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Review Article

Mimicking microbial strategies for the design of mucus-permeating nanoparticles for oral immunization

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ABSTRACT

Dealing with mucosal delivery systems means dealing with mucus. The name mucosa comes from mucus, a dense fluid enriched in glycoproteins, such as mucin, which main function is to protect the delicate mucosal epithelium. Mucus provides a barrier against physiological chemical and physical aggressors (i.e., host secreted digestive products such as bile acids and enzymes, food particles) but also against the potentially noxious microbiota and their products. Intestinal mucosa covers 400 m^2 in the human host, and, as a consequence, is the major portal of entry of the majority of known pathogens. But, in turn, some microorganisms have evolved many different approaches to circumvent this barrier, a direct consequence of natural co-evolution. The understanding of these mechanisms (known as virulence factors) used to interact and/or disrupt mucosal barriers should instruct us to a rational design of nanoparticulate delivery systems intended for oral vaccination and immunotherapy. This review deals with this mimetic approach to obtain nanocarriers capable to reach the epithelial cells after oral delivery and, in parallel, induce strong and long-lasting immune and protective responses.

1. Introduction

The oral administration of bioactive products (i.e., drugs, antigens or immunomodulators) is an attractive and desirable option under diverse points of view: economic, safety (needle-free), easiness and efficiency, particularly for vaccine delivery, taking into account that oral vaccination can induce a systemic, including mucosal, immune response [1]. However, this practice has to face with a hard and very well organized frontier, the mucosa: a mucus secreting epithelium that lines the internal parts of the body. The intestinal mucosa is made up of epithelium, lamina propria, and *muscularis mucosae*. The epithelium is constituted by cells that are held together by tight junctions, which effectively form a seal against the external environment. In addition, there are two extra levels of protection against the outer milieu, the secreted mucus layer and the apical glycocalyx (Fig. 1). Globally considered these layers constitute the mucus that covers the tips of microvilli on the apical surfaces of intestinal enterocytes [2]. Mucus provides a barrier against physical and chemical aggressors, such as food residues, host secreted digestive products (e.g. bile acids and enzymes), but also against the potentially noxious microbiota and their products. Not surprisingly, pathogens have evolved many ways of evading the mucosal barrier. In fact, mucosae cover 400 m^2 in the human host, and as a consequence is the major portal of entry of the majority of known pathogens [3,4].

This review will deal with the generation of nanocarriers, based on microorganism-mimicking approaches, for the oral delivery of either antigens or allergens for vaccination and immunotherapy purposes.

2. Structure and topology of mucus matrix

Mucus is a complex viscous secretion basically formed by water (approx. 95%), salts, lipids and various kinds of macromolecules including the so-called mucins [5,6]. Mucins, secreted by goblet cells, are densely glycosylated proteins in which the protein backbone (apomucin) is linked to a number of carbohydrate chains (50–90% by weight) [7,8]. In addition the carbohydrate structures themselves can be either linear or branched, and can be acidic (containing sialic acid or sulphate groups) or neutral in nature [7,8]. The degree and type of glycosylation differs depending on the type of mucin and its localization throughout the gut [9]. These glycoproteins can be found as oligomers or non-oligomers, and are

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initially classified into three subfamilies: soluble (3–10 nm long), membrane-bound (100–500 nm), and, gel-forming mucins (up to several micrometres). Gel-forming mucins are the major constituent of mucus and responsible for its viscoelastic properties [10].

From a functional point of view, mucus appears as a dense fluid matrix that requires to be ineludibly porous, as a gel, since it needs to allow the diffusion of molecules to both orientations, into the cells (absorption of nutrients) and from the cells (secretion). However, at the same time, it needs to provide an effective physical barrier to foreign particulate matter, including microorganisms. To achieve successfully both functions, mucus is disposed in an arrangement that comprise two different layers: the external, which is named mucus layer, and the internal one or glycocalyx, that corresponds with the glycoproteins attached to the epithelial cell surface [11].

The mucus layer constitutes then the first line of defence against epithelia damage by physical, chemical or biological aggression. It is thick (100–400 μm in the small intestine, 700 μm in the large intestine) and constantly renewed by the host (approx. 5 L/day) [12,13]. Topographically comprise two layers: (i) the outer layer (70–100 μm diameter), which is loosely attached with large functional pores that allow the residence of normal microbiota, and (ii) the inner layer attached to the subjacent glycocalyx and, therefore, densely packed, with a very small functional pore that impede microbial and particle penetration. The predicted model for the physical mucin pore at this level is around 100 nm, although native mucin fibres may aggregate under certain circumstances to create larger pores which allow larger particles to transit [12].

