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Review

Nanoparticles as potential oral delivery systems of proteins and vaccines: A mechanistic approach

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Abstract

Peptides and proteins remain poorly bioavailable upon oral administration. One of the most promising strategies to improve their oral delivery relies on their association with colloidal carriers, e.g. polymeric nanoparticles, stable in gastrointestinal tract, protective for encapsulated substances and able to modulate physicochemical characteristics, drug release and biological behavior. The mechanisms of transport of these nanoparticles across intestinal mucosa are reviewed. In particular, the influence of size and surface properties on their non-specific uptake or their targeted uptake by enterocytes and/or M cells is discussed. Enhancement of their uptake by appropriate cells, *i.e.* M cells by (i) modeling surface properties to optimize access to and transport by M cells (ii) identifying surface markers specific to human M cell allowing targeting to M cells and nanoparticles transcytosis is illustrated. Encouraging results upon *in vivo* testing are reported but low bioavailability and lack of control on absorbed dose slow down products development. Vaccines are certainly the most promising applications for orally delivered nanoparticles.

Keywords: Nanoparticles; Oral delivery; M cells; Proteins; Vaccines; Biodegradable polymers

Contents

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1. Introduction

Oral drug delivery is the choicest route for drug administration because of its non-invasive nature. The oral route presents the advantage of avoiding pain and discomfort associated with injections as well as eliminating contaminations. However, administered bioactive drugs like peptides (>4 a. a.) and proteins must resist the hostile gastric and intestinal environments. They must then persist in the intestinal lumen long enough to adhere to cell apical surface and then, be transcytosed by intestinal cells. Therefore, peptides and proteins remain poorly bioavailable when administrated orally, mainly due to their low mucosal permeability and lack of stability in the gastrointestinal environment, resulting in degradation of the compound prior to absorption.

For many years, many studies have been focused on the improvement of oral delivery of therapeutic peptides and proteins; various strategies have been thus developed to enhance drug and vaccine oral delivery [1–12]. Their association with colloidal carriers, such as polymeric nanoparticles, is one of several approaches proposed to improve their oral bioavailability.

Polymeric nanoparticles are of especial interest from the pharmaceutical point of view. First they are more stable in the gastrointestinal tract than other colloidal carriers, such as liposomes, and can protect encapsulated drugs from gastrointestinal environment. Second, the use of various polymeric materials enable the modulation of physicochemical characteristics (e.g. hydrophobicity, zeta potential), drug release properties (e.g. delayed, prolonged, triggered), and biological behavior (e.g. targeting, bioadhesion, improved cellular uptake) of nanoparticles [12]. Finally, the particle surface can be modified by adsorption or chemical grafting of certain molecules such as poly(ethylene glycol) (PEG), poloxamers, and bioactive molecules (lectins, invasins, …). Moreover, their submicron size and their large specific surface area favor their absorption compared to larger carriers. Consequently, it has already been extensively shown that nanoencapsulation of peptides and protein colloidal particles protects them against the harsh environment of the gastrointestinal tract [13], and enhances their transmucosal transport [14,15].

Different types of cells and structures compose the intestinal epithelium. Epithelium of villi is mainly constituted of enterocytes and goblet cells. One of the main functions of enterocytes is to control the passage of macromolecules and pathogens, and, at the same time, to allow the digestive absorption of dietary nutrients. Goblet cells secrete the mucus gel layer, a viscous fluid composed primarily of highly glycosylated proteins (mucins) suspended in a solution of electrolytes. Dispersed through the intestinal mucosa, lymphoid nodules called O-MALT (Organized Associated Lymphoid Mucosa), individually or aggregated into Peyer's patches, have interested scientists, mainly due to the presence in these structures of particular cells, named M cells [16]. M cells are mainly located within the epithelium of Peyer's patches, called Follicle Associated Epithelium (FAE) (Fig. 1), which is also composed of enterocytes and few goblet cells. M cells deliver samples of foreign material from the lumen to the underlying organized mucosa lymphoid tissues in order to induce immune responses. M cells are specialized for antigen sampling, but they are also exploited as a route of host invasion

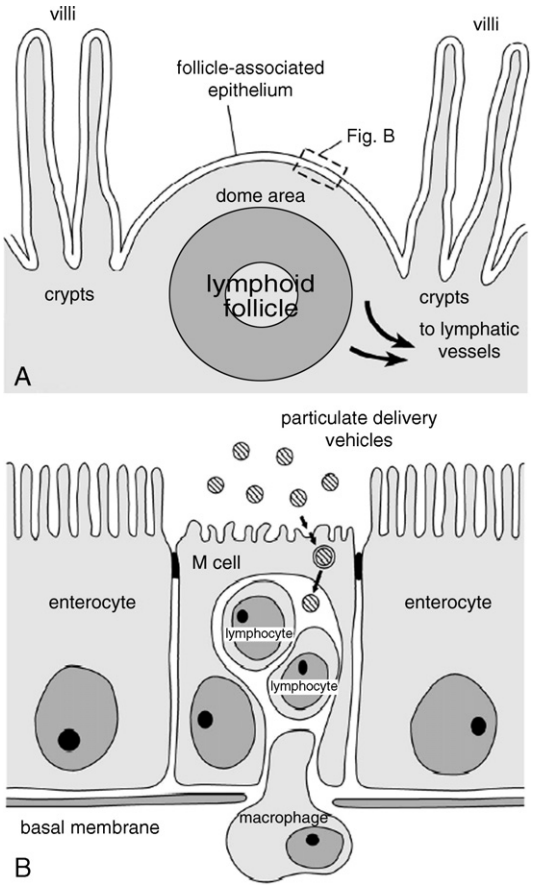

Fig. 1. Schematic transverse sections of a Peyer's patch lymphoid follicle and overlying follicle-associated epithelium (FAE), depicting M cell transport of particulate delivery vehicles. The general structure of intestinal organised mucosa-associated lymphoid tissues (O-MALT) is represented by the schematic transverse section of a Peyer's patch lymphoid follicle and associated structures in (A). The FAE is characterised by the presence of specialised antigen sampling M cells (B). (Reprinted from Adv. Drug. Del. Rev., 50, Clark et al., Exploiting M cells for drug and vaccine delivery, 81–106, Copyright (2001), with permission from Elsevier).

by many pathogens [17,18]. Furthermore, M cells represent a potential portal for oral delivery of peptides and proteins and for mucosal vaccination, since they possess a high transcytotic capacity and are able to transport a broad range of materials, including nanoparticles [19,20]. Uptake of particles, microorganisms and macromolecules by M cells, have been described to occur through adsorptive endocytosis by way of clathrin coated pits and vesicles, fluid phase endocytosis and phagocytosis [21]. In addition, M cells, compared with normal epithelial cells have reduced levels of membrane hydrolase activity, which can influence the uptake of protein-containing or protein-decorated nanoparticles. The relatively sparse nature of the glycocalyx facilitates the adherence of both microorganisms and inert particles to their surfaces [22]. Villous-M cells located outside the FAE have been recently observed [23], but the transport of antigens and microorganisms across the intestinal mucosa is carried out mainly by the FAE-M cells [24]. Although less numerous than enterocytes, M cells present enhanced transcytosis abilities which made them very interesting for oral drug delivery applications.

Diverse strategies have been developed to improve the bioavailability of peptide and protein drugs and vaccines, encapsulated in polymeric nanoparticles. Some focused on M cells, while others target not only M cells but all intestinal cells, enterocytes mainly. Two main approaches prevailed to significantly improve transport: (i) by modifying surface physicochemical properties of nanoparticles, or (ii) by coupling a targeting molecule at the nanoparticle surface. The aim of this review is to discuss the mechanisms of nanoparticle transport across the intestinal barrier and to describe the new approaches developed to enhance their transmucosal transport. Application to therapeutic peptide and protein delivery as well as vaccination will be illustrated.

2. Nanoparticle formulation and physicochemical properties

Polymeric nanoparticles are colloidal carriers ranging in size from 10 to 1000 nm. They are divided into two categories: nanocapsules and nanospheres. The former are vesicular systems with a polymeric shell and an inner core. The latter consist in a polymeric matrix. Their controlled release properties and the protection they offer to the compound of interest make these nanosystems very advantageous in the scope of drug delivery applications [25], particularly in the gastrointestinal (GI) tract, where conditions are very harsh. Therefore, drug encapsulation in protective synthetic colloidal carriers, such as nanoparticles, which can also deliver it in a controlled manner, represents an attractive strategy to successfully orally deliver therapeutic peptides and proteins.

Under this section, the different polymers used to prepare nanoparticles will be described. Then, the different techniques developed to form nanoparticles, linked to the nature of the drug to be encapsulated and of the polymers constituting the carrier, will be mentioned. Finally, strategies employed to improve nanoparticle transport across the intestinal mucosa will be exposed.

2.1. Different polymers developed to formulate nanoparticles

After 30 years of investigation, researchers have at their disposal many polymers and copolymers to develop nanoparticle-based oral delivery systems. The main criteria dictating polymer eligibility for drug delivery have been bioavailability, biocompatibility, straightforward production and degradation rate, which provide a sustained release of drugs encapsulated in nanoparticles. The nature of polymers constituting the formulation significantly influences nanoparticles size and their release profile. Although natural polymers generally provide a relatively quick drug release, synthetic polymers enable extended drug release over periods from days to several weeks. Profile and mechanism of drug release depend on the nature of the polymer, and on all the ensuing physicochemical properties. Some polymers are less sensitive to processing conditions than others, which could be due to their chemical composition, molecular weight and crystallinity [26]. Technologically, a large number of methods exist to manufacture nanoparticles, allowing extensive modulation of their structure, composition, and physiological properties. The choice of the method to prepare nanoparticles essentially depends on polymer and on solubility characteristics of the active compound to be associated with nanoparticles. Since this topic has been recently reviewed [27–29] and is beyond the scope of this review, it will not be further developed.

Polymeric materials used for the formulation of nanoparticles include synthetic (poly(lactic acids) (PLA), poly(lactic-co-glycolic acids) (PLGA), poly(ε-caprolactone) (PCL) [30,31], poly(methyl methacrylates), and poly(alkyl cyanoacrylates)) or natural polymers (albumin, gelatin [32], alginate [33], collagen or chitosan) [12]. Polyesters, alone and in combination with other polymers, are the most commonly used for the formulation of nanoparticles. PLGA [34–38] and PLA [39–41] are highly biocompatible and biodegradable [42,43]. They have been employed since the eighty's for numerous *in vivo* applications (biodegradable implants, controlled drug release) [44–47]. More recently, formulations based on natural polymers have been developed. Up to now, chitosan is the most valued, certainly due to its permeability enhancer abilities and to its properties, allowing the preparation of organic solvent free mucoadhesive particles [48]. Van der Lubben et al. [49] have demonstrated that large amounts of bovine serum albumin (BSA) or vaccine tetanus toxoid (TT) were easily encapsulated in chitosan nanoparticles. Recently, Alonso's group have developed chitosan nanoparticles as carrier systems for transmucosal delivery [50,51]. They have shown on rats and rabbits the potentiality of such chitosan nanoparticles to enhance mucosal absorption.

2.2. Formulation principles

Nanoparticles constituted of synthetic polymers are usually prepared by dispersion of preformed polymers. Several techniques can be used, mainly chosen in function of the hydrophobicity of drugs to be encapsulated (Table 1). The nanoprecipitation method [52] is employed to encapsulate lipophilic

Table 1
Preparation methods of nanoparticles, in function of the polymeric matrice

Polymers	Methods of nanoparticle preparation
Synthetic polymers	
Poly(alkyl cyanoacrylate)	Monomers polymerization
Poly(alkyl methacrylate)	
Poly(styrene)	
Poly(vinylpyridine)	
Poly(ε-caprolactone)	Nanoprecipitation
Poly(lactic acid)	
Poly(lactic-co-glycolic acid)	
Poly(methacrylate)	
Poly(ε-caprolactone)	Solvent evaporation
Poly(lactic acid)	
Poly(lactic-co-glycolic acid)	
Poly(β-hydroxybutyrate)	
Ethyl cellulose	
Cellulose acetate phthalate	Salting out
Poly(alkyl methacrylate)	
Ethyl cellulose	
Poly(lactic acid)	
Poly(lactic-co-glycolic acid)	
Natural polymers	
Albumin	Desolvation, denaturation, ionic gelation
Casein	
Gelatin	
Alginate	
Chitosan	
Ethyl cellulose	

drugs, forming nanospheres. Recently, Bitali et al. [53] have adapted this technique to encapsulate hydrophilic compounds into PLGA and PLA nanoparticles. Small polydispersity indices are easily and rapidly obtained by nanoprecipitation. The solvent evaporation method [54] is used to encapsulate either hydrophobic or hydrophilic drugs. In both cases, polymers are dispersed in a water-immiscible organic solvent such as methylene dichloride, chloroform or ethyl acetate. The solution is then emulsified with an aqueous phase containing a surfactant (poly (vinyl alcohol) (PVA), cholic acid…) (oil-in-water (O/W), hydrophobic drug)) and the organic phase is evaporated. Or, when a hydrophilic drug has to be encapsulated, a first emulsion of aqueous phase in the organic phase of polymer is formed. An outer aqueous phase, supplemented with a surfactant, is added to form the water-in-oil-in-water (W/O/W) emulsion. After total evaporation of the organic solvent the drug-loaded nanospheres are separated from the surfactant and the non-encapsulated drug by centrifugation. A variant, the emulsion–diffusion technique [55], consists in forming an O/W emulsion with a partially water-soluble solvent, containing polymers and an aqueous solution containing a stabilizer. Addition of a large volume of water causes the diffusion of the partially water-soluble solvent into the external phase, and then the formation of nanoparticles. The salting out/solvent displacement method [56] would allow to avoid the use of organic chlorinated solvents and large amount of surfactant during formulation.

Formulation of nanoparticles with natural polymers is performed by iononic gelation (chitosan [48,57]) or by coacervation (chitosan [58], gelatin [59]). They are mild methods, presenting the advantage to produce organic solvent free-formulations.

2.3. Formulation parameters influencing nanoparticle properties

Since particle uptake by intestinal cells is size-dependant, particle size is a critical determinant of orally delivered nanoparticle fate. This is the reason why each parameter influencing nanoparticle size has to be mastered.

Key parameters modulating nanoparticle size during the formulation process have been extensively studied. Factorial designs have been used to investigate the effect of processing parameters on the size of PLGA nanoparticles, like the number of homogenization cycles, the addition of excipient to the inner water phase, the drug concentration and the oil–outer water phase ratio [34,37]. The concentration and nature of the surfactant [37,60] seem also to influence nanoparticle size. For instance, formulations prepared in presence of PVA are smaller than those produced with sodium dodecyl sulfate (SDS). Likewise, a high concentration of surfactant reduces the size of complexes. At last, polymer molecular weight influences also the size of particles: the higher the polymer weight, the smaller and less polydispersed the nanoparticles [61].

Nanoparticle surface properties govern also the extent of nanoparticle uptake. Hydrophobicity and surface charges are greatly influenced by polymer composition. Nanoparticle surface can be modified either by coating or by grafting a molecule whose properties alter nanoparticle surface characteristics. Techniques developed to alter these surface properties are described under Section 2.4.

Integrity of the encapsulated drug has obviously a crucial impact on its bioavailability and pharmacological efficiency. Parameters improving encapsulation and drug stability have been investigated. Particularly, regarding the double emulsion technique, Zambaux et al. [61] demonstrated that process's temperature and solvent evaporation duration did not modify nanoparticle properties but altered encapsulation efficiency. They established that solvent evaporation duration had to be as short as possible and they recommended to formulate nanoparticles at 0 °C to reduce the risk of drug degradation. Similarly, a large internal aqueous phase increases drug loading. Regarding the nanoprecipitation technique, decreasing solubility of water-soluble drugs by pH changing of the aqueous phase, improved encapsulation efficiency [36].

2.4. Modification of nanoparticle surface to improve transport across the intestinal mucosa

Although conclusions regarding optimal nanoparticle surface properties still remain controversial, depending furthermore on the type of intestinal cell (enterocytes versus M cells), it is fully accepted that nanoparticle surface properties are of outmost importance for their uptake by intestinal epithelial cells. Hence, many strategies have been developed to improve mucosal absorption of nanoparticles, either by modifying their surface properties or by coupling a targeting molecule at their surface.

2.4.1. Modification of nanoparticle surface properties

Modification of nanoparticle surface properties can be achieved either by coating nanoparticle surface with hydrophilic stabilizing, bioadhesive polymers or surfactants or by incorporating biodegradable copolymers containing an hydrophilic moiety in the formulation. These modifications mainly change nanoparticle zeta potential, as well as their hydrophobicity, thus influencing formulation colloidal stability, nanoparticle mucoadhesion properties, and protein adsorption at their surface, and finally, oral absorption of the nanoparticles.

Poly(ethylene glycol) (PEG) has been employed as nanoparticle coating in drug delivery applications for its stabilizing properties. Indeed, PEG chains form a steric barrier at nanoparticle surface which stabilizes the complex and prevents opsonisation or interactions with macrophages. Due to these interesting properties numerous PEGylated formulations have been developed. Recently, Yoncheva et al. [62] coated poly (methyl vinyl ether-co-maleic anhydride) (PVM/MA) nanoparticles with PEG_{1000} or PEG_{2000}, enhancing their affinity for rat intestinal mucosa, without increasing interactions with stomach walls. Dong and Feng [63] proposed to physically adsorb montmorillonite, a medicinal clay with mucoadhesive properties, at the surface of PLGA nanoparticles. Due to its mucoadhesive properties, chitosan has been one of the most employed polymer to coat nanoparticle surface [64]. Vila et al. [65] have developed chitosan-coated PLGA nanoparticles adding chitosan in the outer aqueous phase of the double emulsion during the formulation. Chitosan-coated nanocapsules have been obtained incubating "oily nanodroplets" with a solution of chitosan [50]. The same technique was used to coat chitosan nanoparticles with sodium alginate [58]. Adsorption of sodium alginate to nanoparticle surface induced the inversion of coated-nanoparticles zeta potential.

With a same objective, PEGylated copolymers (PLA–PEG, PLGA–PEG and PCL–PEG) have been synthesized and included in formulations, leading to the formation of PEGylated nanoparticles with hydrophilic PEG chains being preferentially located at particle surface. Vangeyte et al. [66] developed a method to synthesize PCL–PEG copolymers by ring-opening polymerization of ε-caprolactone initiated by α-methoxy, ω-hydroxy poly (ethylene oxide). PEGylated PLA/PLGA nanoparticles or nanocapsules are prepared as described under Section 2.2. PEG coating has been assessed by surface analysis techniques or using ^{1}H RMN spectroscopy [67]. As expected, incorporation of these amphiphilic polymers in formulations modified nanoparticle physicochemical properties, such as neutralization of the zeta potential, decrease of the surface hydrophobicity and slight modification of the size of particles.

The ultimate goal of such modifications of nanoparticle surface by addition of hydrophilic polymers such as PEG or chitosan in the formulation is to enhance their transport across the intestinal mucosa via specific interactions between nanocarriers and intestinal epithelium.

2.4.2. Targeted nanoparticles

Another strategy consists in grafting a ligand at nanoparticle surface to specifically target receptors expressed at enterocyte or M cell surface. These receptors and their role in nanocarrier transport across epithelial cells will be described under Section 3.2.2.

Different types of targeting molecules have been tested but the most studied has been the lectin family. Lectins are natural proteins or glycoproteins that bind reversibly and specifically to sugars, and thus agglutinate cells and polysaccharides [68]. They are involved in many cell recognition and adhesion processes. Their conjugation to polymeric nanoparticles significantly increases their transport across the intestinal mucosa by efficiently increasing interactions with mucus [20,69,70] and/or the surface of epithelial cells [71] and by promoting particle translocation [69,72]. The association of lectins with nanoparticles can be achieved by adsorption or covalent coupling, with a definite preference for a covalent linkage, if conjugation does not affect lectin activity and specificity.

Peptidic ligands like the well known RGD (arginine–glycine–aspartate) derivatives, which target, among others, β1 integrins localized at the apical pole of M cells, have also been covalently linked on polymers before the formation of nanoparticles. Covalent binding on PEG chains favors RGD presentation and then targeting [73].

Ligands can also be non-covalently attached to PEG chains. Recently, Gref et al. [74] have grafted biotin molecules on PEG chains and exploited the strongest biological non-covalent interactions to fix, in presence of avidin, biotin-coupled ligands at PCL–PEG–biotin nanoparticle surface. A biotin–lectin was incubated with nanoparticles in presence of avidin, leading to the formation of a nanoparticle–biotin–avidin/biotin–lectin complex. The main advantage of this technique lies in the variety of biotinylated ligands that could be grafted at nanoparticle surface.

Thus, surface properties of nanoparticles can be modified either by improving non-specific interactions with the cell apical surface or by grafting a specific ligand targeting epithelial intestinal cells.

