uptake by macrophages in vitro [26]. The aforementioned “clip and click strategy” allows the production of different functionalized PLGA-PEG with targeting moieties randomly dispersed along the PEG block to produce mannosylated PLGA-PEG copolymer with self-assembling properties [29].

2.3. Analytical methods to evaluate non-peptidic ligand grafting

Due to the uncertainties inherent in self-assembling processes, it is usually difficult to predict where ligands will be localized. Consequently, new characterization methods must be investigated in parallel with conjugation strategies.

The most common test to detect sugar moieties at the particle surface is to provoke the aggregation of sugar-conjugated NPs in the presence of free soluble lectins and further analyze them using isothermal titration calorimetry, enzyme-linked lectin assays (ELLAs) [50], turbidimetry, surface plasmon resonance [55] or affinity column retention assays [53]. NP aggregation upon binding to lectin is clear proof of the presence of bioavailable sugar ligands. Quartz crystal microbalance with dissipation (QCM-D) affinity binding assays have also been conducted, with mannosylated NPs interacting with lectins immobilized on a QCM-D surface [29]. This test provides not only information about the availability of mannose for lectins on a surface but also an opportunity to assess the relevance of the NP-receptor interaction under shear stress, representing a “near to reality” evaluation of targeted NP behavior.

The chemical characterization of the NP surface can be performed by surface chemistry techniques, such as X-ray photoelectron spectroscopy (XPS), but only solids can be analyzed using this technique. NMR could be a good alternative for studying particles in aqueous solution [20, 56, 57]. Indeed, in magnetic resonance, only soluble components are detected. This provides valuable information regarding the bioaccessibility of the constituents of the external shell of NPs, and the micellar stability of the NP. Under appropriate conditions, NMR can also be used for quantitative calculations of ligand accessibility and the lengths of the PEG chains [58].

3. Targeting of nanoparticles to different intestinal cell types

Polymeric carriers protect antigens against degradation and inactivation in the harsh gastro-intestinal environment and have the
ability to enhance their transmucosal transport [1,6,17]. Despite these advantages, the oral delivery of drug- or antigen-loaded NPs remains challenging. The poor efficacy of these particulate systems has been reported, possibly as a consequence of poor particle uptake. Thus, none of these systems has reached the market. Methods for increasing the uptake and transcytosis of orally delivered particles by specific cells represent a promising opportunity for enhancing their efficacy. Due to their physiological functions, M cells are a particularly attractive target for oral drug delivery [1,6,18,59,60]. Enterocytes, which cover most of the gut surface and are responsible for absorption, could also be a good target for NP uptake. Epithelial immune cells that sample particulate antigens and microorganisms can also be exploited for NP uptake. Finally, endocrine cells, which are rarely studied for NP targeting, could be potential new targets, mainly for local drug delivery.

3.1. M cells

M cells are specialized epithelial cells that are located in the follicle-associated epithelium (FAE) of Peyer’s patches or GALT [59–61] and are part of the mucosal immune system. They deliver samples of foreign material from the lumen to the underlying organized mucosa lymphoid tissues to induce immune responses. They have high transcytotic capabilities and are able to transport a broad range of materials, such as bacteria, viruses, antigens and particles, from the intestinal lumen to the underlying lymphoid tissues [1]. In addition, M cells have less glyocalyx and reduced levels of membrane hydrolase activity, which can influence the fate of protein-containing or protein-coated NPs, compared with normal epithelial cells. Villous M cells located outside the FAE have also been observed [62], but the transport of antigens and microorganisms across the intestinal mucosa is carried out mainly by FAE-M cells [1]. Although they are less numerous than enterocytes, M cells present enhanced transcytosis abilities, which makes them particularly interesting for oral drug delivery applications. Therefore, M cells represent a potential portal for the oral delivery of drug-loaded NPs, particularly peptides, proteins and for the mucosal vaccination.

Although the role of M cells in particle uptake is well known, it is commonly believed that their low proportions in the human GI tract (1% of the total intestinal surface) and their variability among species (i.e., they represent 5% to 50% of the FAE surface and express species-specific markers), individuals, physiological state and age decrease the impact they could have on oral drug delivery [1]. However, in light of their particle uptake capabilities, several groups have considered it worthwhile to work on improving NP delivery through M cells, particularly with the aim of compensating for the low number of M cells through more efficient targeting [1,18,32,39,59,60,63–69]. The main strategy has been to coat the NP surface with an M cell-targeting molecule. This task is not trivial, especially considering the limited predictive value of the most commonly employed mouse models and the difficulties in identifying markers that are specific to human M cells.