The glycocalyx consists in long filaments of diverse glycoproteins and glycolipids well attached to the cell surface of enterocytes as a thin but very robust and compact layer (15–30 μm thick in the small intestine and around 100 μm in the large intestine). In fact, this layer would be able to detain any macromolecule above 30 nm [14]. The glycocalyx is renewed every 6–24 h, being then release to the lumen, where is trapped and concentrated at the mucus layers. In addition, epithelial cells actively secrete mucins to block microorganisms in the lumen, before reaching the epithelial cells. Fig. 1 shows a schematic representation of the intestinal mucosa.

Summing up, the structure of a mucin fibre contains hydrophobic domains alternating with hydrophilic glycosidic regions that allow interactions with empathic areas on adjacent mucins or even on other molecules. Consequently, mucin fibres are flexible and sticky. The energy invested by mucosal tissues in the production of mucins, and the finely tuned modulation in response to chemical physical or biological challenges, such as infections, reflects the importance of these glycoproteins. In fact, changes in mucin glycosylation are considered as mechanisms of the innate immune response to mucosal infections [10]. In any case, mucus layers are not insurmountable for the microbial world. Motility and degradative enzymes are main strategies used by many microbial pathogens to penetrate the mucus layers that we will considered in the following section.

3. Strategies of microorganisms to colonize mucosal surfaces

The harsh conditions of the gastrointestinal tract as well as the presence of intense peristaltic wave forces compromise the viability and survival of microorganisms within the gut. Many of them have developed different tricks to interact and even penetrate

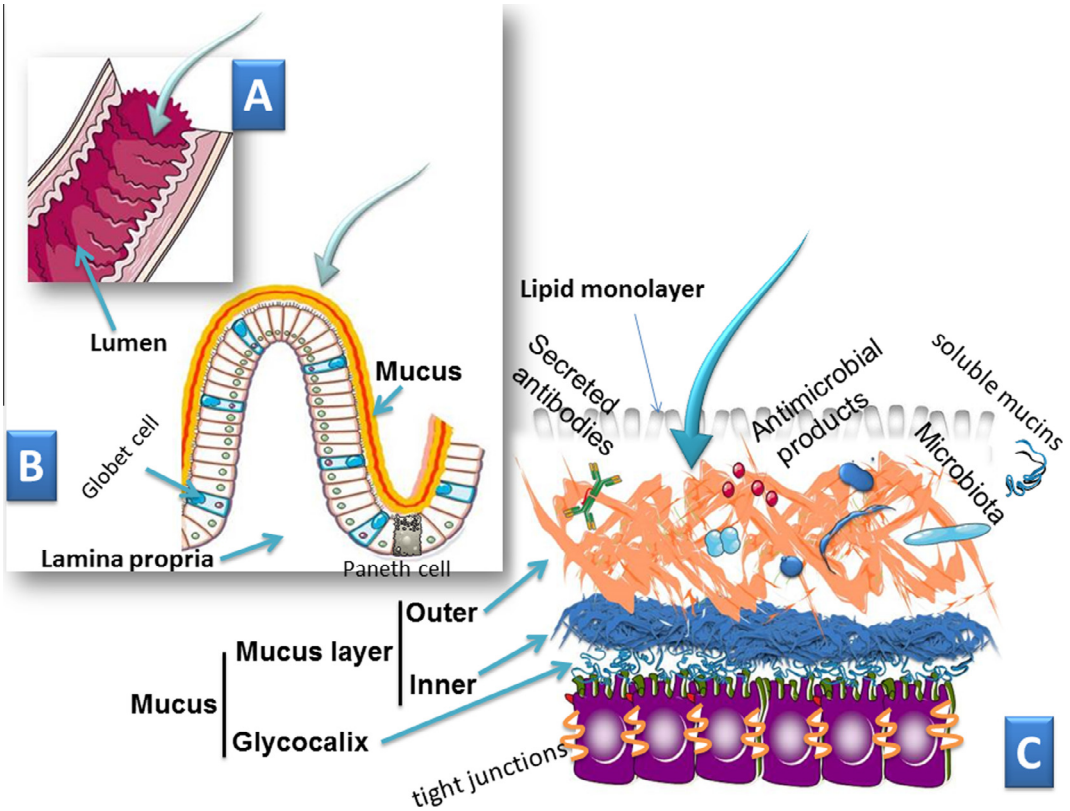

Fig. 1. Schematic representation of intestinal tissues and cell types. The intestinal epithelium is conformed as villi (A). Enterocytes and goblet cells cover most of the villi surface (B). Mucus protects intestinal epithelium presenting a mucus layer (outer layer and inner layer) and the glycocalyx on the cellular membranes (C). The figure displays significant mucosal products and components such as antimicrobial peptides released by Paneth cells, soluble mucins, microbiota and secreted antibodies.