3. Transport of nanoparticles across the intestinal mucosa

Absorption of particulate matters through the intestinal mucosa has been assessed by numerous studies [22,75–80]. For all the advantages presented by the oral route, already described above, huge efforts have been dedicated to the development of oral formulations and on the improvement of their absorption across the intestinal mucosa. However, two stumbling blocks are still pending [22]. First, is the amount of nanoparticles that can reach the blood circulation sufficient to induce a therapeutic effect; second, could experimental data obtained during animal studies be extrapolated to humans?

Most of the studies assessing nanoparticle transport across the intestinal barrier were performed in animals. It is still difficult to conclude on the relevance of animal transport results to predict the efficiency of nanoparticle formulation in humans. Information was provided by studies performed on human *in vitro* models (Caco-2 cells for epithelial cells [81–86] and more recently human FAE model [87,88]), but clinical studies are still cruelly missing.

A macromolecule or a particle can theoretically cross the intestinal epithelium by (i) the paracellular route (between

adjacent cells) and the transcellular route (Fig. 2), the transcellular route being the most explored.

3.1. Paracellular transport

In physiological conditions, the paracellular route is limited, on one hand, by the very small surface area of the intercellular spaces and, on the other hand, by the tightness of the junctions between the epithelial cells (pore diameter between 3 and 10 Å [89]).

A review dealing with the current strategies used to enhance paracellular transport of drugs has been recently published [90]. Briefly, surfactants have been used to enhance absorption of poorly absorbed drugs, but the mode of action relied on mucosal damage in the GI tract [91]. Hydrosoluble polymers such as chitosan, starch and thiolated polymers [92] appeared more successful.

Chitosan and poly(acrylic acids) in solution can enhance paracellular transport of drugs through interactions between the negatively-charged cell membrane and the positive charges of the polymer, or by complexing Ca^{2+} involved in the structure of tight junctions [92,93]. Co-administrated chitosan has been shown to enhance transport of buserelin, vasopressin and insulin, as well as clodronate across Caco-2 cell monolayers [94,95]. Chitosan solution is considered as a permeability enhancer due to its effect on depolymerization of cellular F-actin and on the tight junction protein ZO-1 [96]. Recently, it was described that chitosan acts, at least partly, via an activation of protein kinase C (PKC) [97]. Interestingly, the results of previous studies [98–100] also indicate that the effect of chitosan on Caco-2 cell monolayer is reversible and, hence, that the opening of the cellular barrier is transient. This specific behavior makes a difference in terms of toxicity between chitosan and the classical penetration enhancers that are known to cause irreversible epithelial damage [101]. However, whether this effect remains unaltered when the polymer is present under the form of nanoparticles or attached to them instead of solubilized needs to be further clarified. In the case of poly(methacrylic) derivatives [102], it has been shown that their typical permeation enhancing effect is maintained when they are in the form of nanogels. However, in the case of chitosan based colloidal systems, the maintenance of the inherent capacity of chitosan to open tight junctions has not been fully identified [103].

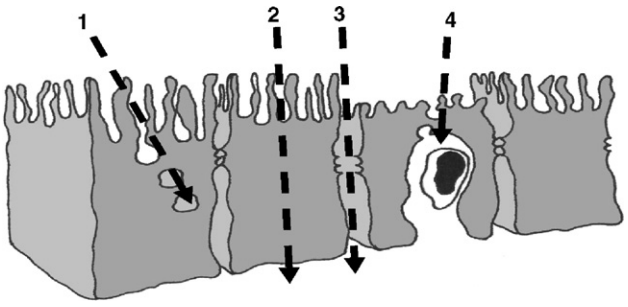

Fig. 2. Particle transport across epithelial cells. Particles can be transcytosed by normal enterocytes (1) as well as by M cells (4). They can reach the basal pole by passive diffusion (2) or by paracellular transport (3). Drawing realized by F. Mathot.

Enhancement of paracellular transport of drugs working with poly(acrylic acid)-based nanoparticles [100,104,105] was also studied. Peppas and co-workers have proven the ability of poly(methacrylic acids) grafted with poly(ethylene glycol) (P(MMA-g-EG)) microparticles (25–212 μm) to improve the transport of proteins such as insulin [106,107] or calcitonin [108] through Caco-2 cell monolayers. The system binds calcium ions, disrupting tight junctions and facilitates paracellular transport [109,110]. Water absorption by a dry and swellable poly(acrylic acids) polymer results in cell dehydration and shrinking and expansion of the spaces between the cells [111].

Thiolated polymers, or thiomers, are another recently introduced category of permeation enhancers that may potentially increase paracellular transport of a variety of drug compounds. Thiomer-based carrier matrix has been proven effective in the transmucosal delivery of protein and polypeptides. Thus, a new enhancer consisting of a thiolated polymer (poly(acrylic acid)-cystein, chitosan-4-thio-butylamidine) and reduced glutathione (GSH) has been shown to increase the paracellular transport of calcitonin, insulin, and heparin across rat intestinal epithelium *in vivo* and freshly excised guinea pig intestinal mucosa *ex vivo* [112]. The permeation-enhancing effect of this system has been attributed to inhibition of protein tyrosine phosphatase (PTP) causing expansion of tight junctions [112].

However, these polymers acting on tight junction indiscriminately allow all content of the intestinal tract, including toxins and biological pathogens, the same access to the systemic bloodstream [91].

Finally, the possible role of dendritic cells (DC) on paracellular transport of bacteria and antigens has been explored [113]. Indeed, *in vitro* experiments [114,115] and *in vivo* observations [116] have shown that DC can open tight junctions between intestinal cells and send their cellular processes into the lumen where they can directly internalize bacteria. Expression of tight junction proteins, such as claudin-1, occludin and *Zonula occludens*-1, enables DC to maintain the integrity of the epithelial barrier while sending their dendrites into the gut lumen. This could be exploited to deliver drug or antigen-loaded nanoparticles, but although this mechanism has been demonstrated, its real relevance for nanoparticles remains to be fully determined.

It was also reported that some intestinal inflammatory diseases, such as the Crohn disease, could modify epithelium permeability [116,117], and thus nanoparticles could be more easily transported. However, this review being restricted to applications in the scope of normal physiological state, oral delivery of drug and vaccines in this particular situation will not be developed.

In conclusion, paracellular transport can be enhanced by some polymers in solution or in the form of nanoparticles, but not without a certain risk of toxicity.

3.2. Transcellular transport

Transcellular transport of nanoparticles occurs by transcytosis, a particular process by which particles are taken up by

cells. This begins with an endocytic process that takes place at the cell apical membrane. Then, particles are transported through the cells and released at the basolateral pole [92].

In the scope of oral drug delivery applications, two types of intestinal cells are concerned: enterocytes, representing the majority of cells lining the gastrointestinal tract, and M cells, mainly located in Peyer's patches, which represent a very small proportion of the intestinal epithelium (5% of the human FAE [118] *i.e. ca.* 1% of the total intestinal surface). Although there has been some controversy in the literature on the extent of particle absorption, there is evidence that particle translocation can occur across enterocytes in the villus part of the intestine [119,120]. However, because of the low endocytic activity of enterocytes, the amount of particles translocated via these routes is usually very low. It is generally believed that the bulk of particle translocation occurs in the FAE [77,121–123]. Consequently, the attention given to Peyer's patches is not surprising, considering that M cells have adapted to absorb a large range of materials.

Transport of nanoparticles by the transcellular pathway depend on several factors: (i) the physicochemical properties of particles, such as size, zeta potential, surface hydrophobicity or the presence of a ligand at the particle surface; (ii) the physiology of the GI tract; (iii) the animal model used to study the uptake [124].

It is generally agreed that nanoparticle transcytosis increases when the particle diameter decreases [22]. Studies on polystyrene latex revealed that maximal number of absorbed nanoparticles occurred with particles ranging 50–100 nm in diameter, while particle above 1 μm being trapped in the Peyer's patches [77,125]. In addition, Lamprecht et al. [126] investigated the size-dependent bioadhesion of particulate carriers to the inflamed colonic mucosa of rats. The highest binding was found for 0.1 μm particles. For 10 μm particles, only fair deposition was observed while 1 μm particles showed higher binding. The percentage of particles associated with the mucus was then size-dependent. Likewise, it is known that charged

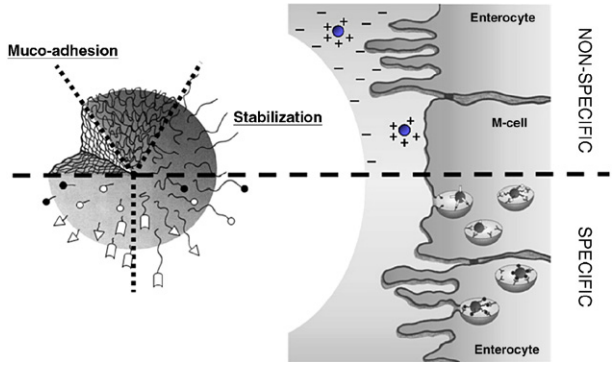

Fig. 3. Nanoparticle surface modifications to enhance their uptake by enterocytes and M cells. Non-specific strategies: mucoadhesive polymers coating or forming nanoparticles (chitosan, poly acrylic acids); PEG chains forming a hydrophilic protective layer stabilizing the colloidal formulation. Specific strategies: Enterocytes: WGA lectin, coupled or not, to a PEG chain (● or ●∿∿); M cells: lectins (○), bacterial adhesins (Δ), antibodies (⌷), coupled or not, to a PEG chain. Drawing realized by F. Mathot.

particles exhibit poor oral bioavailability (through electrostatic repulsion and mucus entrapment). Norris and Sinko [127] demonstrated an inverse relationship, increasing hydrophobicity leading to an increase in permeability through mucin while decreasing translocation across the cell interior [80]. A number of investigators have studied the effect of size, charge and hydrophobicity of a variety of nanoparticles, albeit with discordant results, reflecting the heterogeneity of experimental protocols and animal models involved in the study of oral delivery of nanoparticles. However, they all agreed on the crucial importance of particle size and nanoparticle surface properties.

As already mentioned, intestinal permeability can be influenced by the physiological state of the patient. It has been demonstrated, for instance, that bacterial invasion can induce an up-regulation of particle transport in the FAE covering Peyer's patches [128–130].

Finally, because of the high variability of intestinal cell properties between species, specifically for M cells, the question of the relevance of animal studies was raised. Indeed, how to extrapolate results obtained in rodents to human applications, knowing that their Peyer's patches are composed from 10% to 50% of M cells compared to 5% in men, or to extrapolate conclusions of M cell targeting studies performed in mice, knowing that M cell markers are species specific? However, despite the lack of clinical studies allowing a fit transposition of information gathered during the course of animal experiments, clever and successful strategies have been developed to enhance non-specific as well as specific uptake of nanoparticles. Strategies to enhance unspecifically or specifically the uptake of nanoparticles by enterocytes and M cells are schematized in Fig. 3 and are summarized in Table 2.

3.2.1. Non-specific uptake of nanoparticles

Two main strategies have been explored: (i) adapt polymer properties in order to optimize nanoparticle uptake by cells either M cells or enterocytes; (ii) attach ligands to polymer chains to enhance endocytosis or/and to specifically target cells (Table 2).

To enhance the transport of drug-loaded nanoparticles researchers have played on (i) mucoadhesion, (ii) particle endocytosis and (iii) permeation-enhancing effect. As already mentioned, several physicochemical parameters seem to influence translocation of particles across the epithelium, including surface hydrophobicity, polymer nature and particle size [131]. To ensure enteric protection and to improve bioavailability of peptides and proteins, diverse formulations have been developed, taking into account these parameters.

3.2.1.1. Uptake of nanoparticles by intestinal epithelial cells. The normal absorptive epithelium has the possibility to transcytose particles even though the capacity is considered to be limited. The transcellular transport of nanoparticles generally starts with the uptake by one of these endocytotic mechanisms: pinocytosis, macropinocytosis, or clathrin-mediated endocytosis [132]. All of these are active processes, *i.e.*, energy is required for the particle internalization. Both clathrin-

Table 2
Strategies developed to improve oral absorption of drug-loaded nanoparticles

Target	Parameter	Strategy to enhance transport	Polymer/molecule	Observations	Refs.
Non-specific					
Epithelial cells	Mucoadhesion	NP coated with mucoadhesive polymer/molecule	Polyacrylic acids	Increased NP concentration at the absorption site	[76,146,148,149]
			Chitosan		[63,65,159,164]
			PVA	PVA NP 2.9-fold higher transported than polystyrene NP	[161]
			Vit E TGSP	Vit E TGSP NP 1.4-fold higher transported compared to PVA NP	[86]
			Montmorillonite	Increased the cellular uptake of the PLGA nanoparticles by Caco-2 and HT-29 cells by 57–117% and 11–55%, respectively, in comparison with the PLGA nanoparticles, depending on the amount of MMT and the concentration of the nanoparticles.	[63]
	NP size	Optimal NP size		Optimum size 100–200 nm	[135]
	Hydrophobicity/ hydrophilicity	Better balance between NP surface hydrophobicity/ hydrophilicity		NP hydrophobic more transported than hydrophilic	[103]
		Coating NP surface with hydrophilic polymer	PEG	Good influence on NP bioavailability	[15,141,143,144]
				Greater stability	[65,146]
				Facilitation of NP transport	[65]
				Hydrophilicity increased affinity of NP for Caco-2 cells	[65,142]
	Surface charges	Stabilized NP by cationic DMAB	DMAB	Uptake of DMAB NP was significantly higher (70%, compared to 40 %) than negatively charged PVA NP	[161]
	Temperature			Active transport, inhibited at 4 °C	[86,135]
	NP concentration			Concentration-dependant uptake of NP by Caco-2 cells	[135]
M cells	Size	Optimal NP size		Optimal: 100–200 nm	[77,166]
				<1 μm	[125,165,172]
	Hydrophobicity/ hydrophilicity	Hydrophobic NP	PMMA, PHB, PLA, PLGA> ethyl cellulose or cellulose acetate	Hydrophobic NP more transported than hydrophilic NP	[92,139]
				Influence of NP composition	[139,170,171,174, 177–179,189]
	Surface charges			Charged NP<non ionic hydrophobic NP	[22]
				Negatively charged or neutral NP>positively charged NP	[173]
Specific					
Epithelial cells	Cell targeting	Lectin-coupled NP	WGA	Caco-2 cells and human enterocytes WGA-grafted NP increased by 12-fold their interaction with Caco-2 cells (Control=PEG-NP)	[20,72,74,165,172, 181,184,185]
			Concavalin A	Targeting and uptake of nanoparticles in a range of 50–500 nm	[72]
			LTB		
			TL	4–10 time higher affinity for pig gastric mucin	[184]
			AAL		
		Glucomannan-NP	Glucomannan	Improved stability in the gut and facilitate the NP interactions with mannose receptors of epithelial cells	[187]
M cells	Cell targeting	Lectin-coupled NP	UEA-1	UEA-1-NP bound to M cells 100-fold more than BSA-NP in mice Mouse specific, does not apply to humans	[173,268,269]
			AAL	Targeted M cells (excised murine PP and FAE *in vitro* model)	[191] [194]
			Sambucus nigra *Viscum album*	Selectively targeted the surface of human FAE (not M cell-specific)	[172,195,196]
			WGA	WGA also enhanced NP uptake by M cells, albeit at lower levels than UEA-1	[20,172]

Table 2 (*continued*)

Target	Parameter	Strategy to enhance transport	Polymer/molecule	Observations	Refs.
M cells	Cell targetting	Bacterial adhesins	*Yersinia* *Salmonella*	Adhesin-NP mimicked bioadhesive properties of bacteria (invasin target β1 integrins)	[172,270] [197]
		Peptides	RGD	Target β1 integrins RGD-NP 50-fold more transported by the human FAE model compared with non-coated nanoparticles, whereas no significant increase in transport was observed in the Caco-2 cells	[180,199,201, 203,204]
		Antibodies	IgA	IgA-NP instilled into mouse intestinal loops were taken up by M cells. Selectivity between adsorption and uptake and between the ability of different proteins to facilitate uptake.	[172,207]
		Toxin	B subunit of cholera toxin	Common to both human M cells and enterocytes; CTB-coated particles may confer M-cell selectivity	[268]

mediated endocytosis and phagocytosis are receptor-mediated processes. Clathrin-coated vesicles may internalize particles smaller than 150 nm [133] while during phagocytosis particulate matters up to several μm may be internalized [132]. Macropinocytosis is also an active, actin-dependent process which is in many ways similar to phagocytosis, but is not receptor-mediated [134]. Larger volumes of fluid-containing particles can be internalized by macropinocytosis in vesicles of varying sizes but smaller than 5 μm.

Desai et al. [135] found a size-, concentration-, and temperature-dependent uptake of PLGA nanoparticles (100 nm) by Caco-2 cells, which is compatible with an active internalization process. Win and Feng [86] demonstrated that the binding and uptake of polystyrene nanoparticles by Caco-2 cells were influenced by various parameters such as particle size, duration of incubation, particle concentration, and particle surface properties. They observed a 25–46% decrease of nanoparticle binding and uptake at 4 °C compared to 37 °C by Caco-2 cells, suggesting that nanoparticle binding and uptake by these cells could be an energy-dependant endocytic process. PLGA nanoparticle oral absorption was confirmed by *in vitro* studies in Caco-2 cells [86,136] and by *in vivo* studies in various animal models [136–138].

Several groups modified the surface of nanoparticles in order to improve their uptake. For a number of years, it has been widely accepted that nanoparticles with hydrophobic surface should be taken up more extensively by the intestinal epithelium than those with hydrophilic surface [139]. However, during the course of these studies, other factors, like colloidal stability, mucoadhesion… influencing nanoparticle uptake by intestinal cells have been brought out. Some recent results have suggested that the presence of hydrophilic polymers on the surface of nanoparticles could increase the transport of these systems through mucosal surfaces. For instance, PEG modified polyester nanoparticles were expected to be more stable, with a slower clearance [65,140]. Alonso's group [15,65,140,141] observed that a hydrophilic PEG coating of the nanoparticles had a very positive effect on macromolecule *in vivo* bioavailability. The PEG coating around PLA nanoparticles was designed to make these nanoparticles more stable upon contact with physiological fluids. PEG chains should hinder protein/enzyme adsorption, thereby protecting nanoparticles against degradation and enzyme induced aggregation [140]. As a result, encapsulation in PEG–PLA nanoparticles of the tetanus toxin has enhanced its transport across the intestinal barrier [65]. It is not well defined whether the improvement of drug bioavailability was due to a greater stability of the formulation or whether the presence of PEG could play a role in facilitating nanoparticle transport. Jung et al. [142], observed that the presence of hydrophilic polymers at PLGA nanoparticle surface enhanced affinity of nanoparticles for Caco-2 cells. This hypothesis was supported by Vila et al. [65], whose preliminary results suggested that transport of PEGylated nanoparticles through Caco-2 cells could be promoted by PEG coating. In contrast Behrens et al. [103] described almost no association of PLA–PEG nanoparticles with Caco-2 or goblet-like cells (MTX-E12) and a very low transport, which suggests therefore that PEG-chains could inhibit interactions with cell surface.

Enhancing mucoadhesion properties of nanoparticles can also be a strategy to increase nanoparticle uptake by intestinal enterocytes. Nanoparticles coated with bioadhesive materials were designed to develop mucoadhesion. Adhesion of a carrier system to the mucus may improve residence time and drug contact with the underlying epithelium, thus increasing drug concentration at the site of absorption [143,144]. Hydrophilic polymers such as polyacrylate derivatives and chitosan are examples of such mucoadhesive materials. Several groups demonstrated that coating nanoparticles with mucoadhesive poly(acrylic acids) improved the bioavailability of poorly absorbed drugs [76,143,145–152]. For instance, Sakuma et al. [153] reported an enhancement of oral absorption of salmon calcitonin following its association with poly(*N*-isopropylacrylamide) nanoparticles. Chitosan was also selected as a coating material for hydrophobic nanoparticles or as a component itself of nanosystems [154]. Many examples related the positive influence of chitosan-coating and chitosan-formed nanoparticles on their oral uptake. Formation of a mucoadhesive chitosan coating around PLGA nanoparticles has improved oral absorption of salmon calcitonin [155] and tetanus toxoid [65]. The increasing number of publications (20-fold, in 10 years) related

to chitosan particles underlines the high potential of chitosan to improve mucosal absorption of peptides and proteins [156]. Behrens et al. [103] described a higher cellular association of chitosan nanoparticles with Caco-2 cells, as well as with MTX-E12, compared with PEGylated nanoparticles. Therefore, this polymer could be a perfect candidate for peptide and protein mucosal delivery. It is not yet well defined how chitosan nanoparticles enhance oral uptake, but it has been proposed that either the whole nanoparticle would be able to cross the epithelium, or that chitosan molecules release the drug at the apical pole of epithelial cells, facilitating somehow their transcytosis. According to Behrens et al., the hypothesis assessing that uptake of chitosan nanoparticles was due to interaction of chitosan with tight junctions has been ruled out and it has been proposed that chitosan nanoparticles were most likely transported by adsorptive endocytosis, this process being saturable (30 min approximately) as well as energy and temperature-dependent [103].