The most investigated family of M cell-targeting molecules is the lectins. Lectins constitute a structurally diverse group of proteins and glycoproteins that bind reversibly and with relatively high affinity to specific carbohydrate residues present on cell surface proteins or lipids [18]. In several studies, the Ulex europaeus agglutinin-1 (UEA-1) lectin was grafted to the NP surface to target αvβ6-counergic residues expressed on the apical surface of M cells, yielding improvements in nanoparticle transport across the intestinal barrier [66,67,70]. The conjugation of PLGA particles to Aleuria aurantia lectin (AAL) induced an increased expression of IFNγ upon oral birch pollen immunotherapy [18] and effectiely protects mice against subcutaneous challenge with melanoma or prostate cancer cells [63]. However, the UEA-1 and AAL lectins, in addition to their potential immunoogeneity, are specifically expressed on mouse and not human M cells [60], limiting the translation of these therapies to humans.

Many studies have been dedicated to the search for specific markers of human M cells. Two lectins specific to the human FAE (galectin 9 and sialyl Lewis A antigen (SLAA)) have been identified [65,71]. One anti-SLAA antibody out of the 41 tested lectins and antibodies reacted strongly with human M cells and bound only weakly to FAE enterocytes [59]. Galectin 9 has not been used so far as a targeting molecule for the oral delivery of NP.

To target M cell receptors, it may be advantageous to replace lectins with small molecule mimetics. Lambkin et al. identified UEA-1 lectin mimetics from a combinatorial library [70,72]. These compounds are easy to synthesize and are based on an oligo-lysine scaffold with 1 to 4 lysine units terminated by galloyl entities. The lectin mimetics were coupled to a PEG construct through p-nitrophenyl-activated esters to form a tetragalloyl-β-lysine dendrimer (TGDK) that was used to vectorize a Rhesus CCR5-derived cyclopentapeptide antigen. Misumi et al. conducted in vivo studies on macaques with this TGDK and found a significant stool IgA response and efficient M cell transcytosis of the dendrimer, which induced neutralizing activity against SIV infection [68]. TGDK was efficiently transported from the lumen of the intestinal tract into Rhesus Peyer’s patches by M cells and then accumulated in germinal centers. In addition, TGDK specifically bound to human M-like cells in vitro and was efficiently transcytosed from the apical side to the basolateral side in the M-like cell model (Fig. 3).

M cells are considered the gateways for antigen entry to the underlying mucosal tissues, and they are exploited by various enteric pathogens as a route of entry to the underlying host tissue, predominantly through the hijacking of their endocytic machinery [18]. The invasiveness of these viral and bacterial pathogens is mediated by specific pathogen–host interactions, which could be adapted to deliver drug-loaded NPs into the GALT [60,69]. Some important pathogen recognition receptors (PRRs), such as Toll-like receptor-4 (TLR-4), platelet-activating factor receptor and α5β1 integrins, are expressed on the surfaces of human and mouse M cells [32,39,73]. The specific pathogen–host interactions are crucial for the translocation of bacteria across the lumen. Consequently, targeting PRRs might be a suitable strategy for enhancing the uptake of orally administered NPs by M cells [59].

For instance, M cells take up many enteropathogenic microorganisms, such as Yersinia, via the high-affinity interaction between invasin and the α5β1 integrins that are overexpressed at the apical pole of human M cells [32,74,75]. Integrations with α5β1 integrin occur mainly through RGD sequences. We have demonstrated that grafting RGD to the PEG chains of PLGA-based NPs significantly increases the in vivo transport of these NPs by human M-like cells [32, Fig. 4A] and slightly enhance the IgG immune response after oral immunization [32]. We hypothesized that this low efficiency was due to a partial degradation of the RGD peptide during its trafficking through the GI tract. Therefore, an RGD peptidomimetic (RGDp) was grafted onto PEGylated PLGA-based NPs. RGDp significantly increased the transport of NPs across an in vitro model of human M cells (Fig. 4B), and intraduodenal immunization with RGDp-labeled NPs elicited a higher production of IgG antibodies than the intramuscular injection of free ovalbumin or the intraduodenal administration of either non-targeted or RGD-NPs [39]. NPs conjugated to LDLp also exhibited greater transport by M cells in vitro and showed promising immune responses compared to untargeted NPs, suggesting that these LDL ligands might have bound to β1 integrins on the apical surface of M cells or other integrins homing lymphocytes in the gut [76].