through the mucus layer in order to adhere to and colonize the host mucosa, including the disruption of the balance between mucus erosion and mucus production/secretion or the degradation of mucins by specific proteinases and glycosidases [7]. In view of that, microorganisms have evolved one or several of the following strategies (Fig. 2): (i) interaction with mucin, (ii) alteration of mucin synthesis or mucin assembly into a gel, (iii) degradation of the mucus layer and, (iv) evasion of mucin (alternatives pathways).

3.1. Interaction with mucin

Within mucus, mucins form complex networks that act as a trap for foreign particulates and compounds. The main mechanisms by which mucins interact with foreign bodies are the following: size exclusion, unspecific polyvalent hydrophilic and hydrophobic interactions, and specific bonds.

3.1.1. Size limitations

The mucus gel network is a heterogeneous structure with a wide range of pore sizes, which limit particulates above 200 nm to travel through [12,15,16]. Some great examples of how to avoid size exclusion conferred by the mucus mesh are obtained from enteric virus. In fact, virions that use intestine mucosa to colonize the host have regular sizes below 200 nm (i.e., Rotavirus, Coronavirus, Influenza, and Norwalk). This fact suggests that the mucus barrier has positively selected virus with sizes that better penetrate the mesh to reach efficiently the subjacent target cells. However, some particulates of up to 400 nm are also able to diffuse through mucus [17]. These circumstances are related with the physicochemical properties of mucin, such as the molecular charge, density of anionic and cationic groups, and the numerous hydrophobic domains distributed over the surface of the fibres [16,18]. As a result, adhesive interactions of particles with mucus can be achieved by electrostatic and/or hydrophobic interactions as follows.

3.1.2. Electrostatic interactions

Mucin filaments are covered with glycosylated residues that, in some extent, show an acidic character due to the presence of sialic and sulphate groups (Fig. 3A). Thus, negatively charged mucin can bind with high avidity to positively charged particulates. To counteract this anionic character, a number of enteric virions display an external neutral net charge and, therefore, they are neither repelled nor wrapped into the mucus [11]. Larger organisms, like bacteria, need to use different strategies. For example, *Helicobacter pylori* secretes a glycosulphatase that release sulphate groups from mucin to avoid an electrostatic adsorption and freely migrate through the mucus [19,20].

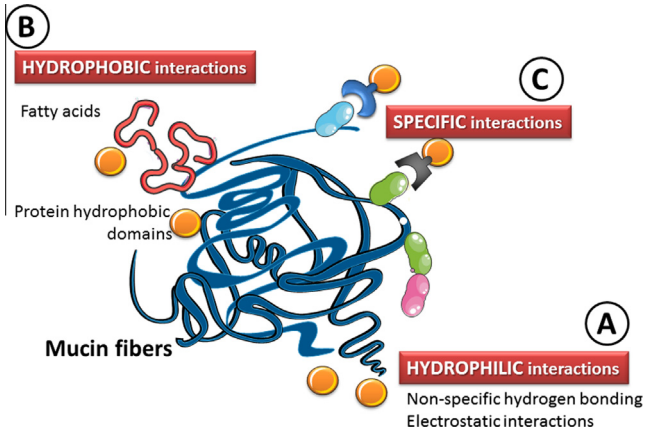

Fig. 3. Mechanisms by which microorganisms/particles may be trapped within the mucin network in the mucus layer: (A) hydrophilic interactions mediated by either electrostatic or non-specific hydrogen bond interactions; (B) hydrophobic interactions with the hydrophobic intrinsic globular domains along mucin or conferred by fatty acids linked to mucin fibres; (C) specific interactions. (For the interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

3.1.3. Hydrophobic interactions

Mucin layers also contain hydrophobic domains along the fibre structure [16]. Thus, and continuing with the virus examples, nature favours enteric naked virus, that means, non-enveloped particles. In contrast, blocking hydrophobic bonds will be established between mucin hydrophobic domains and enveloped viral particles [21] (Fig. 3B). In fact, most microbial cells suffer from this limitation being immobilized by mucus via hydrophobic interactions.