It would be very interesting to compare the influence of PEG coating with chitosan coating of solid nanoparticles (PLA, PLGA) or lipids on oral bioavailability of encapsulated drug. Indeed, up to now, the optimal vehicle to efficiently orally deliver peptides remains to be identified. However it is difficult to find information allowing to conclude which coating is most efficient to promote oral drug delivery. Even so, after nasal administration of antigen-loaded PLA and PLGA nanoparticles, Vila et al. [65] observed a higher transport of chitosan- and PEG-coated PLGA nanoparticles compared to PLA nanoparticles, although PEG coating appeared to be more efficient than chitosan coating in promoting transport of the associated antigen. However chitosan coated lipid nanocapsules increased oral bioavailability of calcitonin whereas PEG coated lipid nanocapsule did not [157]. Association with Caco-2 monolayer was independent of surface coating but chitosan-coated particles caused a concentration dependent reduction in TEER. Changing core composition (liquid *vs.* solid lipid core), influenced uptake by Caco-2 cells but not calcitonin absorption *in vivo* [50]. This could be mainly ascribed to the mucoadhesive character of chitosan coated nanoparticles [158]. PLA–PEG and chitosan nanoparticle transport mechanisms could also differ: PEG–PLA nanoparticles are known to cross the intestinal barrier by a transcellular pathway while the mechanisms of interaction and transport of chitosan nanoparticles remain to be investigated. It seems then that the composition of the nanoparticle core is a key factor to the same extent that nanoparticle surface properties.

However, mucoadhesive nanoparticles have certain limitations. First, they may adhere unspecifically to surfaces they are not intended to (gastric mucosa, gut content, …) or remain trapped within the mucus and then not transcytosed by epithelial cells. Second, as they adhere to mucin, their residence time at the epithelial cell surface should be determined by the physiological turnover time of the mucus layer (50 to 270 min in rat) [159]. Prego et al. [160] developed a new type of nanocapsules composed of chitosan grafted with PEG. They succeeded in finding an equilibrium combining the advantageous properties of chitosan with those of PEG.

Other types of molecules, although less investigated, show a positive effect on drug-loaded nanoparticle oral bioavailability. Coating particles with polyvinyl alcohol (PVA), or vitamin E succinylated polyethylene glycol 1000 (Vitamin E TGSP) can greatly improve their adhesion and absorption by the intestinal cells. Vitamin E TGSP-coated PLGA nanoparticles were found to improve the uptake of nanoparticles 1.4-fold over that of PVA-coated PLGA nanoparticles that already enhanced the uptake by 2.9-fold over that of polystyrene nanoparticles of about the same size [86]. Intestinal uptake of PLGA nanoparticles stabilized by cationic didodecyldimethylammonium bromide (DMAB) was found to be significantly higher (70%, compared to 40%) than negatively charged PVA stabilized ones [161]. Montmorillonite [63], when absorbed at nanoparticle surface, greatly increased the cellular uptake efficiency of PLGA nanoparticles by Caco-2 and HT-29-M6 cells by 57–117% and 11–55%, respectively, in comparison with PLGA nanoparticles, depending on the amount of montmorillonite and the concentration of nanoparticles.

3.2.1.2. Uptake of nanoparticles by intestinal M cells. Nanoparticles administered orally can be absorbed by the numerous gut enterocytes, but also, by way of the membranous epithelial cells (M cells) of Peyer's patches in the gut-associated lymphoid tissue (GALT).

Particle transport by M cells is predominantly transcellular (Fig. 4) and energy-dependent [162]. Uptake of particles, microorganisms and macromolecules by M cells occurs through fluid phase endocytosis, adsorptive endocytosis and phagocytosis [124,163,164]. Factors influencing nanoparticles uptake by M cells are nanoparticle size, hydrophobicity/hydrophilicity balance and the presence of a targeting molecule at the nanoparticle surface. Species variability, age and immuno- and physiopathological state of laboratory animals, as well as a multitude of experimental conditions, are also to be considered when comparing different studies.

It is generally accepted that nanoparticles below 1 μm are taken up by M cells and delivered in the basal medium [165], while particles larger than 5 μm are taken up by M cells

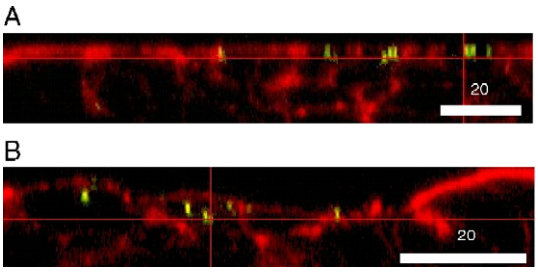

Fig. 4. Localization of nanoparticles in cell monolayers of an *in vitro* model of the human FAE. Cells were stained with rhodamine–phalloidine (red) and beads were yellow-green-labelled. Mono-(A) and co-cultures (B) were fixed and stained after incubation with yellow-green carboxylated 0.2 μm beads at 37 °C during 90 min. Mono-cultures were used as controls. Red lines indicate where, within the cell monolayers, the pictures were taken and analyzed. (Reprinted from Eur.J.Pharm.Sci., 25, des Rieux et al., Transport of nanoparticles across an in vitro model of the human intestinal follicle associated epithelium, 455–465, Copyright (2005), with permission from Elsevier). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

but remain entrapped in Peyer's patches [139]. Even if some controversy remains, the optimal size for a nanoparticle to be transcytosed by a M cell would be below 1 μm [88,125,165], and more precisely below 200 nm [77,166] (Fig. 5).

Together with nanoparticle size, surface hydrophobicity has a strong influence on nanoparticle uptake by M cells, as already observed for enterocytes. Nanoparticles composed of relatively hydrophobic polymers such as polystyrene, poly(methyl methacrylate), poly(hydroxybutyrate), PLA and PLGA are transported by M cells to a larger extent than those consisting of ethyl cellulose or cellulose acetate [92]. Overall, nanoparticle composition could affect not only the intensity but also the mechanism of transport. Indeed, mechanistic studies performed with different types of nanoparticles indicate that hydrophobic nanoparticles are preferentially transported through the GALT [139,166–168], whereas particles with a more hydrophilic nature are transported across normal enterocytes.

Particle interaction with M cell surface can also rely on the nanoparticle surface charge (zeta potential). Indeed, charged nanoparticles are taken up, but to a lower extent than non-ionic hydrophobic nanoparticles [22]. Shakweh et al. [169] described that nanoparticles of negative or neutral zeta potential were better transported by Peyer's patches, compared to positively charged nanoparticles. Thus, according to Jung et al. [142] the best combination favoring absorption through M cells, would be an hydrophobic, negatively charged nanoparticle.

However, since interactions between nanoparticles and M cells are very complex, it is difficult to draw general rules. Depending on animal model and experimental conditions, conclusions fluctuated a lot and could even be contradictory. Indeed, functional differences have arisen from interspecies comparisons. For example, 0.5 μm polystyrene nanoparticles, instilled into ligated gut loops of anaesthetized rabbits, were preferentially endocytosed by rabbit Peyer's patch M cells [170,171]. In contrast, PLGA nanoparticles adhered to rabbit Peyer's patch FAE in much lower amounts, although a high proportion of M cell bound nanoparticles were transcytosed [170]. Similar gut loop studies performed in anaesthetized mice demonstrated that 0.5 μm polystyrene nanoparticles, 3 μm PLA microparticles and 0.4 μm PLGA nanoparticles bound much less efficiently to mouse FAE than to rabbit FAE [172,173]. Despite their suboptimal binding to M cells, PLGA nanoparticles have been shown to be transported into Peyer's patch tissues in diverse species including rodents, rabbits, pigs and chicken [139,170,174–176] and have been shown to be effective vaccine delivery vehicles in animal models [177–179].

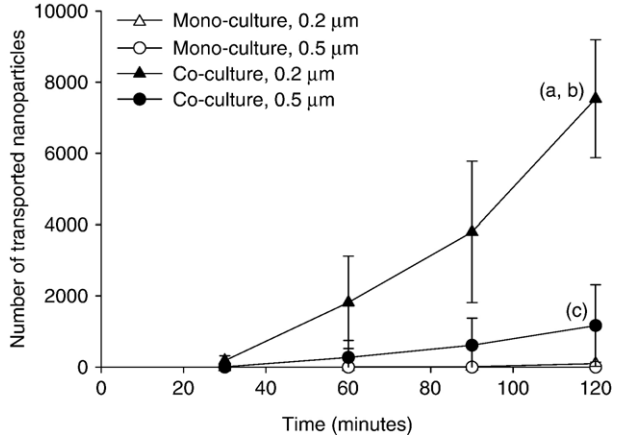

Fig. 5. Influence of particle size on nanoparticle transport [166]. Yellow-green carboxylated nanoparticles of different size (0.2 and 0.5 μm), suspended in buffered HBSS+1% FCS, were added to the apical pole of the cell monolayers and incubated for 90 min at 37 °C. The transport kinetics were compared ($n=4$). (a) $P<0.05$ versus mono-cultures incubated with 0.2 μm nanoparticles; (b) $P<0.05$ versus co-cultures incubated with 0.5 μm nanoparticles,; (c) $P<0.05$ versus mono-cultures incubated with 0.5 μm nanoparticles,. (Reprinted from Eur.J.Pharm.Sci., 25, des Rieux et al., Transport of nanoparticles across an in vitro model of the human intestinal follicle associated epithelium, 455–465, Copyright (2005), with permission from Elsevier).

Therefore, it seems important to find equilibrium between modifying nanoparticle surface to promote cell uptake and choosing an appropriate matrix to guarantee a good stability. Indeed, colloidal instability, leading to flocculation and aggregation, is a key issue given the importance of particle size. Similarly, chemical stability of nanoparticle matrix is vital for biodegradability and release of encapsulated drug [22].

Whether polymer properties (physical (surface charges) and chemical (hydrophobicity)) favor an unspecific uptake by either enterocytes or M cells remains controversial. The lack of comparative studies does not allow to conclude which polymer(s) would be the most efficient for nanoparticle oral delivery. It seems to be well accepted that the smaller the size, the better the uptake both by enterocytes and M cells. However, there is no clear tendency concerning whether or not hydrophobicity favors nanoparticle uptake. The multitude of parameters influencing nanoparticle uptake does not facilitate the establishment of general rules for enhancement of non-specific uptake of nanoparticles by enterocytes or M cells. Although different studies assessed the capability of enterocytes and M cells to transcytose polymeric particulate delivery systems, a high variability between different systems has often been observed and in many cases, the rate of transport remains at a very low level.

3.2.2. Uptake of targeted nanoparticles through the intestinal mucosa

Modifying nanoparticles by coupling a targeting molecule at their surface could represent a more efficient way to enhance oral uptake of nanoparticles. Indeed, to decorate particles by adsorption or covalent attachment of ligands interacting with surface receptors has been an obvious way to target to the epithelium, hoping that such interactions will lead to a greater uptake and delivery of nanoparticles [180].

There are two opposing strategies concerning nanoparticle targeting to the intestinal mucosa. Indeed, some groups work to enhance nanoparticle transport by targeting them to the much more numerous enterocytes while others try to improve transcytosis by M cells, looking for specific ligands.

3.2.2.1. Targeting of intestinal epithelial cells. The most popular approach is to modify the surface of nanoparticles with lectins. Different lectins with different binding specificities have been used. Wheat germ agglutinin (WGA) [72,74,181] from *Tricticum vulgare* specifically binds to *N*-acetyl-D-glucosamine

and sialic acid [182,183]. Concanavalin A (ConA) binds to α-D-mannose. LTB, the binding subunit of *E. coli* heat labile toxin, LT, binds to GM-1 ganglioside and galactose [72]. The tomato lectin (TL) is specific for *N*-acetyl-D-glucosamine, and *Aleuria aurantia* (AAL) lectin for L-fucose [184].WGA was described to bind not only to cell surface of Caco-2 cells and human enterocytes, but also to be taken up into cells by receptor-mediated endocytosis involving the epidermal growth factor receptor that is expressed at a considerable density on enterocytes [185,186]. To take advantage of the cytoadhesive and cytoinvasive properties of WGA, researchers coupled it to nanoparticle surface. WGA-grafted nanoparticles, comparatively to PEGylated ones, increased by 12-fold their interaction with Caco-2 cells [74], through an energy consuming transport process [181]. Nanoparticles coupled with WGA were not toxic towards Caco-2 cells [74,181]. LTB, ConA and WGA, coupled with polystyrene nanoparticles, were able to elicit targeting and nanoparticle uptake in a range size from 50 to 500 nm [72]. TL or AAL-conjugated PLA nanoparticles have demonstrated a 4–10 times higher affinity for pig gastric mucin. All in all, lectin-coupled nanoparticles increase interactions with mucus and Caco-2 cells, as well as stimulate *in vitro* nanoparticles uptake. The level of targeting and uptake was directly proportional to the amount of targeting agent attached to particles. Targeting is also a specific phenomenon as it is greatly reduced in presence of free lectin or specific sugar [72]. A chronic administration of these molecules for oral delivery application is, however, questionable, since all molecules studied so far are highly immunogenic can be cytotoxic or induce an inflammatory response and GI irritation. Besides, the association of these lectins with a delivery system would be contraindicated in persons with known food allergies. An alternative approach could be proposed to reduce this immunogenicity by identifying and coupling to nanoparticles smaller, less immunogenic fragments of lectins that maintain their binding activities [72].

Finally, Cuna et al. [187] produced a different type of nanoparticle made of chitosan in association with the polysaccharide glucomannan. The rationale for the design of this novel colloidal carrier was that glucomannan would improve the stability of nanoparticles in gastrointestinal fluids and also facilitate interactions of nanoparticles with mannose receptors present in the epithelial cells [188,189].

3.2.2.2. Targeting of intestinal M cells. Even if the advantageous properties of M cells for particle uptake are now well known and described, it is commonly accepted that their low proportion in the human GI tract (1% of the total intestinal surface), as well as their variability among species (proportion—from 5% to 50% of the FAE-, specific markers, …), individuals, physiological state, age … decrease the impact they could have on oral drug delivery. However, in the light of their abilities to take up particles, several groups considered worthwhile to work on the improvement of nanoparticle delivery through M cells, trying, especially, to compensate their low number by an efficient targeting. The main strategy is to decorate nanoparticle surface with an M cell targeting molecule. The task is not without difficulty, the lack of a human specific M cell marker being not the slightest.

Indeed, even if some distinct epitopes have been described for individual species, there is no broadly applicable conserved species-independent label [162]. One of the most used ligand is the *Ulex europaeus* agglutinin 1 (UEA-1) lectin, which is highly specific for α-L-fucose, located on the apical membrane of M cells of mice but not of humans. Foster et al. [190] observed that UEA-1 coated polystyrene nanoparticles (0.5 μm diameter) bound to M cells at a level 100-fold greater than BSA-coated nanoparticles, while binding to enterocytes remained unaffected. UEA-1 coating selectively targeted nanoparticles to mouse Peyer's patch M cells, and M cell adherent nanoparticles were rapidly endocytosed. Interestingly, incorporation of WGA also enhanced Peyer's patch uptake of delivery systems, albeit at lower levels than UEA-1 [165]. WGA is not specific to mouse M cells and has been shown to enhance particle uptake in cultured epithelial cells (see under Section 3.2.2.1). To date, no *in vivo* experiment has been realized studying the impact of UEA-1 lectin coupling on oral bioavailability of peptide or protein drug encapsulated in biodegradable nanoparticles.

Another lectin, AAL, could be used to target α-L-fucose receptors. AAL-coated polystyrene particles (2 μm) specifically targeted M cells of murine Peyer's patches, whereas nearly no particle could be observed in adjacent intestinal regions [191]. In addition, a significant increase in cellular uptake of Fluo-Spheres™ functionalized with AAL compared to non-functionalized ones was observed, in Caco-2 cells as well as in the FAE culture model [191]. As an advantage for vaccination, but probably as a drawback for drug delivery applications, AAL, although not toxic, is immunostimulatory.

To conclude with lectins, although studies to date have failed to identify lectins that selectively bind to human M cells, lectins derived from *Sambucus nigra* [173,192] and *Viscum album* [192] were described to selectively label the surface of human FAE, and therefore could be used as ligands to human drug delivery applications.

Another strategy to develop M cell targeting could be to mimic some pathogen bacteria, such as *Yersinia*, *Salmonella*, and *Shigella* species that are able to hijack the mucosal immune system, by using M cells to invade the intestinal mucosa. These bacteria present microbial adhesins at their surface, which are responsible for their binding and internalization by M cells. Salman et al. [193] combined *Salmonella* extract (SE) with polymeric nanoparticles (280 nm). They observed that, when orally administrated to rats, SE-nanoparticles were broadly distributed in Peyer's patches. Similarly, the ability of SE-nanoparticles to be taken up by Peyer's patches was negatively affected by the presence of the bacteria. Purified adhesins have also been used to target M cells. A *Yersinia* adhesin, named invasin, has been one of the most studied. In mouse Peyer's patches [194] and in an *in vitro* model of the human FAE [195,196], invasion of M cells by *Y. enterocolitica* was mediated, at least partly, by the interaction of invasin with cell surface β1 integrins. It was unexpected, since expression of β1 integrins is generally limited to the basolateral membranes of polarized epithelia [197]. But several authors [194–196,198] have reported that M cells could differentiate from enterocytes by the apical expression of β1 integrins (Fig. 6). Gullberg et al.

[199] demonstrated that the β1 integrin expression was higher in both human FAE [88] model and human ileal FAE than in Caco-2 cells and in villus epithelia. It has then been suggested that invasin may permit M cell targeting of mucosal delivery systems. Specifically, Hussain and Florence [200] demonstrated that coating polystyrene nanoparticles (0.5 μm) with invasin-C192 resulted in increased nanoparticle absorption across rat intestinal epithelium. Twenty four hours after a single oral dose, 13% of invasin-C192-coupled nanoparticles were found in the systemic circulation of rats compared to only 2% of control nanoparticle preparations. Histological examination revealed abundant invasin-C192-coupled nanoparticles in the serosal layer of the rat distal ileum. The uptake and transport of integrin-adherent nanoparticles coated with the RGD-peptide motif was studied. In the human FAE model, the number of transported RGD-coated nanoparticles (0.2 μm) increased 50-fold compared with non-coated nanoparticles, whereas no significant increase in transport was observed in the Caco-2 cells [201]. The RGD sequence is known to compete with the *Yersinia* invasin binding to β1 integrin [202], but it recognizes other integrin heterodimers as well [203]. These RGD-coated nanoparticles were readily transported across human FAE mounted in Ussing chambers. Besides, they underlined the role of another cell adhesion protein, CD9, whose expression is also several-folds increased in human FAE. They suggested a role for CD9 in the regulation of integrin activity and, hence, the capacity for antigen transport across the FAE. However, further studies are needed to clarify the function of the CD9-integrin interactions in the FAE.

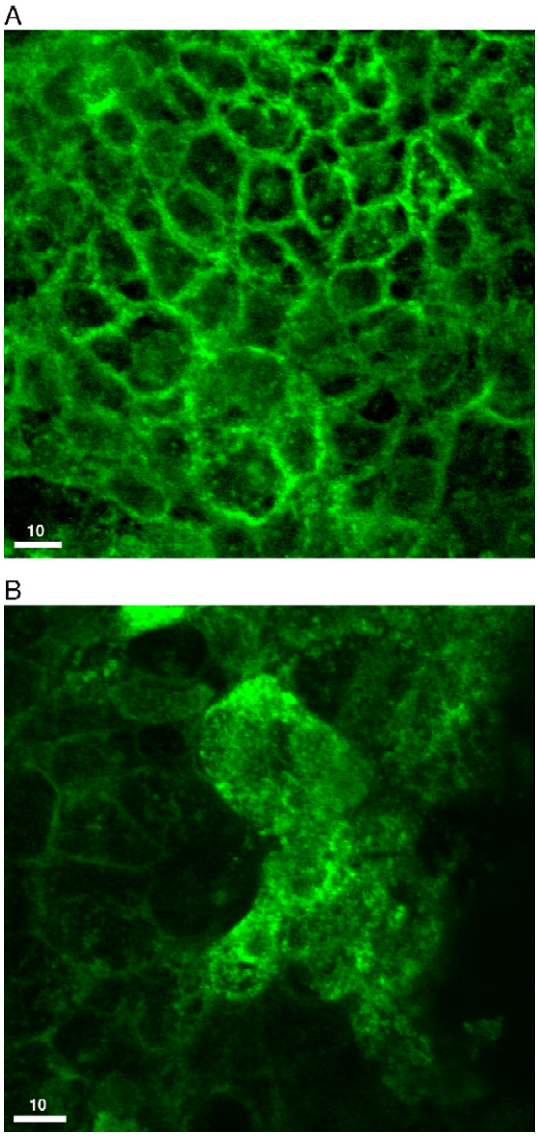

Fig. 6. Apical expression of β1 integrins of an *in vitro* model of the human FAE. Identification and localization of M cells by immunofluorescence staining of β1-integrins in mono-(A) and co-cultures (B), at the apical level, using confocal microscopy. (Reprinted from Eur.J.Pharm.Sci., 25, des Rieux et al., Transport of nanoparticles across an in vitro model of the human intestinal follicle associated epithelium, 455–465, Copyright (2005), with permission from Elsevier).