The Clostridium perfringens enterotoxin (CPE) receptor (claudin 4) is a tight-junction transmembrane protein that plays a role in establishing transepithelial electrical resistance in the mucosal epithelium in addition to its function as a receptor for CPE [77]. Claudin 4 is highly expressed in M cells and is conserved between mouse and human Peyer’s patch. Targeting PLGA NPs to claudin 4 enhanced their in vivo uptake and mucosal IgA responses [77,78].

Recently, Hase et al. [79,80] reported that glycoprotein 2 (GP2) is specifically expressed at the apical surface of human and murine M cells and...
serves as a transcytotic receptor for fimH + bacteria (e.g., *Escherichia coli*, *Salmonella enterica* and *Yersinia*). Thus, the GP2-dependent transcytosis pathway could provide a new target for the development of M cell–targeted nanosystems.

Reovirus in mice and poliovirus and HIV in humans use specific receptors to target and cross the FAE [81–83]. Reovirus can invade intestinal M cells in rodents and rabbits through interactions between its outer capsid protein σ1 with α(2,3)-linked sialic acid containing glycoconjugates of the apical membrane [82]. The incorporation of recombinant σ1 into liposomes or an OVA-σ1 fusion protein enhanced binding to rat Peyer’s patch [84]. HIV-1 can adhere to M cells in mice and rabbits prior to its endocytosis and transport across the epithelial barrier [85]. A lymphotropic (X4) HIV-1 strain crosses M cell monolayers and infects the underlying CD4+ target cells. This transport requires both the lactosyl cerebroside and CXCR4 receptors, which are expressed on the apical surface of Caco-2 and M cells [81]. In contrast, a monotropic (R5) HIV-1 strain is unable to cross M cell monolayers and infect underlying monocytes. Caco-2 and M cells do not express CCR5, but the transfection of these cells with CCR5 cDNA restores the transport of the R5 virus [81].

### 3.2. Enteroocytes

The importance of enteroocytes should not be overlooked; these cells vastly outnumbers M cells and can transcytose many macromolecules, such as cholera toxin (CT) and F4 fimbriae, as well as inert particles [86–89]. For instance, several *Escherichia coli* strains express F4 fimbriae on their surface and bind to specific F4 receptors (F4Rs). The expression of these receptors on the surface of porcine enteroocytes is necessary to induce a protective mucosal immunity following the oral administration of purified F4 fimbriae to piglets [88]. The conjugation of heterologous antigens to F4 fimbriae has been shown to induce enhanced mucosal antigen-specific antibody responses upon oral administration, but to date, no study evaluating the influence of F4 fimbriae targeting on the oral transport of nanocarriers has been reported. The incorporation of flagellin-rich *Salmonella enteritidis* extracts into Gantrez AN NPs induced biodhesion in the ileum during “Salmonella-like” gut colonization [90].

TLR-4, the receptor for LPS, mediates bacterial translocation through enteroocytes [91]. The rat enteroocyte cell line IEC-6 internalized LPS-coated latex beads in a TLR4-dependent manner, indicating that LPS-coated particles may provide yet another alternative for the targeted delivery of NPs to the intestinal epithelium [18].

Some lectins (e.g., wheat germ agglutinin (WGA), concanavalin A (ConA) and tomato-derived lectin) bind to enteroocytes, with a relatively high affinity for the specific carbohydrate residues present on cell surface proteins or lipids [18,92–94]. They have been grafted onto the surfaces of nanoparticles for oral vaccines or drug delivery.

Some authors have proposed using certain metabolic pathways as routes for NP uptake. This strategy was attempted with vitamin B12 (VB12) or B1 (thiamine)-coated NPs. VB12 forms a complex with the intrinsic factor (IF) in the stomach, which is subsequently recognized by IF receptors on ileal epithelial cells, resulting in the endocytosis of VB12. VB12-targeted micelles exhibited better in vitro uptake and transport of a hydrophobic drug in a model intestinal cell monolayer in comparison to untreated micelles [30,95]. Similarly, Salman et al. also produced thiamine-poly(anhydride) NPs, which could be administered at a higher daily dose than VB12. Encouraging immunization results were also obtained with these thiamine-coated vehicles in mice, although no direct comparison with VB12 NPs was made [96].