3.1.4. Specific interactions

The complex chemical composition of mucin facilitates the specific linkages via conformational interactions (Fig. 3C). In fact, the

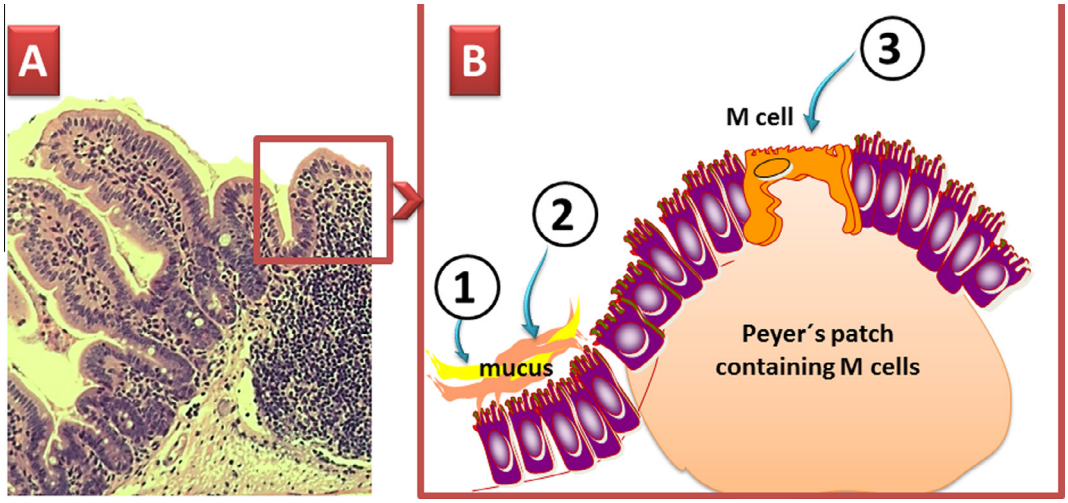

Fig. 2. The gut epithelium and associated lymphoid tissue. The villi, apart from enterocytes and goblet cells, also contain some discrete lymphoid regions (follicle associated epithelium, FAE) such as the Peyer's patches, covered with M cells specialized for antigen sampling. M cells are not so protected by mucus and have a high capacity for particle transcytosis. (A) Thin section of mouse intestinal epithelium showing mucus (yellow), villi and a Peyer's patch. (B) Microorganisms/Particles may interact or even penetrate the mucus layer by alteration of the mucin synthesis or the mucin assembly into a gel (1), degradation of the mucus layer (2) and evasion of mucin/mucus (through M cells or other alternative pathway) (3). (For the interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

selective pressure to handle pathogens may have modulated the ample variety of mucin glycosylation patterns [22]. Some examples are found in bacteria and protozoa for which the adherence to mucin could be a desirable strategic step to avoid natural peristaltic flowing.

Campylobacter jejuni is a motile bacterium that colonizes the intestine of vertebrates, being a main cause of human acute bacterial gastroenteritis. The evolutionary adaptation to mucosa is such that mucins are chemoattractants for *Campylobacter* [23] and the bacterium binds avidly to them through specific ligands, including lipopolysaccharide (LPS) [24,25]. Similarly, the intestinal pathogenic protozoa *Cryptosporidium parvum* and *Giardia duodenalis* express surface lectins to adhere to the intestinal mucins [26,27]. In a similar way, *H. pylori* attaches to mucin carbohydrates by the blood-group antigen-binding and the sialic acid binding adhesins at neutral pH. However, when the mucus gel is released into the acidic gastric milieu the interaction is weak, allowing the bacteria to detach and go further to the epithelium [28].

A similar approach has been described for *Candida* and *Salmonella* species. Once the specific contact between *Candida albicans* and mucins has been established, the microorganism releases an aspartyl proteinase to degrade the surrounding glycoprotein and to move deeper into the mucus layer [29]. On the other hand, *Salmonella* Typhimurium binds specifically to sialomucins. Then, it expresses a sialidase to degrade the mucins, which liquefies the mucus, facilitating its penetration into the protective layer [30,31].

3.2. Alteration of mucin synthesis or mucin assembly into a gel

As stated above, a sign on the importance of mucin in the host defence is that its secretion is enhanced in response to intestinal microbes. In order to solve this drawback, some specialized intestinal pathogens are capable of expressing virulence factors to either alter mucin production (decreasing or increasing) or modify mucin assembly.