At last, other ligands possibly targeting M cells were also considered, although in a lesser extent. Immunoglobulins, particularly IgA, can specifically interact with M cell surface [204,205]. In order to demonstrate that the IgA-specific receptors present on the apical surface of M cells [206] could be useful for M cell targeting, uptake of IgA-coated polystyrene particles by M cells was evaluated in a mouse intestinal loop model [207]. Latex beads coated with IgA instilled into mouse intestinal loops were taken up to 20–30 times more readily than BSA-coated beads did. Coating beads with BSA causes a fourfold reduction in adsorption and a twenty fold reduction in uptake. Results demonstrate selectivity between adsorption and uptake and between the ability of different proteins to facilitate uptake.

Another apical membrane receptor was pursued as a target for drug-loaded particles: ganglioside GM1, the B subunit of cholera toxin (CTB) receptor. It is ubiquitous and displayed apically on most cells along the GI [208]. Since this receptor is common to human M cells and enterocytes, CTB-coated particles may confer M cell selectivity due to the thinner glycocalyx and reduced overlying mucous layer of M cells. Several other receptors were described in the literature and could certainly be investigated as new targets to enhance M cell targeting [162,208,209].

In summary, there is a growing interest to discover a specific marker of the apical pole of human M cells, or even better, a common set to different species of conserved apical membrane target proteins of FAE and M cells. Indeed, even representing a low proportion of intestinal cells, M cells present a real interest in drug and vaccine delivery, but only if it would be possible to concentrate nanoparticles at their surface. Already, encouraging results have been obtained for oral peptide and vaccine formulations.

4. Nanoparticles as potential oral delivery systems of peptides and vaccines

4.1. Oral delivery of therapeutic peptides and proteins

A number of studies, although not many, have demonstrated that nanoparticles can enhance the oral bioavailability of encapsulated therapeutic peptides and proteins.

Daily subcutaneous injections of insulin are a tedious treatment for patients with insulin-dependent diabetes. Considering the number of patients affected by this disease and the oral delivery route being really more convenient for chronic administration, a lot of efforts have been put to make possible oral delivery of insulin. Couvreur and co-workers developed insulin loaded poly(isobutylcyanoacrylate) nanocapsules that upon oral administration, gave results suggesting that nanocapsules could deliver insulin directly to the blood [210] and caused a dramatic reduction of blood glycaemia, following oral administration to diabetic rats (Table 3) [211]. Pinto-Alphandary et al. [76] have shown, based on TEM observations, that the same nanocapsules were absorbed by the intestinal epithelial cells, leading to the transport of insulin through the intestinal mucosa. However, nanocapsules were highly degraded upon transport across M cell containing epithelium. Nevertheless, even if impressive results were obtained, Cournarie et al. [212] underlined the high variability in insulin concentration crossing the intestinal barrier. Orally administrated nanosphere-based insulin delivery system (poly(fumaric anhydre)/poly(lactide-co-glycolide)) can maintain normoglycaemia in the face of a glucose challenge [91] and, chitosan–insulin nanoparticles which, upon oral administration of insulin doses of either 50 U or 100 U/kg, were effective at lowering the serum glucose level of streptozotocin-induced diabetic rats [96]. This effectiveness was attributed to the local effect of insulin in the intestine [213]. Finally, Alonso co-workers tested the efficacy of insulin-loaded chitosan–glucomannan nanoparticles following oral administration to normal rats. Interestingly, the carriers were able to elicit a delayed hypoglycemic response at 14 h post-administration, and this response was maintained for ≥ 10 h.

The enhancement of oral bioavailability of encapsulated insulin in nanoparticles and nanocapsules has been clearly demonstrated in animal studies. However, the question as why they were not followed by industrial development and clinical trials is still open. It seems that the high variability of the insulin concentration delivered to the blood, the still low bioavailability of encapsulated insulin requiring the administration of high doses, as well as the lack of information allowing to adapt these results to humans, represent heavy handicaps for the development of an oral formulation of insulin to treat the human insulin-dependent diabetes.

Table 3
Oral administration of therapeutic peptides and proteins encapsulated in nanoparticles

Molecule	Polymer	Size (nm)	Species	Observations	Refs.
Increased therapeutic peptide or protein bioavailability					
Insulin	Poly(isobutylcyanoacrylate)	300	Rat	Decreased of glycaemia from 300 mg/dl to a normal level of 125 mg/dl	[210,211]
Insulin Humalog®	Poly(isobutylcyanoacrylate)	400	Rat	High variability in the concentration of insulin crossing the intestinal barrier, and the absence of modification of the glycaemia	[212]
Insulin	Poly(fumaricanhydre)/poly (lactide co-glycolide)	<1 μm	Rat	Maintained normoglycaemia in the face of a glucose challenge	[91]
Insulin	Chitosan	270–340	Rat	Insulin doses of 50 U/kg and/or 100 U/kg were effective at lowering the glycaemia of diabetic rats	[96]
Insulin	Chitosan-glucomannan		Rat	Delayed hypoglycemic response at 14h post administration maintained for >10 h.	[271]
Calcitonin	Chitosan-coated PLGA	200–300	Rat	Reduced blood calcium over 12 h after administration of the NP at doses of 125 IU/Kg and 250 IU/Kg.	[155]
Calcitonin	PEG-chitosan	160–250	Rat	Chitosan–PEG nanocapsules enhanced and prolonged the intestinal absorption of salmon calcitonin. 0.5 % PEG-CS >1% PEG-CS >CS coating >PEG coating=lipidic nanoparticles	[160]
Cyclosporin A	Chitosan HCL	100–150	Beagle dog	1.8-fold increase of AUC when compared with Neoral® microemulsion.	[216]
Cyclosporin A	Poly(methacrylic acid and methacrylate) copolymers: Eudragit® E100, L100, L100-55 & S100	37–107	Rat	Bioavailability increased (32.5% to 13.6%) compared with the Neoral microemulsion	[272]
Dalargin	Tween 80 and PEG 20 000 coated poly (butylcyanoacrylate) (PBCA-NDSs)	100	Mouse	Analgesia with double-coated PBCA-NDSs compared to single-coated PBCA-NDSs (either Tween 80 or PEG)	[218]
mEpo DNA	Chitosan	70–150	Mouse	Rapid increase of hematocrite, sustained for a week	[217]
Treatment of local diseases					
AZT	Poly(isohexylcyanoacrylate	250	Rat	Nanospheres efficiently concentrated AZT in Peyer's patches-specifically, 4 fold greater than the solution.	[226]
HIV-1 protease inhibitors (peptidomimetic)	PLA	300	Mice	Plasma concentrations lowered and fluctuated over time	[224]
	Eudragit® L100-55, L100 and S100	250	Mice	Eudragit® L100-55 nanoparticles provided plasma concentrations that were 10–100-fold higher than the in vitro IC90 for approximately 8 h. Using Eudragit® S100 nanoparticles, the plasma concentrations decreased rapidly after 2.5 h.	

Moreover high doses of insulin will be required due to its low bioavailability.

The improvement of oral bioavailability of several other therapeutic peptides by encapsulation in polymeric nanoparticles was also studied. For instance, nanoparticles have been shown to enhance the absorption of calcitonin. When calcitonin was incorporated in nanoparticles, oral absorption was enhanced in rats and consequently calcium concentration in blood was decreased compared to oral administration of a calcitonin solution [142,214]. Takeuchi et al. [215] evaluated the effectiveness of mucoadhesive polymeric nanospheres in the absorption of calcitonin, developing Elcatonin-loaded PLGA nanospheres coated with chitosan. The reduction of blood calcium was significantly prolonged over 12 h after administration of chitosan-coated nanospheres at doses of 125 IU/Kg and 250 IU/Kg. Prego et al. [50] confirmed these results observing an enhanced hypocalcemic response, compared to a salmon calcitonin solution.

Chitosan nanoparticles were also explored for their efficacy to increase systemic absorption of hydrophobic peptides such as ciclosporin A [216]. Chitosan nanoparticles administered orally to beagle dogs provided an improved absorption compared to the currently available ciclosporin A microemulsion (Neoral®). Finally, chitosan was also able to successfully encapsulate a mEpo gene (coding for Erythropoietin) and protect it from DNase degradation. In addition, a rapid increase of hematocrite, sustained for a week, was observed when mEpo-loaded chitosan nanoparticles (70–150 nm) were administrated orally to mice [217]. Then, oral chitosan–DNA nanoparticles can efficiently deliver genes to enterocytes and may be used as a useful tool for gene transfer.

Das and Lin [218] developed double-coated (Tween 80 and PEG 20000) poly(butylcyanoacrylate) nanoparticulate delivery systems (PBCA) for oral delivery and brain targeting of dalargin. Even if they did not elucidate the mechanisms of PBCA nanoparticle transport from gastrointestinal tract to brain, they observed a significant dalargin-induced analgesia with double-coated PBCA nanoparticles compared to single-coated PBCA nanoparticles (either Tween 80 or PEG). Earlier studies described similar approaches to protect and orally deliver ciclosporin A [219–221], LHRH [222], vasopressin [147], INF [223] and peptidomimetics [224].

The lack of comparative studies makes it difficult to determine the most efficient formulations to orally deliver therapeutic peptides or proteins in the systemic circulation. However, studies performed mainly by Alonso's group on calcitonin delivery in rat allow some useful comparison between different carriers in same conditions. They have concluded that the core of nanocapsules (liquid *vs.* solid lipid) had no effect on oral bioavailability of calcitonin. However, the coating of the lipid nanoparticles had an influence. Prego et al. [160] demonstrated the ability of chitosan coated nanoparticles to decrease serum calcitonin levels [50] by about 27%. However due to cell toxicity (30%), they developed and tested a new type of nanocapsules composed of chitosan chemically modified with PEG (0.5% and 1% PEGylation degree) [160]. The two formulations elicited a significant reduction of serum calcium levels compared to salmon calcitonin solution (20%), but lower than the response induced by chitosan coated nanoparticles. The most important response was obtained in nanocapsules prepared with the lowest degree of substitution of chitosan (0.5%). They suggest that the presence of PEG improved the stability of nanocapsules in gastrointestinal fluids and reduced nanocapsule cytotoxicity. Their relative lower efficiency, compared to chitosan nanoparticles, could be explained by the partial shielding by PEG moieties of the positive charge of chitosan amino groups, thereby altering their interaction with the negatively charged mucosa, and reducing chitosan mucoadhesive properties. Therefore, by modulating the PEGylation degree of chitosan, it could be possible to find equilibrium between good stability, low cytotoxicity, and enhanced absorption properties. This new carrier seems then to be close to the ideal carrier combining an adequate size (160–250 nm), a good encapsulation efficiency (44–50%), stability and mucoadhesion, providing a good *in vivo* response.

However, as mentioned above, the projection of conclusions drawn from animal studies to human applications is not as straightforward as researchers would like and, unfortunately, quite often, the accuracy of the delivered dose, as well as the bioavailability of the formulation, remaining too low to reach therapeutic doses in patients, greatly limit therapeutic applications of these oral formulations of peptides. Nevertheless, such strategies could still remain interesting for highly potent drugs, with a low dose-response, as well as for local drug delivery. Indeed, in certain cases, instead of concentrating efforts to deliver peptides to the systemic circulation, perhaps more emphasis should be placed on delivery to the epithelial cells or to the lymphoid tissues themselves. For instance, since it was described that intestinal lymphoid tissues can be the reservoir of viruses such as HIV-1 [225], it could be worth of it to specifically target anti-viral loaded nanosystems to M cells, in order to induce a local action and to decrease viral stores in these tissues. Therefore, a formulation of AZT-loaded poly(isohexylcyanoacrylate) nanospheres has been developed and orally administered to rats. It was observed that nanospheres efficiently concentrated AZT in Peyer's patches specifically, 4 fold greater than the solution. In this regard, this finding supports the view that nanoparticles may represent a promising carrier for local treatment of the GI reservoir of HIV [226].

As a conclusion, the oral delivery strategies described above need to be further investigated and improved, as far as the systemic administration of therapeutic peptides is concerned, in order to reach a comparable efficiency and safety to which the parenteral route provides.

4.2. Oral delivery of vaccines

Peptide and protein encapsulation in polymeric nanocarriers has also been applied to oral vaccination applications. Compared to systemic administration, mucosal vaccination avoids pain and risks of infection associated with injections. In general, mucosal vaccination, and more particularly oral vaccination, led also to lower costs and could make large-population immunization more feasible. Vaccination at the site of infection is highly desirable to obtain a local mucosal defense at the entry pathways of pathogens. Indeed, mucosal delivery is the only

vaccination route that induces both mucosal and systemic immune responses, especially if the vaccine contains adjuvants, immunostimulators or carriers. Locally produced secretory IgA constitute over 80% of all antibodies produced in mucosa associated tissues [227,228] and are considered to be among the most important protective humoral immune factors [49]. They have been recognized for a long time as the first barrier against pathogen entrance [58]. Furthermore, a fascinating feature of mucosal immunology is that administration of antigen in one mucosal site can lead to generation of immune responses not only locally but also at distant mucosal sites, a phenomenon referred to common mucosal immune system. Finally, mucosal immunization has also the potential to elicit an immune response against infectious diseases for which current parenteral vaccines either have a low efficiency or are inexistent, such as vaccines against HIV and tuberculosis [229,230].

Induction of secretory immune response to ingested antigens is mainly achieved by the Gut Associated Lymphoid Tissues [121], organized into Peyer's patches. Oral immunity is induced after sampling, uptake of antigens from the intestinal lumen and their transport by M cells to lymphocytes, macrophages and dendritic cells enfolded in pockets in the basolateral surfaces of M cells [17].

In the scope of oral vaccination, it is particularly interesting to favor the uptake of antigen-loaded nanoparticles by M cells. There is a consensus that Peyer's patch M cells represent a key entry portal for some bacteria–viruses and prions, thus playing a key role in initiating the cascade of mucosal immunity [209]. Indeed, M cells deliver nanoparticles from the lumen directly to intraepithelial lymphoid cells and to O-MALT that are designed to process antigens and initiate immune responses. Particles are considered to be foreign bodies for the cells of the immune system, which implies that they are taken up by antigen presenting cells (APCs) and processed to elicit an immune response. Some polymers are even able to elicit an immune response by themselves when administrated orally, such as chitosan, which can enhance a naturally Th2/Th3-biased microenvironment at the mucosal level in absence of antigen [231]. Furthermore, nanoparticles can protect antigens from degradation and provide a depot-effect [232]. In addition, adjuvants can be co-encapsulated with the antigen to increase and direct the immune response.

4.2.1. Immunization following vaccine-loaded microparticle and nanoparticle oral administration

Based on data published by Eldridge et al. [174], asserting that microspheres smaller than 5 μm in diameter were transported by M cells through the efferent lymphatic macrophages, numerous microparticulate systems were developed to reach the exiting goal of oral immunization [190].

Among the vehicles developed during these last years, polymeric biodegradable microparticles, mainly composed of PLA or PLGA, have been probably the most extensively studied. A great number of proteins has been successfully encapsulated in PLGA microparticles with a full maintenance of structural and immunologic integrity [233–237]. Data obtained after oral immunization by microencapsulated antigens have recently been reviewed by Mutwiri et al. [238]. In general, ovalbumin, peptides, bacterial toxoids, inactivated bacteria and, more recently, DNA plasmids [239–243] entrapped in PLGA microparticles induced both mucosal and systemic immune responses following oral or intragastric administration. A possible denaturation of antigens during their encapsulation in PLA or PLGA polymers has to be pointed out, as a consequence of exposure to organic solvent, elevated temperature and aqueous–organic interfaces [92]. As alternatives to polyesters, microspheres made from a variety of others polymers have also showed their potential for oral vaccination. Mucosal immunity was achieved after oral administration of antigen-loaded microparticles composed of sodium alginate in mice, rabbits, chicken and poultry. Microparticles can be also composed of chitosan. However, as discussed above, particles in the nanoscale size rather than microscale size are more adapted for cellular uptake in the GI tract.

Recently, data have emerged showing that latex and PLGA particles in ~ 500 nm range may be taken up better than the generally regarded optimal size range of 1–5 μm [92]. Therefore, although there is no compelling evidence to clearly demonstrate that these nanoparticles are more effective that the traditionally evaluated larger microparticles in oral delivery of vaccines, more and more nanoparticulate systems are being developed to achieve oral delivery of vaccines, taking advantage of the better uptake of nanoscale carrier by M cells to induce a better mucosal immunization. Non targeted polymeric nanoparticles were first investigated.

Up to now, few studies have examined the capacity of biodegradable loaded-nanoparticles to induce an *in vivo* immune response after oral administration (Table 4). Jung et al. [241] used a poly(vinyl alcohol)-co-PLGA to reach a high level of tetanus toxoid loading by adsorption. Nanoparticles given *per os* to mice induced seric IgG and IgA immune responses, IgA titers being significantly higher than control (intra peritoneal administration). Particle size was found to significantly affect the induction of antibody production; smaller particles inducing higher titers. In addition, cholera toxin B subunit (CTB) entrapped in nanoparticles (420 nm) caused comparable immunogenicity than the potent oral adjuvant, cholera toxin [244]. The influence of the dose on serum IgG response after oral delivery of BSA at a dosing range from 50 to 200 μg, entrapped in 1 μm PLGA nanoparticles has elicited a systemic IgG dose/response relationship [245]. The influence of particle size on immune response after oral delivery of BSA entrapped in 200, 500 and 1000 nm PLGA particles have also been studied [246]. In contrast to results found in the literature over the more extensive intestinal absorption of smaller particles, a higher serum IgG antibody level and a similar IgG2a/IgG1 ratio have been observed working with 1 μm particles than with the 200 and 500 nm particles.

Kim et al. [242] administrated PLGA nanoparticles containing *Helicobacter pylori* lysates to stimulate *H. pylori*-specific mucosal and systemic immune responses and induce Th2-type responses in Balb/c mice. They observed that serum IgG and gut washing IgA responses were significantly higher than in control (immunized with soluble antigens alone). However, antibody

Table 4
Non-exhaustive list of immune responses by following oral administration of antigen-loaded polymer nanoparticles

Antigen	Polymer	Size	Species	Observations	Refs.
Tetanus toxoid (TT)	PVAL-PLGA	0,1; 0,5; 1,5	Mice	Circulating toxin-specific antibodies and a concurrent sIgA anti-toxoid response in saliva, gut-wash fluid and bronchial alveolar wash fluid detected. The induction of IgG and IgA antibody responses strongly influenced by the NP size distribution. Highest antibody titers found in case of 100 nm NP.	[241]
cholera toxin B subunit (CTB)	PLGA	0,42	Mice	The responses induced by microencapsulated CTB were comparable to those induced by oral immunization with CTB mixed with whole cholera toxin. Microencapsulated CTB induced Serum antibodies against intact CTB and not against monomeric fragments.	[244]
BSA (50 μg to 200 μg)	PLGA	1	Mice	Elicited a systemic IgG dose/response relationship.	[245]
BSA	PLGA	0,2; 0,5; 1	Mice	Higher serum IgG antibody levels and a similar IgG2a/IgG1 ratio with 1 μm particles than with the 200 and 500 nm particles.	[246]
Protective antigen from *B. pertussis*	PLGA	0,2–0,6	Mice	Three oral doses of antigen loaded particles elicited IgG serum and conferred a high level of protection against *B. pertussis* challenge. Induction of spleen cells (secretion of IL-5) and of antigen-specific IgA in the lungs.	[249]
Helicobacter pylori lysates	PLGA	0,32	Mice	Significantly *H. pylori*-specific mucosal IgA response as well as serum IgG responses (predominantly IgG1 and IgG2b).	[242]
DNA coding for a dominant peanut allergen	Chitosan	0,2	Mice	Transduced gene expression in the intestinal epithelium. Production of secretory IgA, serum IgG2a and substantial reduction in allergen-induced anaphylaxis (reduction of IgE, plasma histamine and vascular leakage).	[247]
Dermatophagoides pteronyssiums (Der p1)/plasmid DNA	Chitosan	0,5	Mice	Specific IgG2a and IgA antibodies in the systemic circulation. No specific IgA antibodies detected in the fecal extract and urine.	[273]
Toxoplasma grondii GRA1 protein/ DNA	Chitosan	0,4	Mice	Priming with GRA1 protein loaded chitosan NP and boosting with GRA1 pDNA vaccine resulted in high anti-GRA1 antibodies (mixed IgG2a/IgG1 ratio). The type of immune response largely depend on the prime/boost regimen and the type of vaccine used.	[253]
Rotavirus antigen VP6 nd VP7 DNA	PLGA	0,5	Mice	Intestinal IgA antibodies protected mice against rotavirus challenge.	[254,255]

titers of the group immunized with *H. pylori*-loaded PLGA nanoparticles were lower than those measured in the group immunized with the free *H. pylori* protein associated with the cholera toxin. These results suggested that *H. pylori*-loaded PLGA nanoparticles might be a safer adjuvant than cholera toxin.