### 3.3. Goblet cells

Goblet cells represent the second largest population of intestinal epithelial cells, but they are rarely chosen as a target for orally delivered nanocarriers. Recently, a CKS peptide identified using a random phage display technique was found to have a specific affinity for goblet cells [97]. Orally administered insulin-loaded trimethyl chitosan chloride NPs that were modified with the CKS targeting peptide induced enhanced transport via clathrin- and caveolea–dependent endocytosis and produced a better hypoglycemic effect than non-targeted NPs [98].

Furthermore, *Listeria monocytogenes* is transcytosed across the intestinal barrier by binding to E-cadherin, which is luminally accessible.
on goblet cells, suggesting that targeting E-cadherin could be a promising strategy for delivering NP to goblet cells [99].

3.4. Dendritic cells

Dendritic cells (DCs) represent the most potent APCs. They are found throughout the intestine and can be divided into two major subsets, which can be distinguished based on the expression of CD103 (the αE chain of the αEβ7 integrin), the receptor for the epithelial cell adhesion molecule E-cadherin, and C3CR1 [100]. Intestinal DCs have been proposed to be involved in the induction of protective immunity against pathogens, tolerance to commensal bacteria and tolerance to food antigens and self-antigens. Thus, they represent a potent target for oral vaccination strategies because vaccine interactions with DCs can be enhanced by targeting DC surface molecules [18,101]. There are very few, if any, DC-specific markers, but these cells possess a broad spectrum of cell surface receptors that are involved in endocytosis and the induction of immune responses, such as C-type lectins, scavenger receptors, TLRs and Fc receptors (FcRs: FcgR, FcaR and FceR, which bind to IgG, IgA and IgE, respectively) [102].

The outcome of the immune response can differ depending on the targeted receptor. TLR ligands induce strong DC activation and thus have potent adjuvant properties. The C-type lectin DEC-205 and mannose receptors are more involved in enhancing endocytosis, although DC activation has been achieved with DEC-205 targeting [101]. The FcγRs are a family of membrane glycoproteins that bind the Fc fragment of IgG and activate a signaling pathway that can regulate the adaptive immune response when cross-linked with antigen–antibody immune complexes [26, 54, 100, 103].

Mannan and other mannosylated structures enhance antigen endocytosis by DCs and induce immune responses when grafted to a vaccine carrier surface [104, 105]. Mannosylated liposomes bind to DC-SIGN and the mannose receptor CD206, resulting in enhanced antigen-specific cell proliferation relative to antigen alone or non-targeted liposomes, and they protect mice from lethal challenge when delivered intraperitoneally. Mannosylated, PEGylated PLGA NPs elicited higher antigen-specific IgG serum responses in mice upon intraduodenal administration than non-grafted NPs [39]. They also enhanced NP uptake in a mouse model of colitis [106].

Targeting intestinal DCs enhances the endocytosis of antigen-loaded carriers and can thus improve immune responses, but most of the studies investigating this phenomenon have been performed in vitro or in murine models. Therefore, further studies are required to i) evaluate vaccine carriers targeting intestinal DCs of higher species (pigs and primates), ii) analyze the behavior of antigen-loaded carriers in the GI tract and iii) deepen our understanding of the interactions between vaccine carriers and intestinal DCs.

3.5. Enteroendocrine cells and the identification of novel potential ligands for nanoparticle delivery

The enteroendocrine system constitutes the largest endocrine organ. Enteroendocrine cells are scattered throughout the GI tract in the epithelium among enterocytes. These cells are typically conically shaped, with a large base from which gut hormones are released into the blood from secretory granules. These cells are distributed along the GI tract, and the apical pole facing the gut lumen possesses microvilli. Strikingly, these cells are among the least understood cells in the body. However, new molecular genetic techniques have led to important advances, thereby highlighting novel aspects of enteroendocrine biology [107]. Enteroendocrine cells represent approximately 1% of all epithelial cells in the intestine and are subdivided into more than 10 different cell types based on their main secretory products and localization along the GI tract (e.g., ileal/colonic L cells). Multiple biological functions are physiologically regulated by gut hormones (e.g., food intake, gastric emptying, gut motility, gut barrier function and glucose metabolism). Among the enteroendocrine cells, L cells have attracted particular interest because of the pleiotropic effects of their secreted peptides.