However, as promising as these results look, to date there are very few commercially available oral vaccines and, despite numerous articles demonstrating the success of biodegradable antigen carriers, none of them has achieved commercial status. There is no appraisal of the quantity of antigen loaded particles that are required to be taken up by M cells to induce a significant immune response. Up to now, numerous efforts have been made to target nanoparticles to enterocytes or M cells (as described under Section 3.2.2) but few *in vivo* immunization studies have been carried on. Then their efficiency to induce an immune response after an oral delivery was not assessed *in vivo*. Immunization studies will be then required to assess the real capabilities of these systems to induce immunological responses after oral administration. A preliminary study demonstrated that a protein antigen could be transcytosed by M-like cells in a form that could be processed and presented to underlying T lymphocytes, thus inducing immunological response [242,247]. AAL coated PLGA particles, encapsulating birch pollen antigens, have been administrated to mice as a potential oral immunotherapy against allergens [248]. Allergy is a TH2-dominated immune response with increased production of IgG1 and IgE antibodies. The authors hypothesized that it could be possible to counterbalance this phenomenon, e.g. through a support of a TH1-response, where IgG2 antibodies are predominant. The type of immune response induced in mice fed with birch pollen-loaded microparticles coated with AAL was compared to the one elicited by non-coated microparticles. It appeared that feeding mice with functionalized (AAL) birch pollen-loaded microparticles resulted in a significant production of IgG2a antibodies (TH1 response), in the contrary of non-functionalized microspheres, but no IgG1 antibodies have been detected. Then, AAL-functionalized particles allow to accumulate antigens at the desired mucosal site after administration and to achieve a specific TH1 antibody response. This allergen delivery system may thus be suitable for oral immunotherapy of TH2-dominated allergies.

Despite numerous studies providing evidence that oral delivery of encapsulated antigens can efficiently elicit immune responses, up to now, less studies report a protection induced by antigen loaded particles administrated by the oral route against a challenge with the pathogen (highlight in tables) [190]. Fattal et al. [234] achieved the protection of mice against *Salmonella typhimurium* following oral administration of *S. typhimurium* phosphorylcholine antigen encapsulated in PLGA particles. Conway et al. [249] have investigated the immunogenicity and protective efficacy of orally delivered *Bordetella pertussis* antigen entrapped in either PLGA microparticles or nanoparticles against a murine respiratory challenge model. Orally administered encapsulated antigens elicited not only mucosal responses but also systemic responses, leading to a protection against *B.*

pertussis. Specific IgA and IgG responses have been induced by oral immunization with the encapsulated antigen. The demonstration that a single oral dose of encapsulated *B. pertussis* fimbria can confer protection is a testimony that, for certain antigens, oral vaccination is very promising, even if up to now, no study has reported a protective immunity induced in humans by PLGA-entrapped antigens.

Although M cell particle uptake has been repeatedly demonstrated *in vivo* in rodents and rabbits, it remains uncertain as to whether this is the case in man [209]. Only two rather disappointing oral vaccine Phase 1 human trials using PLGA-entrapped enterotoxigenic *Escherichia coli* [250] antigens have been published in the last 10 years [250,251]. Tacket et al. [251] used orally administrated *E. coli* colonization factor antigen Π (CFA Π) entrapped in PLGA microspheres to study human immune responses after their oral administration. However, in the 10 volunteers examined, vaccine efficacy was only about 30%. A more recent study [250] reported a significant increase of IgA, IgG and antibody secreting cells in human volunteers, following orally administered CS6 (a common *E. coli* CFA) entrapped in PLGA microspheres. Although, in this study, only 4–5 volunteers per group were tested (in a nonplacebo controlled trial), the authors demonstrated that all the formulations of the CS6 vaccine were safe and well tolerated. All the formulations elicited an immune response (at least one response in each group), which was similar in magnitude to the response induced by the pathogenic strain of enterotoxigenic *E. coli* expressing CS6 and by a transcutaneous administration of CS6.

4.2.2. DNA encapsulation for oral vaccination applications

DNA plasmids can be encapsulated in nanoparticles with significant retention of biological function, and an oral dose of encapsulated DNA can elicit systemic and mucosal antibody responses to the encoded protein [12].

Oral administration of chitosan nanoparticles (200 nm) complexed with DNA coding for a dominant peanut allergen elicited elevated secretory IgA and serum IgG2a titers [247]. Oral feeding of DNA-loaded chitosan nanoparticles can raise immune responses against native dust mite allergen Der p 1 in mice, whereas intramuscular immunization alone can not; this results confirmed those obtained by Wolfowicz et al. [252]. No humoral response in control mice, fed with naked DNA embedded in jelly was detected, suggesting that DNA protection by chitosan nanoparticles was crucial. Nanoparticles might also facilitate mucoadhesion and DNA uptake by the host cells, leading to enhanced transfection efficiency. Bivas-Benita et al. [247,253] compared the potential of chitosan nanoparticles (500 nm) loaded with *Toxoplasma gondii* GRA1 encoding DNA plasmid (pDNA) and chitosan microparticles loaded with recombinant GRA-1 protein to elicit GRA-1-specific immune responses after intragastric administration using different prime/boost regimen. Boosting with GRA1 DNA vaccine resulted in high anti-GRA1 antibody levels, characterized by a mixed IgG2a/IgG1 ratio. These results showed that oral delivery of vaccines using chitosan as a carrier efficiently induced an immune response against *T. gondii*. The type of immune response, however, will largely depend on the prime/boost regimen and the type of vaccine used. Chen et al. [254] have also employed PLGA nanoparticles (500 nm) to encapsulate a rotavirus VP6 DNA vaccine. Following oral administration to mice, a single immunization with the encapsulated vaccine was sufficient to elicit rotavirus-specific serum IgG, IgM, and IgA as well as intestinal IgA [254]. Moreover, after challenge with an homologous rotavirus, fecal rotavirus antigen was significantly reduced compared with controls. Similar results were obtained when VP4 and VP7 vaccines were encapsulated in PLGA nanoparticles and orally administrated to mice [255].

4.2.3. Antigen-loaded particles induce oral tolerance

Systemic unresponsiveness to orally delivered antigens (oral tolerance) may adversely affect oral vaccination or, conversely, could be used as a possible therapy in patients who hyper respond to antigens, or respond to normally innocuous environmental antigens (e.g., food allergens or commensal bacteria) [190]. Oral administration of free antigens has been for a long time recognized as a method to induce antigen-specific peripheral tolerance [256]. Oral tolerance, which has been expertly reviewed elsewhere [257,258], is mediated by two main mechanisms, depending on the dose of administrated antigens. Repeated administrations of low doses of antigen induce active suppression. The antigen-specific regulatory T-cells that mediate active suppression are triggered and secrete suppressive cytokines such as TGF-β, IL-4 or IL-10, a Th2/Th3 cytokine pattern [259] which have been shown to antagonize pro-inflammatory responses. In contrast, higher doses of antigen induce T cell clonal deletion and/or anergy, characterized by both antibody and cell-mediated immune response inhibition [260]. Many studies have demonstrated that oral administration of auto antigens led to active suppression in several autoimmune and transplantation experimental models (Table 5). These results suggest that inducing oral tolerance may be an efficient way to treat human inflammatory autoimmune diseases [259] such as allergic encephalomyelitis, rheumatoid arthritis (RA), experimental uveitis and diabetes (in the non-obese diabetic mouse) [256,260–262] or down-regulate alloreactivity associated with transplantation [259]. However several factors have been shown to affect the effectiveness of tolerance induction [263]: nature of antigen (soluble or particulate), antigen dose, genetic background and antigen uptake [264]. In particular, the effectiveness of oral tolerance induction is largely dependent on the presence in the circulation of an immunologically relevant antigen. Biodegradable microparticles, first promoted for vaccine development, now appear attractive to induce oral tolerance. In this view some authors have studied the possibility to induce oral tolerance after oral administration of antigen loaded-particles. In 2002, Kim et al. [264] working with an *in vivo* model of arthritis, have shown that a single administration of PLGA nanoparticles entrapping type Π collagen (CΠ) could induce oral tolerance more efficiently than repeated oral administrations of intact CΠ. Conjugation of polyethylene glycol (PEG) to a peptide could be an alternative method. Through PEG modification, proteins could be rendered not only non-immunogenic but also tolerogenic. The proportion of IL-4 and CD4 cells was greatly expanded in Peyer's patches of

Table 5
Non-exhaustive list of oral tolerance induced by oral administration of antigen-loaded polymer micro and nanoparticles

Antigen	Polymer	Size (μm)	Species	Observations	Refs.
Type Π collagen (CΠ)	PLGA	0,3	Rheumatoid arthritis mice	Single oral administration suppressed the development of arthritis, autoimmune responses to CΠ and induced TGFβ mRNA expression in PP.	[264]
PEG conjugated-epitope peptide CΠ	PLGA	~ 0,34	Rheumatoid arthritis mice	Single administration triggered the production of IL-4 and IL-10 in T cells of PP.	[263]
Â lactoglobulin (BLG)	PLGA	6,2–7,2	Mice	Reduced 10,000-times the amount of protein necessary to decrease both specific anti-BLG IgE and DTH response, compared with soluble BLG.	[234]
Ovalbumin	PLA	4; 7; 10	Mice	Efficiently induced oral tolerance. Size of the carrier-beads regulated the nature of immune response: an appropriate diameter induced mucosal tolerance (larger failed to induce an immune response and smaller could only systemically prime the mice).	[259]
Ovalbumin	PLA	4; 7; 18	Mice	Elicited INF-γ production, small increase of IgA and was as effective as 100 mg of soluble antigen to suppress Ova-specific IgG and DTH response. Smaller MS enhanced IgG titers and not secretory IgA antibodies.	[274]
Bich pollen allergen	PLGA	1–3	Mice	Higher production of IgG2a antibody but not of IgG1. In contrast, feedings with birch pollen protein alone induced IgG2a and IgG1, a typical antibody subclass of the Th2-type response in mice.	[275]

nanoparticles/PEG-pep2-fed mice, which was comparable to that in mice fed with 100 μg of CΠ peptides.

5. Conclusion

Currently, resulting from significant progresses in biotechnology, many protein or peptide-based drugs have been or are being developed. The oral route remains the preferred way to administrate them, but due to physicochemical and enzymatic barriers, they still have to be administered parenterally.

To improve patient's quality of life, many groups have been working on the development of peptide and protein oral formulations, based on the encapsulation of molecules in biodegradable and biocompatible polymeric nanoparticles. They benefited from three decades of research focused on parenteral drug release, leading to the development of numerous systems, allowing an efficient protection of the molecule as well as a controlled release. So, nowadays, there is almost no limitation concerning the possibilities to encapsulate a peptide or a protein, and researchers can find an adapted system for almost any molecule. Thus, numerous polymeric nanoparticle-based formulations have been developed for oral delivery applications. They all succeed in protecting the encapsulated peptide, protein or vaccine and their absorption through the intestinal mucosa has been demonstrated. All these formulations are able to deliver the molecule they contained. In addition, nanoparticles offer several advantages over other forms. When compared to single-unit preparations, multiparticulate systems such as nanoparticles distribute more uniformly in the GI tract, resulting in more uniform drug absorption and a reduced risk of local irritation [12]. In comparison with microspheres, the smaller size of nanoparticules is considered as the key parameter. Indeed microparticles larger than 10 μm do not penetrate the mucus layer [126]. Moreover nanoparticles are taken up better than microparticles by intestinal cells [84] and the uptake exclusion was evident for particles larger than 4 μm [12]. Finally, nanoparticles have shown to be more stable than liposomes in biological fluids [265] and, if endocytosis of intact liposomes by the intestinal cells occur at all, it remains a rare event [266], thus limiting the potential application of these carriers.

Numerous articles have been published up to now in literature about oral delivery of nanoparticulate systems, providing encouraging results, whether they focused on "normal" enterocytes or M cells. However, these observations are based on data obtained in different experimental conditions (different *in vitro* and *in vivo* models, different proteins, concentrations, polymers, nanoparticle size,…), which raise some questions. It is awkward to compare results from different studies, to draw general conclusions about the best characteristics a nanoparticle requires to be optimally endocytosed, and even more difficult, to transpose conclusions to human applications, above all concerning studies implying M cells. From that point of view, clinical studies are cruelly missing to really conclude on the optimal efficiency of the developed systems. However, some general conclusions can be still drawn about factors influencing the uptake of nanoparticles by intestinal cells. Nanoparticle size and nanoparticle surface properties are criteria of first importance. Almost all authors agreed on the optimal size (100–500 nm) but not on the optimal surface properties (charge, hydrophobicity, mucoadhesion, pegylation,…), the latter being extremely influenced by the polymer nature. Thus, the choice of the polymeric matrix and the development of new ones, better adapted to cell requirements, are crucial in the matter of therapeutic peptide and protein oral delivery.

Concerning the efficiency of these colloidal carriers, very encouraging results have been reached, but especially for drug delivery applications, the required administered dose to obtain a systemic action is often too high to be realistic. Then, we believe that coupling a targeting molecule at the nanoparticle surface, allowing to concentrate nanoparticles at their site of

uptake, is almost mandatory and consists in a very promising strategy, which has already produced really encouraging results, especially those dealing with M cells. Preliminary results of β1 integrin targeting could be considered as top candidate for the targeted delivery of mucosal systems to human FAE. Then, it can be reasonably assumed that, once a marker specific to human M cells would be identified, nanoparticle targeting to human M cells should give good results and that a successful oral delivery of therapeutic peptides and proteins, encapsulated in polymeric nanoparticles, is within reach. However, very few studies have demonstrated the ability of these targeted delivery systems to elicit *in vivo* enhanced pharmacologic or immunologic responses.

Speaking more precisely about peptide and protein delivery applications, some nanoparticulate systems seem to give encouraging results when tested *in vivo* but the still low bioavailability and the lack of control of the absorbed dose slow down the development of products destined to the human market. Indeed, the doses to administrate would remain very high. It is the reason why, as things stand as the moment, it is more realistic to address therapeutic peptide/protein local delivery or vaccine applications. For instance, local deliverance of anti-HIV drugs to the GALT structures, reservoir of HIV-1 virions, or of drugs treating gut inflammatory diseases, like antibodies against TNF α receptors, could be very interesting challenges. Considering that in certain pathologies intestinal absorption and permeability is reduced (HIV), studies on nanoparticle uptake in such conditions could be worthwhile.

Currently, vaccines are certainly the most promising applications for orally delivered nanoparticulate systems. Indeed, immunological stimulation does not require a dose as high as those required to obtain a pharmacologic effect, and control of time release profile could be less critical. Many research groups provided very interesting *in vitro* and *in vivo* results in this field, tempered by the low number of systems that have gone into clinical trials and the absence of vaccines delivered by nanoparticles on the market.

In our opinion, one strategy to increase the efficiency of orally delivered nanoparticulate vaccines would be to enhance the uptake of nanoparticles by appropriate cells, *i.e.* M cells. Two research axes are to be explored: (i) to model nanoparticle surface properties to optimize their access to and their transport by M cells, reaching the best compromise between surface hydrophobicity, charge, mucoadhesion, …, (ii) to identify surface markers specific to human M cell that may allow the targeting of nanoparticles to M cells, and thus increase the proportion of nanoparticles transcytosed to GALT immunologic cells.

Finally, new ways of antigen uptake have been described recently, especially by dendritic cells (DCs) in the intestine. Antigens can be captured by M cells of the FAE, and then taken up by sub-epithelial DCs. Alternative pathways rely on cells present in the villi: the recently described villous M cells [23] and DCs of the lamina propria sending dendrites through the epithelial layer to DCs sampling of antigen directly in the intestinal lumen. DCs then migrate to T-cell zones of Peyer's patches or to mesenteric lymph nodes to present antigens [267]. The question is do M cells and DCs sample the same antigens or do they sense the microbes in different ways [267]? However that may be, it could be worth to explore the possibilities DCs offer in matter of oral vaccination or tolerance [113].

In conclusion, we believe that in the future, the challenges will not be how to protect the drug anymore but, how to adapt delivery system properties to cell requirements and to deepen our understanding of particles uptake by intestinal cells, whichever they might be.

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References

[1] B. Steffansen, C.U. Nielsen, B. Brodin, A.H. Eriksson, R. Andersen, S. Frokjaer, Intestinal solute carriers: an overview of trends and strategies for improving oral drug absorption, Eur. J. Pharm. Sci. 21 (2004) 3–16.
[2] H.J. Lee, Protein drug oral delivery: the recent progress, Arch. Pharm. Res. 25 (2002) 572–584.
[3] U.B. Kompella, V.H. Lee, Delivery systems for penetration enhancement of peptide and protein drugs: design considerations, Adv. Drug Deliv. Rev. 46 (2001) 211–245.
[4] G.J. Russell-Jones, Use of vitamin B12 conjugates to deliver protein drugs by the oral route, Crit. Rev. Ther. Drug Carr. Syst. 15 (1998) 557–586.
[5] A. Fasano, Modulation of intestinal permeability: an innovative method of oral drug delivery for the treatment of inherited and acquired human diseases, Mol. Genet. Metab. 64 (1998) 12–18.
[6] J.P. Bai, L.L. Chang, J.H. Guo, Targeting of peptide and protein drugs to specific sites in the oral route, Crit. Rev. Ther. Drug Carr. Syst. 12 (1995) 339–371.
[7] J.A. Fix, Oral controlled release technology for peptides: status and future prospects, Pharm. Res. 13 (1996) 1760–1764.
[8] W. Wang, Oral protein drug delivery, J. Drug Target. 4 (1996) 195–232.
[9] L.M. Sanders, Drug delivery systems and routes of administration of peptide and protein drugs, Eur. J. Drug Metab. Pharmacokinet. 15 (1990) 95–102.
[10] J.H. Hamman, G.M. Enslin, A.F. Kotze, Oral delivery of peptide drugs: barriers and developments, BioDrugs 19 (2005) 165–177.
[11] C.M. Lehr, Bioadhesion technologies for the delivery of peptide and protein drugs to the gastrointestinal tract, Crit Rev. Ther. Drug Carrier Syst. 11 (1994) 119–160.
[12] S.A. Galindo-Rodriguez, E. Allemann, H. Fessi, E. Doelker, Polymeric nanoparticles for oral delivery of drugs and vaccines: a critical evaluation of in vivo studies, Crit. Rev. Ther. Drug Carr. Syst. 22 (2005) 419–464.
[13] P.J. Lowe, C.S. Temple, Calcitonin and insulin in isobutylcyanoacrylate nanocapsules: protection against proteases and effect on intestinal absorption in rats, J. Pharm. Pharmacol. 46 (1994) 547–552.
[14] E. Mathiowitz, J.S. Jacob, Y.S. Jong, G.P. Carino, D.E. Chickering, P. Chaturvedi, C.A. Santos, K. Vijayaraghavan, S. Montgomery, M. Bassett, C. Morrell, Biologically erodable microspheres as potential oral drug delivery systems, Nature 386 (1997) 410–414.
[15] M. Tobio, R. Gref, A. Sanchez, R. Langer, M.J. Alonso, Stealth PLA–PEG nanoparticles as protein carriers for nasal administration, Pharm. Res. 15 (1998) 270–275.
[16] J. Mestecky, M.E. Lamm, W. Stroder, J. Bienenstock, J.R. McGhee, E.L. Mayer, Mucosal Immunity (2005).
[17] A. Gebert, H.J. Rothkotter, R. Pabst, M cells in Peyer's patches of the intestine, Int. Rev. Cytol. 167 (1996) 91–159.

[18] J.P. Kraehenbuhl, M.R. Neutra, Epithelial M cells: differentiation and function, Annu. Rev. Cell Dev. Biol. 16 (2000) 301–332.
[19] A. Frey, M.R. Neutra, Targeting of mucosal vaccines to Peyer's patch M cells, Behring-Inst.-Mitt. (1997) 376–389.
[20] M.A. Clark, B.H. Hirst, M.A. Jepson, Lectin-mediated mucosal delivery of drugs and microparticles, Adv. Drug Deliv. Rev. 43 (2000) 207–223.
[21] A. Buda, C. Sands, M.A. Jepson, Use of fluorescence imaging to investigate the structure and function of intestinal M cells, Adv. Drug Deliv. Rev. 57 (2005) 123–134.
[22] A.T. Florence, Nanoparticle uptake by the oral route: fulfilling its potential? Drug Discov. Today. Technol. 2 (2005) 75–81.
[23] M.H. Jang, M.N. Kweon, K. Iwatani, M. Yamamoto, K. Terahara, C. Sasakawa, T. Suzuki, T. Nochi, Y. Yokota, P.D. Rennert, T. Hiroi, H. Tamagawa, H. Iijima, J. Kunisawa, Y. Yuki, H. Kiyono, Intestinal villous M cells: an antigen entry site in the mucosal epithelium, Proc. Natl. Acad. Sci. U. S. A. 101 (2004) 6110–6115.
[24] M.R. Neutra, N.J. Mantis, J.P. Kraehenbuhl, Collaboration of epithelial cells with organized mucosal lymphoid tissues, Nat. Immunol. 2 (2001) 1004–1009.
[25] L. Brannon-Peppas, Recent advances on the use of biodegradable microparticles and nanoparticles in controlled drug delivery, Int. J. Pharm. 116 (1995) 1–9.
[26] D. Lemoine, C. Francois, F. Kedzierewicz, V. Preat, M. Hoffman, P. Maincent, Stability study of nanoparticles of poly(epsilon-caprolactone), poly(DL-lactide) and poly(D,L-lactide-co-glycolide), Biomaterials 17 (1996) 2191–2197.
[27] K.S. Soppimath, T.M. Aminabhavi, A.R. Kulkarni, W.E. Rudzinski, Biodegradable polymeric nanoparticles as drug delivery devices, J. Control. Release 70 (2001) 1–20.
[28] P. Couvreur, G. Barratt, E. Fattal, P. Legrand, C. Vauthier, Nanocapsule technology: a review, Crit. Rev. Ther. Drug Carr. Syst. 19 (2002) 99–134.
[29] I. Montasser, S. Briancon, J. Lieto, H. Fessi, Methods of obtaining and formation mechanisms of polymer nanoparticles, J. Pharm. Belg. 55 (2000) 155–167.
[30] Z.H. Gan, D.H. Yu, Z.Y. Zhong, Q.Z. Liang, X.B. Jing, Enzymatic degradation of poly(epsilon-caprolactone)/poly(DL-lactide) blends in phosphate buffer solution, Polymer 40 (1999) 2859–2862.
[31] V.M. Leroueil-Le, L. Fluckiger, Y.I. Kim, M. Hoffman, P. Maincent, Preparation and characterization of nanoparticles containing an antihypertensive agent, Eur. J. Pharm. Biopharm. 46 (1998) 137–143.
[32] S. Balthasar, K. Michaelis, N. Dinauer, B.H. von, J. Kreuter, K. Langer, Preparation and characterisation of antibody modified gelatin nanoparticles as drug carrier system for uptake in lymphocytes, Biomaterials 26 (2005) 2723–2732.
[33] F.A. Johnson, D.Q. Craig, A.D. Mercer, Characterization of the block structure and molecular weight of sodium alginates, J. Pharm. Pharmacol. 49 (1997) 639–643.
[34] K. Dillen, J. Vandervoort, M.G. Van den, L. Verheyden, A. Ludwig, Factorial design, physicochemical characterisation and activity of ciprofloxacin-PLGA nanoparticles, Int. J. Pharm. 275 (2004) 171–187.
[35] C. Fonseca, S. Simoes, R. Gaspar, Paclitaxel-loaded PLGA nanoparticles: preparation, physicochemical characterization and in vitro anti-tumoral activity, J. Control. Release 83 (2002) 273–286.
[36] T. Govender, S. Stolnik, M.C. Garnett, L. Illum, S.S. Davis, PLGA nanoparticles prepared by nanoprecipitation: drug loading and release studies of a water soluble drug, J. Control. Release 57 (1999) 171–185.
[37] D. Lemoine, V. Preat, Polymeric nanoparticles as delivery system for influenza virus glycoproteins, J. Control. Release 54 (1998) 15–27.
[38] M.N. Ravi Kumar, U. Bakowsky, C.M. Lehr, Preparation and characterization of cationic PLGA nanospheres as DNA carriers, Biomaterials 25 (2004) 1771–1777.
[39] H.-J. Krause, A. Schwarz, P. Rohdewald, Polylactic acid nanoparticles, a colloidal drug delivery system for lipophilic drugs, Int. J. Pharm. 27 (1985) 145–155.
[40] E. Leo, B. Brina, F. Forni, M.A. Vandelli, In vitro evaluation of PLA nanoparticles containing a lipophilic drug in water-soluble or insoluble form, Int. J. Pharm. 278 (2004) 133–141.
[41] M.F. Zambaux, F. Bonneaux, R. Gref, E. Dellacherie, C. Vigneron, Preparation and characterization of protein C-loaded PLA nanoparticles, J. Control. Release 60 (1999) 179–188.
[42] M.G. Anderson, J.D. Miller, Therapeutic effects of leuprorelin microspheres on endometriosis and uterine leiomyomata, Adv. Drug Deliv. Rev. 28 (1997) 139–155.
[43] R.S. Langer, N.A. Peppas, Present and future applications of biomaterials in controlled drug delivery systems, Biomaterials 2 (1981) 201–214.
[44] S.P. Ayalasomayajula, U.B. Kompella, Subconjunctivally administered celecoxib-PLGA microparticles sustain retinal drug levels and alleviate diabetes-induced oxidative stress in a rat model, Eur. J. Pharm. 511 (2005) 191–198.
[45] J.H. Kou, C. Emmett, P. Shen, S. Aswani, T. Iwamoto, F. Vaghefi, G. Cain, L. Sanders, Bioerosion and biocompatibility of poly(*d*,*l*-lactic-co-glycolic acid) implants in brain, J. Control. Release 43 (1997) 123–130.
[46] S.Y. Lee, J.H. Oh, J.C. Kim, Y.H. Kim, S.H. Kim, J.W. Choi, In vivo conjunctival reconstruction using modified PLGA grafts for decreased scar formation and contraction, Biomaterials 24 (2003) 5049–5059.
[47] T. Zhou, H. Lewis, R.E. Foster, S.P. Schwendeman, Development of a multiple-drug delivery implant for intraocular management of proliferative vitreoretinopathy, J. Control. Release 55 (1998) 281–295.
[48] K.A. Janes, M.P. Fresneau, A. Marazuela, A. Fabra, M.J. Alonso, Chitosan nanoparticles as delivery systems for doxorubicin, J. Control. Release 73 (2001) 255–267.
[49] I. van der Lubben, J.C. Verhoef, G. Borchard, H.E. Junginger, Chitosan for mucosal vaccination, Adv. Drug Deliv. Rev. 52 (2001) 139–144.
[50] C. Prego, M. Garcia, D. Torres, M.J. Alonso, Transmucosal macromolecular drug delivery, J. Control. Release 101 (2005) 151–162.
[51] A. Vila, A. Sanchez, K. Janes, I. Behrens, T. Kissel, J.L. Vila Jato, M.J. Alonso, Low molecular weight chitosan nanoparticles as new carriers for nasal vaccine delivery in mice, Eur. J. Pharm. Biopharm. 57 (2004) 123–131.
[52] H. Fessi, F. Puisieux, J.P. Devissaguet, N. Ammoury, S. Benita, Nanocapsule formation by interfacial polymer deposition following solvent displacement, Int. J. Pharm. 55 (1989) R1–R4.
[53] U. Bilati, E. Allemann, E. Doelker, Development of a nanoprecipitation method intended for the entrapment of hydrophilic drugs into nanoparticles, Eur. J. Pharm. Sci. 24 (2005) 67–75.
[54] R. Gurny, N.A. Peppas, D.D. Harrington, G.S. Banker, Development of biodegradable and injectable latices for controlled release of potent drugs, Drug Dev. Ind. Pharm. 7 (1981) 1–25.
[55] J.C. Leroux, E. Alleman, E. Doelker, R. Gurny, New approach for the preparation of nanoparticles by an emulsion–diffusion method, Eur. J. Pharm. Biopharm. 41 (1995) 14–18.
[56] H. Ibrahim, C. Bindschaedler, E. Doelker, P. Buri, R. Gurny, Aqueous nanodispersions prepared by a salting-out process, Int. J. Pharm. 87 (1992) 239–246.
[57] A.M. De Campos, A. Sanchez, M.J. Alonso, Chitosan nanoparticles: a new vehicle for the improvement of the delivery of drugs to the ocular surface. Application to cyclosporin A, Int. J. Pharm. 224 (2001) 159–168.
[58] O. Borges, G. Borchard, J.C. Verhoef, S.A. de, H.E. Junginger, Preparation of coated nanoparticles for a new mucosal vaccine delivery system, Int. J. Pharm. 299 (2005) 155–166.
[59] E. Leo, M.A. Vandelli, R. Cameroni, F. Forni, Doxorubicin-loaded gelatin nanoparticles stabilized by glutaraldehyde: involvement of the drug in the cross-linking process, Int. J. Pharm. 155 (1997) 75–82.
[60] H. Jeffery, S.S. Davis, D.T. O'Hagan, The preparation and characterisation of poly(lactide-co-glycolide) microparticles. I: Oil-in-water emulsion solvent evaporation, Int. J. Pharm. 77 (1991) 169–175.
[61] M.F. Zambaux, F. Bonneaux, R. Gref, P. Maincent, E. Dellacherie, M.J. Alonso, P. Labrude, C. Vigneron, Influence of experimental parameters on the characteristics of poly(lactic acid) nanoparticles prepared by a double emulsion method, J. Control. Release 50 (1998) 31–40.
[62] K. Yoncheva, E. Lizarraga, J.M. Irache, Pegylated nanoparticles based on poly(methyl vinyl ether-co-maleic anhydride): preparation and evaluation of their bioadhesive properties, Eur. J. Pharm. Sci. 24 (2005) 411–419.

[63] Y. Dong, S.S. Feng, Poly(*d*,*l*-lactide-co-glycolide)/montmorillonite nanoparticles for oral delivery of anticancer drugs, Biomaterials 26 (2005) 6068–6076.
[64] H. Takeuchi, J. Thongborisute, Y. Matsui, H. Sugihara, H. Yamamoto, Y. Kawashima, Novel mucoadhesion tests for polymers and polymer-coated particles to design optimal mucoadhesive drug delivery systems, Adv. Drug Deliv. Rev. 57 (2005) 1583–1594.
[65] A. Vila, A. Sanchez, M. Tobio, P. Calvo, M.J. Alonso, Design of biodegradable particles for protein delivery, J. Control. Release 78 (2002) 15–24.
[66] P. Vangeyte, S. Gautier, R. Jerome, About the methods of preparation of poly(ethylene oxide)-b-poly([var epsilon]-caprolactone) nanoparticles in water: Analysis by dynamic light scattering, Colloids Surf., A Physicochem. Eng. Asp. 242 (2004) 203–211.
[67] J.S. Hrkach, M.T. Peracchia, A. Domb, N. Lotan, R. Langer, Nanotechnology for biomaterials engineering: structural characterization of amphiphilic polymeric nanoparticles by 1H NMR spectroscopy, Biomaterials 18 (1997) 27–30.
[68] I. Goldstein, R.C. Hughes, s.M. Mo, T. Osawa, N. Sharon, What should be called a lectin? Nature 285 (1980) 66.
[69] J.M. Irache, C. Durrer, D. Duchene, G. Ponchel, Preparation and characterization of lectin–latex conjugates for specific bioadhesion, Biomaterials 15 (1994) 899–904.
[70] J.M. Irache, C. Durrer, D. Duchene, G. Ponchel, Bioadhesion of lectin–latex conjugates to rat intestinal mucosa, Pharm. Res. 13 (1996) 1716–1719.
[71] C.M. Lehr, J.A. Bouwstra, W. Kok, A.B. Noach, A.G. De Boer, H.E. Junginger, Bioadhesion by means of specific binding of tomato lectin, Pharm. Res. 9 (1992) 547–553.
[72] G.J. Russell-Jones, H. Veitch, L. Arthur, Lectin-mediated transport of nanoparticles across Caco-2 and OK cells, Int. J. Pharm. 190 (1999) 165–174.
[73] W.J. Kim, J.W. Yockman, M. Lee, J.H. Jeong, Y.H. Kim, S.W. Kim, Soluble Flt-1 gene delivery using PEI-g-PEG-RGD conjugate for anti-angiogenesis, J. Control. Release 106 (2005) 224–234.
[74] R. Gref, P. Couvreur, G. Barratt, E. Mysiakine, Surface-engineered nanoparticles for multiple ligand coupling, Biomaterials 24 (2003) 4529–4537.
[75] N.W. Thomas, P.G. Jenkins, K.A. Howard, M.W. Smith, E.C. Lavelle, J. Holland, S.S. Davis, Particle uptake and translocation across epithelial membranes, J. Anat. 189 (Pt 3) (1996) 487–490.
[76] H. Pinto-Alphandary, M. Aboubakar, D. Jaillard, P. Couvreur, C. Vauthier, Visualization of insulin-loaded nanocapsules: in vitro and in vivo studies after oral administration to rats, Pharm. Res. 20 (2003) 1071–1084.
[77] P. Jani, G.W. Halbert, J. Langridge, A.T. Florence, Nanoparticle uptake by the rat gastrointestinal mucosa: quantitation and particle size dependency, J. Pharm. Pharmacol. 42 (1990) 821–826.
[78] A.T. Florence, A.M. Hillery, N. Hussain, P.U. Jani, Nanoparticles as carriers for oral peptide absorption: studies on particle uptake and fate, J. Control. Release 36 (1995) 39–46.
[79] F. Delie, Evaluation of nano- and microparticle uptake by the gastrointestinal tract, Adv. Drug Deliv. Rev. 34 (1998) 221–233.
[80] N. Hussain, V. Jaitley, A.T. Florence, Recent advances in the understanding of uptake of microparticulates across the gastrointestinal lymphatics, Adv. Drug Deliv. Rev. 50 (2001) 107–142.
[81] C. Jumarie, C. Malo, Caco-2 cells cultured in serum-free medium as a model for the study of enterocytic differentiation in vitro, J. Cell. Physiol. 149 (1991) 24–33.
[82] Z. Zhang, S.S. Feng, The drug encapsulation efficiency, in vitro drug release, cellular uptake and cytotoxicity of paclitaxel-loaded poly(lactide)-tocopheryl polyethylene glycol succinate nanoparticles, Biomaterials (2006).
[83] P. Pietzonka, B. Rothen-Rutishauser, P. Langguth, H. Wunderli-Allenspach, E. Walter, H.P. Merkle, Transfer of lipophilic markers from PLGA and polystyrene nanoparticles to Caco-2 monolayers mimics particle uptake, Pharm. Res. 19 (2002) 595–601.
[84] S. McClean, E. Prosser, E. Meehan, D. O'Malley, N. Clarke, Z. Ramtoola, D. Brayden, Binding and uptake of biodegradable poly-lactide micro- and nanoparticles in intestinal epithelia, Eur. J. Pharm. Sci. 6 (1998) 153–163.
[85] A.M. Koch, F. Reynolds, H.P. Merkle, R. Weissleder, L. Josephson, Transport of surface-modified nanoparticles through cell monolayers, ChemBioChem 6 (2005) 337–345.
[86] K.Y. Win, S.S. Feng, Effects of particle size and surface coating on cellular uptake of polymeric nanoparticles for oral delivery of anticancer drugs, Biomaterials 26 (2005) 2713–2722.
[87] S. Kerneis, A. Bogdanova, J.P. Kraehenbuhl, E. Pringault, Conversion by Peyer's patch lymphocytes of human enterocytes into M cells that transport bacteria, Science 277 (1997) 949–952.
[88] E. Gullberg, M. Leonard, J. Karlsson, A.M. Hopkins, D. Brayden, A.W. Baird, P. Artursson, Expression of specific markers and particle transport in a new human intestinal M-cell model, Biochem. Biophys. Res. Commun. 279 (2000) 808–813.
[89] H.N. Nellans, (B) Mechanisms of peptide and protein absorption: (1) paracellular intestinal transport: modulation of absorption, Adv. Drug Deliv. Rev. 7 (1991) 339–364.
[90] N. Salamat-Miller, T.P. Johnston, Current strategies used to enhance the paracellular transport of therapeutic polypeptides across the intestinal epithelium, Int. J. Pharm. 294 (2005) 201–216.
[91] G.P. Carino, E. Mathiowitz, Oral insulin delivery, Adv. Drug Deliv. Rev. 35 (1999) 249–257.
[92] M. Shakweh, G. Ponchel, E. Fattal, Particle uptake by Peyer's patches: a pathway for drug and vaccine delivery, Expert. Opin. Drug Deliv. 1 (2004) 141–163.
[93] I. van der Lubben, J.C. Verhoef, G. Borchard, H.E. Junginger, Chitosan and its derivatives in mucosal drug and vaccine delivery, Eur. J. Pharm. Sci. 14 (2001) 201–207.
[94] J. Raiman, S. Tormalehto, K. Yritys, H.E. Junginger, J. Monkkonen, Effects of various absorption enhancers on transport of clodronate through Caco-2 cells, Int. J. Pharm. 261 (2003) 129–136.
[95] A.F. Kotze, B.J. de Leeuw, H.L. en, A.G. De Boer, J.C. Verhoef, H.E. Junginger, Chitosans for enhanced delivery of therapeutic peptides across intestinal epithelia: in vitro evaluation in Caco-2 cell monolayers, Int. J. Pharm. 159 (1997) 243–253.
[96] Z. Ma, T.M. Lim, L.Y. Lim, Pharmacological activity of peroral chitosan–insulin nanoparticles in diabetic rats, Int. J. Pharm. 293 (2005) 271–280.
[97] J.M. Smith, M. Dornish, E.J. Wood, Involvement of protein kinase C in chitosan glutamate-mediated tight junction disruption, Biomaterials 26 (2005) 3269–3276.
[98] V. Dodane, K.M. Amin, J.R. Merwin, Effect of chitosan on epithelial permeability and structure, Int. J. Pharm. 182 (1999) 21–32.
[99] J. Smith, E. Wood, M. Dornish, Effect of chitosan on epithelial cell tight junctions, Pharm. Res. 21 (2004) 43–49.
[100] N.G. Schipper, K.M. Varum, P. Artursson, Chitosans as absorption enhancers for poorly absorbable drugs. 1: influence of molecular weight and degree of acetylation on drug transport across human intestinal epithelial (Caco-2) cells, Pharm. Res. 13 (1996) 1686–1692.
[101] C. Prego, D. Torres, M.J. Alonso, The potential of chitosan for the oral administration of peptides, Expert. Opin. Drug Deliv. 2 (2005) 843–854.
[102] M. Torres-Lugo, M. Garcia, R. Record, N.A. Peppas, Physicochemical behavior and cytotoxic effects of p(methacrylic acid-g-ethylene glycol) nanospheres for oral delivery of proteins, J. Control. Release 80 (2002) 197–205.
[103] I. Behrens, A.I. Pena, M.J. Alonso, T. Kissel, Comparative uptake studies of bioadhesive and non-bioadhesive nanoparticles in human intestinal cell lines and rats: the effect of mucus on particle adsorption and transport, Pharm. Res. 19 (2002) 1185–1193.
[104] M.A. Hurni, A.B. Noach, M.C. Blom-Roosemalen, A.G. De Boer, J.F. Nagelkerke, D.D. Breimer, Permeability enhancement in Caco-2 cell monolayers by sodium salicylate and sodium taurodihydrofusidate: assessment of effect-reversibility and imaging of transepithelial transport routes by confocal laser scanning microscopy, J. Pharmacol. Exp. Ther. 267 (1993) 942–950.
[105] A. Fasano, S. Uzzau, Modulation of intestinal tight junctions by Zonula occludens toxin permits enteral administration of insulin and other macromolecules in an animal model, J. Clin. Invest. 99 (1997) 1158–1164.