In the gut, the posttranslational processing of proglucagon in endocrine L cells gives rise to the major proglucagon-derived peptides (GLP-1, GLP-2, oxyntomodulin and glicentin) [108]. These peptides are rapidly secreted in response to food intake, and their production is modulated according to the nutrient (i.e., lipid, carbohydrate, and protein) [109]. L cells secrete another anorexigenic peptide, PYY. Similar to GLP-1 administration, PYY injection delays gastric emptying and pancreatic and gastric secretions. GLP-2 is co-secreted by L cells with GLP-1. GLP-2 assists in the maintenance of the physiological gut barrier function (i.e., protects against gut permeability) and facilitates the digestion and absorption of ingested nutrients. In addition, GLP-2 regulates the stimulation of intestinal epithelial cell proliferation [110] and constitutes a key target in the maintenance of gut barrier function.

Given the location of these cells (the ileum and colon), targeting L cells and thereby the endogenous production of these hormones remains challenging. Therefore, strategies devoted to targeting specific receptors involved in L cell physiology, thus leading to the production of hormones, such as GLP-1, GLP-2 and PYY, could be a promising potential method to locally influence the mechanisms involved in
high-impact diseases, such as diabetes, obesity and inflammatory diseases.

Recent studies have shown that L cells express various G-protein-coupled receptors (GPCRs) that are activated by a wide variety of endogenous ligands found in the gut lumen. GPCRs are involved in a large number of physiological processes and could serve as targets for coated NPs. Potential ligands for these receptors include specific lipids from short-chain fatty acids (i.e., GPR43, GPR41 and GPR109a) [111,112] and long-chain fatty acids (GPR40 and GPR120) [113]. Interestingly, GPR43 and GPR41 expression is well conserved across species (human, pigs, and rodents) [114]; both receptors are expressed in L cells and have been shown to directly control GLP-1 and PYY secretion (Fig. 5) [115]. Although a direct link between GPR43 or GPR41 and GLP-2 secretion has not been established, we have demonstrated that changing the composition of the gut microbiota using specific nutrients that increase short-chain fatty acids (SCFA) (fermentable carbohydrate) stimulate endogenous GLP-2 production (Fig. 5) and protect against gut permeability and associated inflammation [116]. Moreover, a similar treatment increases PYY and GLP-1 secretion.

Bioactive lipids belonging to the N-acyethanolamine family that are part of the endocannabinoid system [117,118] could also activate the L cell-specific receptor GPR119, thereby increasing GLP-1, GLP-2 and PYY secretion. Finally, TGR5 (also known as M-BAR, GPBAR-1 or GPR131) can be activated by bile acids [119].

SCFAs are present in the gut lumen (ileum and colon) at a high concentration (approximately 100 mM) [120]. These fatty acids are mainly produced through the metabolic activity of the gut microbiota (undigested carbohydrate fermentation). Over the last 30 years, numerous roles have been attributed to SCFAs, including the harvesting of energy from undigested food, the regulation of epithelial cell proliferation, electrolyte uptake and smooth muscle contraction [112], and SCFAs could thus be used as NP ligands to act on L cell metabolism.

Another potential target is the GPR109A receptor, which binds to the ketone body β-hydroxybutyrate and butyrate [114,115]. The GPR109A receptor is highly expressed in the gut lumen, in which the concentration of butyrate reaches approximately 20 mM, thereby activating this receptor. The intestinal GPR109A receptor could therefore be targeted by butyrate-labeled NPs.

The postprandial satiety effect of dietary lipids and free fatty acids stimulates several gut peptides that control food intake. Growing evidence suggests that these effects are mediated through two different receptors, GPR40 and GPR120. Both receptors are activated by medium- to long-chain free fatty acids that stimulate gut peptide secretion [113]. It is worth noting that similar strategies and ligands might be used to target L cells (i.e., GLP-2 production) and improve gut barrier function.