[106] E. Scott Swenson, W.J. Curatolo, (C) Means to enhance penetration: (2) intestinal permeability enhancement for proteins, peptides and other polar drugs: mechanisms and potential toxicity, Adv. Drug Deliv. Rev. 8 (1992) 39–92.
[107] J.E. Lopez, N.A. Peppas, Cellular evaluation of insulin transmucosal delivery, J. Biomater. Sci., Polym. Ed. 15 (2004) 385–396.
[108] M. Torres-Lugo, N.A. Peppas, Transmucosal delivery systems for calcitonin: a review, Biomaterials 21 (2000) 1191–1196.
[109] F. Madsen, N.A. Peppas, Complexation graft copolymer networks: swelling properties, calcium binding and proteolytic enzyme inhibition, Biomaterials 20 (1999) 1701–1708.
[110] H.L. Luessen, C.-M. Lehr, C.-O. Rentel, A.B.J. Noach, A.G. De Boer, J.C. Verhoef, H.E. Junginger, Bioadhesive polymers for the peroral delivery of peptide drugs, J. Control. Release 29 (1994) 329–338.
[111] J. Haas, C.M. Lehr, Developments in the area of bioadhesive drug delivery systems, Expert Opin. Biol. Ther. 2 (2002) 287–298.
[112] A. Bernkop-Schnurch, C.E. Kast, D. Guggi, Permeation enhancing polymers in oral delivery of hydrophilic macromolecules: thiomer/GSH systems, J. Control. Release 93 (2003) 95–103.
[113] M. Rimoldi, M. Rescigno, Uptake and presentation of orally administered antigens, Vaccine 23 (2005) 1793–1796.
[114] M. Rescigno, Dendritic cells and the complexity of microbial infection, Trends Microbiol. 10 (2002) 425–461.
[115] M. Rimoldi, M. Chieppa, M. Vulcano, P. Allavena, M. Rescigno, Intestinal epithelial cells control dendritic cell function, Ann. N.Y. Acad. Sci. 1029 (2004) 66–74.
[116] P. Hofman, Pathological interactions of bacteria and toxins with the gastrointestinal epithelial tight junctions and/or the zonula adherens: an update, Cell. Mol. Biol. (Noisy.-le-grand) 49 (2003) 65–75.
[117] C.A. Cuvelier, J. Quatacker, H. Mielants, V.M. De, E. Veys, H. Roels, M cells are damaged and increased in number in inflamed human ileal mucosa, Eur. J. Morphol. 31 (1993) 87–91.
[118] P.J. Giannasca, K.T. Giannasca, A.M. Leichtner, M.R. Neutra, Human intestinal M cells display the sialyl Lewis A antigen, Infect. Immun. 67 (1999) 946–953.
[119] P.U. Jani, A.T. Florence, D.E. McCarthy, Further histological evidence of the gastrointestinal absorption of polystyrene nanospheres in the rat, Int. J. Pharm. 84 (1992) 245–252.
[120] K. Kataoka, J. Tabata, M. Yamamoto, T. Toyota, The association of gap junctions with large particles in the crypt epithelium of the rat small intestine, Arch. Histol. Cytol. 52 (1989) 81–86.
[121] E.C. Lavelle, S. Sharif, N.W. Thomas, J. Holland, S.S. Davis, The importance of gastrointestinal uptake of particles in the design of oral delivery systems, Adv. Drug Deliv. Rev. 18 (1995) 5–22.
[122] D.T. O'Hagan, Intestinal translocation of particulates–implications for drug and antigen delivery, Adv. Drug Deliv. Rev. 5 (1990) 265–285.
[123] J. Mestecky, Z. Moldoveanu, S.M. Michalek, C.D. Morrow, R.W. Compans, D.P. Schafer, M.W. Russell, Current options for vaccine delivery systems by mucosal routes, J. Control. Release 48 (1997) 243–257.
[124] A.T. Florence, Issues in oral nanoparticle drug carrier uptake and targeting, J. Drug Target. 12 (2004) 65–70.
[125] P. Jani, G.W. Halbert, J. Langridge, A.T. Florence, The uptake and translocation of latex nanospheres and microspheres after oral administration to rats, J. Pharm. Pharmacol. 41 (1989) 809–812.
[126] A. Lamprecht, U. Schäfer, C.M. Lehr, Size-dependent bioadhesion of micro- and nanoparticulate carriers to the inflamed colonic mucosa, Pharm. Res. 18 (2001) 788–793.
[127] D.A. Norris, P.J. Sinko, The role of surface hydrophobicity in the transport of polystyrene microspheres through Caco-2 cell monolayers and intestinal mucin, Proceedings of the Controlled Release Society, 1997, pp. 17–18.
[128] A. Gebert, I. Steinmetz, S. Fassbender, K.H. Wendlandt, Antigen transport into Peyer's patches: increased uptake by constant numbers of M cells, Am. J. Pathol. 164 (2004) 65–72.
[129] H.M. Meynell, N.W. Thomas, P.S. James, J. Holland, M.J. Taussig, C. Nicoletti, Up-regulation of microsphere transport across the follicle-associated epithelium of Peyer's patch by exposure to *Streptococcus pneumoniae* R36a, FASEB J. 13 (1999) 611–619.
[130] C. Borghesi, M.J. Taussig, C. Nicoletti, Rapid appearance of M cells after microbial challenge is restricted at the periphery of the follicle-associated epithelium of Peyer's patch, Lab. Invest. 79 (1999) 1393–1401.
[131] D.T. O'Hagan, Microparticles and polymers for the mucosal delivery of vaccines, Adv. Drug Deliv. Rev. 34 (1998) 305–320.
[132] S.D. Conner, S.L. Schmid, Regulated portals of entry into the cell, Nature 422 (2003) 37–44.
[133] S.-H. Liu, W.G. Mallet, F.M. Brodskt, Clatherin-mediated endocytosis, in: M. Marsh (Ed.), Endocytosis, Oxford University Press, New York, 2001, pp. 1–25.
[134] M. Maniak, Macropinocytosis, in: M. Marsh (Ed.), Endocytosis, Oxford University Press, New York, 2001, pp. 78–93.
[135] M.P. Desai, V. Labhasetwar, G.L. Amidon, R.J. Levy, Gastrointestinal uptake of biodegradable microparticles: effect of particle size, Pharm. Res. 13 (1996) 1838–1845.
[136] Y.H. Sang, P.T. Gwan, Biodegradable nanoparticles containing protein–fatty acid complexes for oral delivery of salmon calcitonin, J. Pharm. Sci. 93 (2004) 488–495.
[137] Y. Jiao, N. Ubrich, M. Marchand-Arvier, C. Vigneron, M. Hoffman, T. Lecompte, P. Maincent, In vitro and in vivo evaluation of oral heparin-loaded polymeric nanoparticles in rabbits, Circulation 105 (2002) 230–235.
[138] A. Saez, M. Guzman, J. Molpeceres, M.R. Aberturas, Freeze-drying of polycaprolactone and poly(D,L-lactic-glycolic) nanoparticles induce minor particle size changes affecting the oral pharmacokinetics of loaded drugs, Eur. J. Pharm. Biopharm. 50 (2000) 379–387.
[139] J.H. Eldridge, C.J. Hammond, J.A. Meulbroek, J.K. Staas, R.M. Gilley, T.R. Tice, Controlled vaccine release in the gut-associated lymphoid tissues. I. Orally administered biodegradable microspheres target the Peyer's patches, J. Control. Release 11 (1990) 205–214.
[140] M. Tobio, A. Sanchez, A. Vila, I.I. Soriano, C. Evora, J.L. Vila-Jato, M.J. Alonso, The role of PEG on the stability in digestive fluids and in vivo fate of PEG–PLA nanoparticles following oral administration, Colloids Surf., B Biointerfaces 18 (2000) 315–323.
[141] A. Vila, H. Gill, O. McCallion, M.J. Alonso, Transport of PLA–PEG particles across the nasal mucosa: effect of particle size and PEG coating density, J. Control. Release 98 (2004) 231–244.
[142] T. Jung, W. Kamm, A. Breitenbach, E. Kaiserling, J.X. Xiao, T. Kissel, Biodegradable nanoparticles for oral delivery of peptides: is there a role for polymers to affect mucosal uptake? Eur. J. Pharm. Biopharm. 50 (2000) 147–160.
[143] A.G. Mikos, E. Mathiowitz, R. Langer, N.A. Peppas, Interaction of polymer microspheres with mucin gels as a means of characterizing polymer retention on mucus, J. Colloid Interface Sci. 143 (1991) 366–373.
[144] J.D. Smart, The basics and underlying mechanisms of mucoadhesion, Adv. Drug Deliv. Rev. 57 (2005) 1556–1568.
[145] H. Takeuchi, H. Yamamoto, T. Niwa, T. Hino, Y. Kawashima, Enteral absorption of insulin in rats from mucoadhesive chitosan-coated liposomes, Pharm. Res. 13 (1996) 896–901.
[146] Y. Akiyama, N. Nagahara, T. Kashihara, S. Hirai, H. Toguchi, In vitro and in vivo evaluation of mucoadhesive microspheres prepared for the gastrointestinal tract using polyglycerol esters of fatty acids and a poly (acrylic acid) derivative, Pharm. Res. 12 (1995) 397–405.
[147] C.M. Lehr, J.A. Bouwstra, W. Kok, A.G. De Boer, J.J. Tukker, J.C. Verhoef, D.D. Breimer, H.E. Junginger, Effects of the mucoadhesive polymer polycarbophil on the intestinal absorption of a peptide drug in the rat, J. Pharm. Pharmacol. 44 (1992) 402–407.
[148] M.A. Longer, H.S. Ch'ng, J.R. Robinson, Bioadhesive polymers as platforms for oral controlled drug delivery III: oral delivery of chlorothiazide using a bioadhesive polymer, J. Pharm. Sci. 74 (1985) 406–411.
[149] C. Pimienta, V. Lenaerts, C. Cadieux, P. Raymond, J. Juhasz, M.A. Simard, C. Jolicoeur, Mucoadhesion of hydroxypropylmethacrylate nanoparticles to rat intestinal ileal segments in vitro, Pharm. Res. 7 (1990) 49–53.
[150] C. Pimienta, F. Chouinard, A. Labib, V. Lenaerts, Effect of various poloxamer coatings on in vitro adhesion of isohexylcyanoacrylate nanospheres to rat ileal segments under liquid flow, Int. J. Pharm. 80 (1992) 1–8.

[151] K.V.R. Rao, P. Buri, A novel in situ method to test polymers and coated microparticles for bioadhesion, Int. J. Pharm. 52 (1989) 265–270.
[152] H. Takeuchi, H. Yamamoto, T. Niwa, T. Hino, Y. Kawashima, Mucoadhesion of polymer-coated liposomes to rat intestine in vitro, Chem. Pharm. Bull. 42 (1994) 1954–1956.
[153] S. Sakuma, M. Hayashi, M. Akashi, Design of nanoparticles composed of graft copolymers for oral peptide delivery, Adv. Drug Deliv. Rev. 47 (2001) 21–37.
[154] L. Illum, Chitosan and its use as a pharmaceutical excipient, Pharm. Res. 15 (1998) 1326–1331.
[155] Y. Kawashima, H. Yamamoto, H. Takeuchi, Y. Kuno, Mucoadhesive DL-lactide/glycolide copolymer nanospheres coated with chitosan to improve oral delivery of elcatonin, Pharm. Dev. Technol. 5 (2000) 77–85.
[156] M.M. Issa, M. Koping-Hoggard, P. Artursson, Chitosan and the mucosal delivery of biotechnology drugs, Drug Discov. Today. Technol. 2 (2005) 1–6.
[157] M. Garcia-Fuentes, D. Torres, M.J. Alonso, New surface-modified lipid nanoparticles as delivery vehicles for salmon calcitonin, Int. J. Pharm. 296 (2005) 122–132.
[158] C. Prego, M. Fabre, D. Torres, M.J. Alonso, Efficacy and mechanism of action of chitosan nanocapsules for oral peptide delivery, Pharm. Res. 23 (2006) 549–556.
[159] C.M. Lehr, F.G.J. Poelma, H.E. Junginger, J.J. Tukker, An estimate of turnover time of intestinal mucus gel layer in the rat in situ loop, Int. J. Pharm. 70 (1991) 235–240.
[160] C. Prego, D. Torres, E. Fernandez-Megia, R. Novoa-Carballal, E. Quinoa, M.J. Alonso, Chitosan-PEG nanocapsules as new carriers for oral peptide delivery effect of chitosan pegylation degree, J. Control. Release (2006).
[161] S. Hariharan, V. Bhardwaj, I. Bala, J. Sitterberg, U. Bakowsky, M.N. Ravi Kumar, Design of estradiol loaded PLGA nanoparticulate formulations: a potential oral delivery system for hormone therapy, Pharm. Res. (2005).
[162] D.J. Brayden, M.A. Jepson, A.W. Baird, Keynote review: intestinal Peyer's patch M cells and oral vaccine targeting, Drug Discov. Today 10 (2005) 1145–1157.
[163] R.L. Owen, Sequential uptake of horseradish peroxidase by lymphoid follicle epithelium of Peyer's patches in the normal unobstructed mouse intestine: an ultrastructural study, Gastroenterology 72 (1977) 440–451.
[164] M.R. Neutra, T.L. Phillips, E.L. Mayer, D.J. Fishkind, Transport of membrane-bound macromolecules by M cells in follicle-associated epithelium of rabbit Peyer's patch, Cell Tissue Res. 247 (1987) 537–546.
[165] M.A. Clark, H. Blair, L. Liang, R.N. Brey, D. Brayden, B.H. Hirst, Targeting polymerised liposome vaccine carriers to intestinal M cells, Vaccine 20 (2001) 208–217.
[166] A. des Rieux, E.G.E. Ragnarsson, E. Gullberg, V. Preat, Y.J. Schneider, P. Artursson, Transport of nanoparticles across an in vitro model of the human intestinal follicle associated epithelium, Eur. J. Pharm. Sci. 25 (2005) 455–465.
[167] M.E. LeFevre, J.B. Warren, D.D. Joel, Particles and macrophages in murine Peyer's patches (with 1 color plate), Exp. Cell Biol. 53 (1985) 121–129.
[168] A.M. Hillery, A.T. Florence, The effect of adsorbed poloxamer 188 and 407 surfactants on the intestinal uptake of 60-nm polystyrene particles after oral administration in the rat, Int. J. Pharm. 132 (1996) 123–130.
[169] M. Shakweh, M. Besnard, V. Nicolas, E. Fattal, Poly (lactide-co-glycolide) particles of different physicochemical properties and their uptake by Peyer's patches in mice, Eur. J. Pharm. Biopharm. 61 (2005) 1–13.
[170] M.A. Jepson, N.L. Simmons, D.T. O'Hagan, B.H. Hirst, Comparison of poly(DL-lactide-co-glycolide) and polystyrene microsphere targeting to intestinal M cells, J. Drug Target. 1 (1993) 245–249.
[171] M.A. Jepson, N.L. Simmons, T.C. Savidge, P.S. James, B.H. Hirst, Selective binding and transcytosis of latex microspheres by rabbit intestinal M cells, Cell Tissue Res. 271 (1993) 399–405.
[172] M.A. Clark, M.A. Jepson, B.H. Hirst, Exploiting M cells for drug and vaccine delivery, Adv. Drug Deliv. Rev. 50 (2001) 81–106.
[173] M.A. Jepson, M.A. Clark, N. Foster, C.M. Mason, M.K. Bennett, N.L. Simmons, B.H. Hirst, Targeting to intestinal M cells, J. Anat. 189 (Pt 3) (1996) 507–516.
[174] J.H. Eldridge, J.A. Meulbroek, J.K. Staas, T.R. Tice, R.M. Gilley, Vaccine-containing biodegradable microspheres specifically enter the gut-associated lymphoid tissue following oral administration and induce a disseminated mucosal immune response, Adv. Exp. Med. Biol. 251 (1989) 191–202.
[175] T.H. Ermak, E.P. Dougherty, H.R. Bhagat, Z. Kabok, J. Pappo, Uptake and transport of copolymer biodegradable microspheres by rabbit Peyer's patch M cells, Cell Tissue Res. 279 (1995) 433–436.
[176] A.M. Torche, H. Jouan, P. Le Corre, E. Albina, R. Primault, A. Jestin, R. Le Verge, Ex vivo and in situ PLGA microspheres uptake by pig ileal Peyer's patch segment, Int. J. Pharm. 201 (2000) 15–27.
[177] D.T. O'Hagan, H. Jeffery, K.J. Maloy, A.M. Mowat, D. Rahman, S.J. Challacombe, Biodegradable microparticles as oral vaccines, Adv. Exp. Med. Biol. 371 (1995) 1463–1467.
[178] D.T. O'Hagan, D. Rahman, J.P. McGee, H. Jeffery, M.C. Davies, P. Williams, S.S. Davis, S.J. Challacombe, Biodegradable microparticles as controlled release antigen delivery systems, Immunology 73 (1991) 239–242.
[179] J.H. Eldridge, J.K. Staas, J.A. Meulbroek, J.R. McGhee, T.R. Tice, R.M. Gilley, Biodegradable microspheres as a vaccine delivery system, Mol. Immunol. 28 (1991) 287–294.
[180] N. Hussain, Ligand-mediated tissue specific drug delivery, Adv. Drug Deliv. Rev. 43 (2000) 95–100.
[181] A. Weissenbock, M. Wirth, F. Gabor, WGA-grafted PLGA-nanospheres: preparation and association with Caco-2 single cells, J. Control. Release 99 (2004) 383–392.
[182] F. Gabor, M. Stangl, M. Wirth, Lectin-mediated bioadhesion: binding characteristics of plant lectins on the enterocyte-like cell lines Caco-2, HT-29 and HCT-8, J. Control. Release 55 (1998) 131–142.
[183] F. Gabor, E. Bogner, A. Weissenboeck, M. Wirth, The lectin–cell interaction and its implications to intestinal lectin-mediated drug delivery, Adv. Drug Deliv. Rev. 56 (2004) 459–480.
[184] M.J. Montisci, G. Giovannuci, D. Duchene, G. Ponchel, Covalent coupling of asparagus pea and tomato lectins to poly(lactide) microspheres, Int. J. Pharm. 215 (2001) 153–161.
[185] N. Lochner, F. Pittner, M. Wirth, F. Gabor, Wheat germ agglutinin binds to the epidermal growth factor receptor of artificial Caco-2 membranes as detected by silver nanoparticle enhanced fluorescence, Pharm. Res. 20 (2003) 833–839.
[186] A. Pusztai, S.W. Ewen, G. Grant, D.S. Brown, J.C. Stewart, W.J. Peumans, E.J. Van Damme, S. Bardocz, Antinutritive effects of wheat-germ agglutinin and other *N*-acetylglucosamine-specific lectins, Br. J. Nutr. 70 (1993) 313–321.
[187] M. Cuna, M. Alonso-Sande, C. Remunan-Lopez, M.J. Alonso, Development of chitosan/glucomannan nanoparticles as carrier for oral protein administration, Proceedings of the Controlled Release Society, 2002, p. 136.
[188] N. Cruz, X. Alvarez, R.D. Specian, R.D. Berg, E.A. Deitch, Role of mucin, mannose, and beta-1 integrin receptors in *Escherichia coli* translocation across Caco-2 cell monolayers, Shock 2 (1994) 121–126.
[189] H. Tomizawa, Y. Aramaki, Y. Fujii, T. Hara, N. Suzuki, K. Yachi, H. Kikuchi, S. Tsuchiya, Uptake of phosphatidylserine liposomes by rat Peyer's patches following intraluminal administration, Pharm. Res. 10 (1993) 549–552.
[190] N. Foster, B.H. Hirst, Exploiting receptor biology for oral vaccination with biodegradable particulates, Adv. Drug Deliv. Rev. 57 (2005) 431–450.
[191] F. Roth-Walter, B. Bohle, I. Scholl, E. Untersmayr, O. Scheiner, G. Boltz-Nitulescu, F. Gabor, D.J. Brayden, E. Jensen-Jarolim, Targeting antigens to murine and human M-cells with *Aleuria aurantia* lectin-functionalized microparticles, Immunol. Lett. 100 (2005) 182–188.
[192] R. Sharma, E.J. Van Damme, W.J. Peumans, P. Sarsfield, U. Schumacher, Lectin binding reveals divergent carbohydrate expression in human and mouse Peyer's patches, Histochem. Cell Biol. 105 (1996) 459–465.

[193] H.H. Salman, C. Gamazo, M.A. Campanero, J.M. Irache, Salmonella-like bioadhesive nanoparticles, J. Control. Release 106 (2005) 1–13.
[194] M.A. Clark, B.H. Hirst, M.A. Jepson, M-cell surface beta1 integrin expression and invasin-mediated targeting of *Yersinia pseudotuberculosis* to mouse Peyer's patch M cells, Infect. Immun. 66 (1998) 1237–1243.
[195] N. Hamzaoui, S. Kerneis, E. Caliot, E. Pringault, Expression and distribution of beta1 integrins in in vitro-induced M cells: implications for Yersinia adhesion to Peyer's patch epithelium, Cell. Microbiol. 6 (2004) 817–828.
[196] R. Schulte, S. Kerneis, S. Klinke, H. Bartels, S. Preger, J.P. Kraehenbuhl, E. Pringault, I.B. Autenrieth, Translocation of *Yersinia entrocolitica* across reconstituted intestinal epithelial monolayers is triggered by Yersinia invasin binding to beta1 integrins apically expressed on M-like cells, Cell. Microbiol. 2 (2000) 173–185.
[197] J.F. Beaulieu, Differential expression of the VLA family of integrins along the crypt-villus axis in the human small intestine, J. Cell Sci. 102 (Pt 3) (1992) 427–436.
[198] P. Tyrer, A.R. Foxwell, A.W. Cripps, M.A. Apicella, J.M. Kyd, Microbial pattern recognition receptors mediate M-cell uptake of a gram-negative bacterium, Infect. Immun. 74 (2006) 625–631.
[199] E. Gullberg, A. Velin Keita, SY. Salim, M. Andersson, K. Caldwell, JD. Söderholm, P. Artursson, Increased CD9 and B1-integrin expression in human follicule associated epithelium is linked with selective transport into human Peyer's patches, Particle Transcytosis Across the Human Intestinal Epithelium (Thesis), (2005).
[200] N. Hussain, A.T. Florence, Invasin-induced oral uptake of nanospheres: utilising bacterial mechanisms of epithelial cell entry, J. Control. Release 41 (1996) S3–S4.
[201] M. Andersson, K. Fromell, E. Gullberg, P. Artursson, K.D. Caldwell, Characterization of surface-modified nanoparticles for in vivo biointeraction. A sedimentation field flow fractionation study, Anal. Chem. 77 (2005) 5488–5493.
[202] G.F. Dawson, G.W. Halbert, The in vitro cell association of invasin coated polylactide-co-glycolide nanoparticles, Pharm. Res. 17 (2000) 1420–1425.
[203] E. Ruoslahti, RGD and other recognition sequences for integrins, Annu. Rev. Cell Dev. Biol. 12 (1996) 697–715.
[204] M.J. Roy, M. Varvayanis, Development of dome epithelium in gut-associated lymphoid tissues: association of IgA with M cells, Cell Tissue Res. 248 (1987) 645–651.
[205] R. Weltzin, P. Lucia-Jandris, P. Michetti, B.N. Fields, J.P. Kraehenbuhl, M.R. Neutra, Binding and transepithelial transport of immunoglobulins by intestinal M cells: demonstration using monoclonal IgA antibodies against enteric viral proteins, J. Cell Biol. 108 (1989) 1673–1685.
[206] N.J. Mantis, M.C. Cheung, K.R. Chintalacharuvu, J. Rey, B. Corthesy, M.R. Neutra, Selective adherence of IgA to murine Peyer's patch M cells: evidence for a novel IgA receptor, J. Immunol. 169 (2002) 1844–1851.
[207] C. Porta, P.S. James, A.D. Phillips, T.C. Savidge, M.W. Smith, D. Cremaschi, Confocal analysis of fluorescent bead uptake by mouse Peyer's patch follicle-associated M cells, Exp. Physiol. 77 (1992) 929–932.
[208] D.J. Brayden, A.W. Baird, Microparticle vaccine approaches to stimulate mucosal immunisation, Microbes Infect. 3 (2001) 867–876.
[209] D.J. Brayden, A.W. Baird, Apical membrane receptors on intestinal M cells: potential targets for vaccine delivery, Adv. Drug Deliv. Rev. 56 (2004) 721–726.
[210] C. Damge, C. Michel, M. Aprahamian, P. Couvreur, New approach for oral administration of insulin with polyalkylcyanoacrylate nanocapsules as drug carrier, Diabetes 37 (1988) 246–251.
[211] C. Damge, C. Michel, M. Aprahamian, P. Couvreur, J.P. Devissaguet, Nanocapsules as carriers for oral peptide delivery, J. Control. Release 13 (1990) 233–239.
[212] F. Cournarie, D. Auchere, D. Chevenne, B. Lacour, M. Seiller, C. Vauthier, Absorption and efficiency of insulin after oral administration of insulin-loaded nanocapsules in diabetic rats, Int. J. Pharm. 242 (2002) 325–328.
[213] Y. Pan, Y.J. Li, H.Y. Zhao, J.M. Zheng, H. Xu, G. Wei, J.S. Hao, F.D. Cui, Bioadhesive polysaccharide in protein delivery system: chitosan nanoparticles improve the intestinal absorption of insulin in vivo, Int. J. Pharm. 249 (2002) 139–147.
[214] S. Sakuma, N. Suzuki, H. Kikuchi, K.i. Hiwatari, K. Arikawa, A. Kishida, M. Akashi, Oral peptide delivery using nanoparticles composed of novel graft copolymers having hydrophobic backbone and hydrophilic branches, Int. J. Pharm. 149 (1997) 93–106.
[215] H. Takeuchi, H. Yamamoto, Y. Kawashima, Mucoadhesive nanoparticulate systems for peptide drug delivery, Adv. Drug Deliv. Rev. 47 (2001) 39–54.
[216] M.H. El-Shabouri, Positively charged nanoparticles for improving the oral bioavailability of cyclosporin-A, Int. J. Pharm. 249 (2002) 101–108.
[217] J. Chen, W.L. Yang, G. Li, J. Qian, J.L. Xue, S.K. Fu, D.R. Lu, Transfection of mEpo gene to intestinal epithelium in vivo mediated by oral delivery of chitosan-DNA nanoparticles, World J. Gastroenterol. 10 (2004) 112–116.
[218] D. Das, S. Lin, Double-coated poly (butylcynanoacrylate) nanoparticulate delivery systems for brain targeting of dalargin via oral administration, J. Pharm. Sci. 94 (2005) 1343–1353.
[219] S. Bonduelle, C. Foucher, J.C. Leroux, F. Chouinard, C. Cadieux, V. Lenaerts, Association of cyclosporin to isohexylcyanoacrylate nanospheres and subsequent release in human plasma in vitro, J. Microencapsul 9 (1992) 173–182.
[220] S. Bonduelle, C. Pimienta, J.P. Benoit, V. Lenaerts, Body distribution in mice of intravenously injected radiolabelled cyclosporin associated with polyisohexylcyanoacrylate nanocapsules or nanospheres, Eur. J. Pharm. Biopharm. 41 (1995) 27–30.
[221] S. Bonduelle, M. Carrier, C. Pimienta, J.P. Benoit, V. Lenaerts, Tissue concentration of nanoencapsulated radiolabelled cyclosporin following peroral delivery in mice or ophthalmic application in rabbits, Eur. J. Pharm. Biopharm. 42 (1996) 313–319.
[222] A.M. Hillery, I. Toth, A.T. Florence, Co-polymerised peptide particles II: oral uptake of a novel co-polymeric nanoparticulate delivery system for peptides, J. Control. Release 42 (1996) 65–73.
[223] J.E. Eyles, H.O. Alpar, B.R. Conway, M. Keswick, Oral delivery and fate of poly(lactic acid) microsphere-encapsulated interferon in rats, J. Pharm. Pharmacol. 49 (1997) 669–674.
[224] J.C. Leroux, R. Cozens, J.L. Roesel, B. Galli, F. Kubel, E. Doelker, R. Gurny, Pharmacokinetics of a novel HIV-1 protease inhibitor incorporated into biodegradable or enteric nanoparticles following intravenous and oral administration to mice, J. Pharm. Sci. 84 (1995) 1387–1391.
[225] P.D. Smith, G. Meng, J.F. Salazar-Gonzalez, G.M. Shaw, Macrophage HIV-1 infection and the gastrointestinal tract reservoir, J. Leukoc. Biol. 74 (2003) 642–649.
[226] A. Dembri, M.J. Montisci, J.C. Gantier, H. Chacun, G. Ponchel, Targeting of 3′-azido 3′-deoxythymidine (AZT)-loaded poly(isohexylcyanoacrylate) nanospheres to the gastrointestinal mucosa and associated lymphoid tissues, Pharm. Res. 18 (2001) 467–473.
[227] J.R. McGhee, J. Mestecky, M.T. Dertzbaugh, J.H. Eldridge, M. Hirasawa, H. Kiyono, The mucosal immune system: from fundamental concepts to vaccine development, Vaccine 10 (1992) 75–88.
[228] J. Mestecky, J.R. McGhee, Immunoglobulin A (IgA): molecular and cellular interactions involved in IgA biosynthesis and immune response, Adv. Immunol. 40 (1987) 153–245.
[229] L. Illum, I. Jabbal-Gill, M. Hinchcliffe, A.N. Fisher, S.S. Davis, Chitosan as a novel nasal delivery system for vaccines, Adv. Drug Deliv. Rev. 51 (2001) 81–96.
[230] K. Regnstrom, E.G. Ragnarsson, M. Koping-Hoggard, E. Torstensson, H. Nyblom, P. Artursson, PEI — a potent, but not harmless, mucosal immuno-stimulator of mixed T-helper cell response and Fasl-mediated cell death in mice, Gene Ther. 10 (2003) 1575–1583.
[231] C. Porporatto, I.D. Bianco, S.G. Correa, Local and systemic activity of the polysaccharide chitosan at lymphoid tissues after oral administration, J. Leukoc. Biol. 78 (2005) 62–69.
[232] T. Storni, T.M. Kundig, G. Senti, P. Johansen, Immunity in response to particulate antigen-delivery systems, Adv. Drug Deliv. Rev. 57 (2005) 333–355.

[233] K. laoui-Attarki, S. Pecquet, E. Fattal, S. Trolle, E. Chachaty, P. Couvreur, A. Andremont, Protective immunity against *Salmonella typhimurium* elicited in mice by oral vaccination with phosphorylcholine encapsulated in poly(DL-lactide-co-glycolide) microspheres, Infect. Immun. 65 (1997) 853–857.

[234] E. Fattal, S. Pecquet, P. Couvreur, A. Andremont, Biodegradable microparticles for the mucosal delivery of antibacterial and dietary antigens, Int. J. Pharm. 242 (2002) 15–24.

[235] D.H. Jones, B.W. McBride, C. Thornton, D.T. O'Hagan, A. Robinson, G.H. Farrar, Orally administered microencapsulated *Bordetella pertussis* fimbriae protect mice from *B. pertussis* respiratory infection, Infect. Immun. 64 (1996) 489–494.

[236] N. Kofler, C. Ruedl, C. Rieser, G. Wick, H. Wolf, Oral immunization with poly-(D,L-lactide-co-glycolide) and poly-(L-lactic acid) microspheres containing pneumotropic bacterial antigens, Int. Arch. Allergy Immunol. 113 (1997) 424–431.

[237] K. laoui-Attarki, E. Fattal, S. Pecquet, S. Trolle, E. Chachaty, P. Couvreur, A. Andremont, Mucosal immunogenicity elicited in mice by oral vaccination with phosphorylcholine encapsulated in poly (D,L-lactide-co-glycolide) microspheres, Vaccine 16 (1998) 685–691.

[238] G. Mutwiri, T.L. Bowersock, L.A. Babiuk, Microparticles for oral delivery of vaccines, Expert Opin. Drug Deliv. 2 (2005) 791–806.

[239] S.J. Challacombe, D. Rahman, D.T. O'Hagan, Salivary, gut, vaginal and nasal antibody responses after oral immunization with biodegradable microparticles, Vaccine 15 (1997) 169–175.

[240] I. Esparza, T. Kissel, Parameters affecting the immunogenicity of microencapsulated tetanus toxoid, Vaccine 10 (1992) 714–720.

[241] T. Jung, W. Kamm, A. Breitenbach, K.D. Hungerer, E. Hundt, T. Kissel, Tetanus toxoid loaded nanoparticles from sulfobutylated poly(vinyl alcohol)-graft-poly(lactide-co-glycolide): evaluation of antibody response after oral and nasal application in mice, Pharm. Res. 18 (2001) 352–360.

[242] S.Y. Kim, H.J. Doh, M.H. Jang, Y.J. Ha, S.I. Chung, H.J. Park, Oral immunization with *Helicobacter pylori*-loaded poly(D,L-lactide-co-glycolide) nanoparticles, Helicobacter 4 (1999) 33–39.

[243] K.J. Maloy, A.M. Donachie, D.T. O'Hagan, A.M. Mowat, Induction of mucosal and systemic immune responses by immunization with ovalbumin entrapped in poly(lactide-co-glycolide) microparticles, Immunology 81 (1994) 661–667.

[244] O'Hagan. Cholera toxin B subunit (CTB) entrapped in microparticles shows comparable immunogenicity to CTB mixed with whole cholera toxin following oral immunization. J.P.McGee, M. Lindblad and J. Holmgren. Int. J. Pharm. 119, 251-255(5). 9-6-1995.

[245] I. Gutierro, R.M. Hernandez, M. Igartua, A.R. Gascon, J.L. Pedraz, Influence of dose and immunization route on the serum Ig G antibody response to BSA loaded PLGA microspheres, Vaccine 20 (2002) 2181–2190.

[246] I. Gutierro, R.M. Hernandez, M. Igartua, A.R. Gascon, J.L. Pedraz, Size dependent immune response after subcutaneous, oral and intranasal administration of BSA loaded nanospheres, Vaccine 21 (2002) 67–77.

[247] K. Roy, H.Q. Mao, S.K. Huang, K.W. Leong, Oral gene delivery with chitosan-DNA nanoparticles generates immunologic protection in a murine model of peanut allergy, Nat. Med. 5 (1999) 387–391.

[248] F. Roth-Walter, I. Scholl, E. Untersmayr, A. Ellinger, G. Boltz-Nitulescu, O. Scheiner, F. Gabor, E. Jensen-Jarolim, Mucosal targeting of allergen-loaded microspheres by *Aleuria aurantia* lectin, Vaccine 23 (2005) 2703–2710.

[249] M.A. Conway, L. Madrigal-Estebas, S. McClean, D.J. Brayden, K.H. Mills, Protection against *Bordetella pertussis* infection following parenteral or oral immunization with antigens entrapped in biodegradable particles: effect of formulation and route of immunization on induction of Th1 and Th2 cells, Vaccine 19 (2001) 1940–1950.

[250] D.E. Katz, A.J. DeLorimier, M.K. Wolf, E.R. Hall, F.J. Cassels, J.E. van Hamont, R.L. Newcomer, M.A. Davachi, D.N. Taylor, C.E. McQueen, Oral immunization of adult volunteers with microencapsulated enterotoxigenic *Escherichia coli* (ETEC) CS6 antigen, Vaccine 21 (2003) 341–346.

[251] C.O. Tacket, R.H. Reid, E.C. Boedeker, G. Losonsky, J.P. Nataro, H. Bhagat, R. Edelman, Enteral immunization and challenge of volunteers given enterotoxigenic *E. coli* CFA/II encapsulated in biodegradable microspheres, Vaccine 12 (1994) 1270–1274.

[252] C.B. Wolfowicz, T. HuangFu, K.Y. Chua, Expression and immunogenicity of the major house dust mite allergen Der p 1 following DNA immunization, Vaccine 21 (2003) 1195–1204.

[253] M. Bivas-Benita, M. Laloup, S. Versteyhe, J. Dewit, B.J. De, E. Jongert, G. Borchard, Generation of *Toxoplasma gondii* GRA1 protein and DNA vaccine loaded chitosan particles: preparation, characterization, and preliminary in vivo studies, Int. J. Pharm. 266 (2003) 17–27.

[254] S.C. Chen, D.H. Jones, E.F. Fynan, G.H. Farrar, J.C. Clegg, H.B. Greenberg, J.E. Herrmann, Protective immunity induced by oral immunization with a rotavirus DNA vaccine encapsulated in microparticles, J. Virol. 72 (1998) 5757–5761.

[255] J.E. Herrmann, S.C. Chen, D.H. Jones, A. Tinsley-Bown, E.F. Fynan, H.B. Greenberg, G.H. Farrar, Immune responses and protection obtained by oral immunization with rotavirus VP4 and VP7 DNA vaccines encapsulated in microparticles, Virology 259 (1999) 148–153.

[256] H.L. Weiner, A. Friedman, A. Miller, S.J. Khoury, A. al-Sabbagh, L. Santos, M. Sayegh, R.B. Nussenblatt, D.E. Trentham, D.A. Hafler, Oral tolerance: immunologic mechanisms and treatment of animal and human organ-specific autoimmune diseases by oral administration of autoantigens, Annu. Rev. Immunol. 12 (1994) 809–837.

[257] P. Garside, A.M. Mowat, Oral tolerance, Semin. Immunol. 13 (2001) 177–185.

[258] A.M. Mowat, Anatomical basis of tolerance and immunity to intestinal antigens, Nat. Rev. Immunol. 3 (2003) 331–341.

[259] K. Masuda, K. Horie, R. Suzuki, T. Yoshikawa, K. Hirano, Oral-antigen delivery via a water-in-oil emulsion system modulates the balance of the Th1/Th2 type response in oral tolerance, Pharm. Res. 20 (2003) 130–134.

[260] D.E. Trentham, R.A. Dynesius-Trentham, E.J. Orav, D. Combitchi, C. Lorenzo, K.L. Sewell, D.A. Hafler, H.L. Weiner, Effects of oral administration of type II collagen on rheumatoid arthritis, Science 261 (1993) 1727–1730.

[261] R.B. Nussenblatt, R.R. Caspi, R. Mahdi, C.C. Chan, F. Roberge, O. Lider, H.L. Weiner, Inhibition of S-antigen induced experimental autoimmune uveoretinitis by oral induction of tolerance with S-antigen, J. Immunol. 144 (1990) 1689–1695.

[262] Z.J. Zhang, L. Davidson, G. Eisenbarth, H.L. Weiner, Suppression of diabetes in nonobese diabetic mice by oral administration of porcine insulin, Proc. Natl. Acad. Sci. U. S. A. 88 (1991) 10252–10256.

[263] W.K. Lee, J.Y. Park, S. Jung, C.W. Yang, W.U. Kim, H.Y. Kim, J.H. Park, J.S. Park, Preparation and characterization of biodegradable nanoparticles entrapping immunodominant peptide conjugated with PEG for oral tolerance induction, J. Control. Release 105 (2005) 77–88.

[264] W.U. Kim, W.K. Lee, J.W. Ryoo, S.H. Kim, J. Kim, J. Youn, S.Y. Min, E.Y. Bae, S.Y. Hwang, S.H. Park, C.S. Cho, J.S. Park, H.Y. Kim, Suppression of collagen-induced arthritis by single administration of poly(lactic-co-glycolic acid) nanoparticles entrapping type II collagen: a novel treatment strategy for induction of oral tolerance, Arthritis Rheum. 46 (2002) 1109–1120.

[265] P. Couvreur, Polyalkylcyanoacrylates as colloidal drug carriers, Crit. Rev. Ther. Drug Carr. Syst. 5 (1988) 1–20.

[266] C. Chia-Ming, N. Weiner, Gastrointestinal uptake of liposomes.I. In vitro and in situ studies, Int. J. Pharm. 37 (1987) 75–85.

[267] F. Niedergang, M.N. Kweon, New trends in antigen uptake in the gut mucosa, Trends Microbiol. 13 (2005) 485–490.

[268] D.J. Brayden, Oral vaccination in man using antigens in particles: current status, Eur. J. Pharm. Sci. 14 (2001) 183–189.

[269] N. Foster, M.A. Clark, M.A. Jepson, B.H. Hirst, *Ulex europaeus* 1 lectin targets microspheres to mouse Peyer's patch M-cells in vivo, Vaccine 16 (1998) 536–541.

[270] P. Tyrer, A. Ruth Foxwell, J. Kyd, M. Harvey, P. Sizer, A. Cripps, Validation and quantitation of an in vitro M-cell model, Biochem. Biophys. Res. Commun. 299 (2002) 377–383.

[271] M. Alonso-Sande, D. Teijeiro, C. Remunan-lopez, M.J. Alonso, Chitosan/Glucomannan nanoparticles as oral delivery systems for insulin, 2nd Pharmaceutical Sciences World Congress, 2004, p. 239.

[272] M.H. El-Shabouri, Positively charged nanoparticles for improving the oral bioavailability of cyclosporin-A, Int. J. Pharm. 249 (2002) 101–108.
[273] J.L. Chew, C.B. Wolfowicz, H.Q. Mao, K.W. Leong, K.Y. Chua, Chitosan nanoparticles containing plasmid DNA encoding house dust mite allergen, Der p 1 for oral vaccination in mice, Vaccine 21 (2003) 2720–2729.
[274] Y. Matsunaga, Y. Wakatsuki, Y. Tabata, H. Kawasaki, T. Usui, M. Yoshida, T. Itoh, S. Habu, T. Kita, Oral immunization with size-purified microsphere beads as a vehicle selectively induces systemic tolerance and sensitization, Vaccine 19 (2000) 579–588.
[275] F. Roth-Walter, I. Scholl, E. Untersmayr, R. Fuchs, G. Boltz-Nitulescu, A. Weissenbock, O. Scheiner, F. Gabor, E. Jensen-Jarolim, M cell targeting with *Aleuria aurantia* lectin as a novel approach for oral allergen immunotherapy, J. Allergy Clin. Immunol. 114 (2004) 1362–1368.