Bile acids are not only byproducts of cholesterol metabolism but also key metabolic regulators that act through TGR5 [121]. For instance, Katsuma et al. discovered that bile acids promote GLP-1 secretion through a TGR5-dependent mechanism [122], thereby suggesting a novel role of bile acids in energy metabolism and glucose homeostasis. Interestingly, a recent study has demonstrated the relevance of targeting TGR5 in experimental colitis [123]. TGR5 activation has been shown to exert a peripheral immune-modulatory effect in macrophages, and this study also showed that TGR5 activation through a targeting strategy restores tight-junction protein distribution, leading to reduced gut permeability [123]. For decades, ciprofloxacin has been used for the treatment of Gram-negative bacterial infections occurring in the context of Crohn's disease. Cipriani et al. have demonstrated that ciprofloxacin also acts as a TGR5 agonist, thereby contributing to improvement of the inflammatory status [123]. This last study supports the concept that the TGR5 receptor might be targeted by not only specific bile acids but also synthetic molecules, such as ciprofloxacin. While they are effective for treating infections in Crohn's disease patients, the wide use of antibiotics plays a minor role in the maintenance therapy for these patients. Therefore, we propose that targeting TGR5 using NPs carrying a lower dose of ciprofloxacin might be useful to target colon cells or macrophages to treat inflammatory bowel disorder, preventing the major adverse effects linked to the antibiotic activities of this compound.

In conclusion, although endogenous non-peptidic ligands have helped to identify the different GPCRs described here, numerous
synthetic agonists are also currently being studied and may constitute a source of therapeutic agents that merit future consideration [124]. Moreover, it is worth noting that in addition to their roles in the control of gut peptide secretion, most of these receptors are also expressed in immune cells, such as macrophages. Therefore, we propose that the oral delivery of NPs with non-peptidic ligands targeting GPCRs of L cells could lead to new treatments, acting locally on obesity, type 2 diabetes, and intestinal inflammation. Thus, by exploring the wide variety of L cell stimulation-dependent effects (e.g., GLP-1, PYY secretion) on GI tract physiology and peripheral metabolism (energy and glucose homeostasis), we propose to provide valuable insight into some novel beneficial therapeutic targets.

4. Conclusions regarding the potential biomedical applications of the oral delivery of nanoparticles targeted with non-peptidic ligands

Orally administered targeted NPs have a large number of potential biomedical applications and exhibit several putative advantages for oral drug delivery, such as the protection of fragile drugs or the potential for the modification of drug pharmacokinetics. Despite these advantages, the oral delivery of drugs by NPs remains challenging. To achieve efficient drug delivery, NPs must i) avoid rapid mucus clearance; ii) penetrate the mucus layer; and iii) be extensively taken up by the intestinal epithelium. The optimization of particle size and surface properties and the targeting of specific cells by ligand grafting have been shown to enhance NP transport across the intestinal epithelium.

In particular, targeted NPs with novel non-peptidic ligands for oral delivery have been investigated. The main advantage of these particles is that their targeting properties are improved by the use of ligands that are not degraded in the GI tract, unlike peptidic/proteinaceous ligands, and are not limited to receptors, which interact only with proteins. The use of targeted NPs will also depend on the type of cells/receptors targeted by cell-specific targeting.

To the best of our knowledge, no clinical studies on the oral delivery of antigens or drugs with targeted NPs are ongoing because until recently, the potential benefits of these particles have been outweighed by the associated pitfalls. In particular, the sophisticated design and associated high cost of the synthesis of the grafted polymer and the high cost of NP production might be major limitations for their development as pharmaceuticals. Due to their high cost of manufacture, targeted NPs must provide significant added value for unmet pharmaceutical and medical needs. For example, they will not be used for the solubilization of BCS class II or IV drugs; rather, their use will likely be limited to punctual applications (e.g., vaccines) or the treatment of diseases that are currently lacking satisfactory therapies. To a lesser extent, the lack of control/robustness of NP absorption might also hinder their use in several biomedical applications. Thus, they should be used for applications that do not require a fine-tuning of the delivered dose (e.g., vaccines and type 2 diabetes), and they would likely not be suitable for insulin delivery for type 1 diabetes treatment.

Based on the literature review, we propose (as illustrated in Fig. 1) that the oral delivery of NPs that are targeted using non-peptidic ligands would be useful to further investigate i) oral vaccination targeting M cells; ii) the oral delivery of therapeutic proteins and peptides by targeting enterocytes; and iii) the specific targeting of L cells for the treatment of type 2 diabetes, obesity and inflammatory diseases. Anne des Rieux, Patrice D. Cani and Jacqueline Marchand-Brynaert are Research Associates of the Fonds National de la Recherche Scientifique (Belgium).

References