3.2.1. Decreasing mucin synthesis

Outer exposed bacterial components, such as exotoxins, flagellin, LPS or lipoteichoic acid, are known modulators of mucin production. For example, *H. pylori* is able to decrease mucin biosynthesis by LPS and a cytosolic phospholipase A2 [32,33], whereas *Clostridium difficile* uses the so-called toxin A to obtain the same effect [34].

3.2.2. Increasing mucin synthesis

Some other pathogens cause mucin hypersecretion in order to produce mucus depletion. The well-known *Vibrio cholerae* enterotoxin, which increases the intracellular levels of adenosine 3′,5′-cyclic monophosphate, activates mucus secretion mechanisms in intestinal goblet cells [35]. *Listeria monocytogenes* produces the exotoxin listeriolysin O to promote the synthesis and secretion of mucins with the same purpose [36].

3.2.3. Alteration of mucin assembly

For the colonization of gastric and duodenum mucosae, *H. pylori* bacteria release urease that neutralizes the acid pH by generating ammonium from urea. This increase of the pH triggers the transition from mucin-gel to mucin-solution, allowing the bacteria to swim through the mucus [20]. In addition, its outer membrane LPS can also inhibit mucin glycosylation which may have deleterious effects on mucin assembly [37].

3.3. Degradation of mucus layer

An obvious direct mechanism to freely move through the mucus barrier is to degrade it. Thus, some symbiotic intestinal bacteria have mucolytic activity by glycosidases and proteases, with the purpose of getting monomers to be used as a source of energy [38]. In turn, some intestinal pathogens use similar specific enzymes to open small breaches in the mucin network with the purpose of disassemble the oligomerized mucin. For example, some intestinal protozoa (e.g. *Tritrichomonas*, *Giardia lamblia* and *Entamoeba histolytica*) may express several mucin-degrading enzymes [39]. Thus, *E. histolytica* secretes glycosidases [40] and proteases that cleave mucin in the non-glycosylated oligomerization domains, breaking down the macromolecular structure and reducing mucus viscosity [41].

Bacteria also carry specific weapons against mucins. In this way, *H. pylori* releases a glycosulphatase to disrupt the oligomeric structure of mucin [42], whereas *V. cholerae* uses a TagA protease for the same purpose [43]. Similarly, *Salmonella* Typhimurium possesses a sialidase [30,31] and *Pseudomonas aeruginosa* and the enterotoxigenic *Escherichia coli* strains (the most common causes of diarrhoea in children) secrete specific proteases [44,45]. Finally, in the subcellular world, we can also find interesting examples such as the reovirus that also release mucolytic proteases to facilitate their penetration through the protective mucus barrier [46].

4. Avoidance of the mucus barrier

Some enteric pathogens are capable of reaching epithelial cells, travelling through the follicle-associated epithelium (FAE). FAE overlays the gut-associated lymphoid tissue (GALT), in which Peyer's patches (PP) and isolated lymphoid follicles are integrated, as a part of the mucosal-associated lymphoid tissue (MALT) of the intestine. In order to mount an efficient immune response against luminal antigens, microfold (M) cells are strategically sited in the dome epithelium of PP. These M cells are specialized for "antigen sampling", presenting a reduced density of microvilli [3,47]. In addition, this dome epithelium lacks goblet cells, thus making a specialized sampling area where the mucus barrier is minimal. Another related special feature of M cells is that they present a deep invagination at the basolateral side, forming an intraepithelial pocket containing immunocompetent cells. In spite of this, penetration of the gut mucosa by pathogens is believed to occur mainly through M cells [48].

As a first step, previous to the invasion, pathogens interact with different pattern recognition receptors (PRR) that recognize molecules that are broadly shared by pathogens but distinguishable from host molecules (pathogen-associated molecular patterns, PAMPs) [48,49]. These PRR include Toll-like receptors (TLR), NOD-like receptors and C-type lectin receptors [49]. The TLR family is particularly expressed by M cells and they detect, for example, LPS from Gram negative bacteria (TLR4 mediated), lipoteichoic acid from Gram positive bacteria (TLR-2), or bacterial flagellin (TLR-5), among many others PAMPs [50,51]. Enteric pathogens exploit those receptors for invading and colonizing the host.

Other receptors localized on M-cells that are used by microorganisms include specific glycoconjugates and the complement component 5a receptor (C5aR). Thus, reovirus specifically targets M cells through the interaction between σ1 hemagglutinin with glycoconjugates terminated in sialic acid residues [52], whereas *E. coli* and *S.* Typhimurium use the fimbriae adhesins FimH+ to specifically interact with the glycoprotein-2 also expressed on M cells [53]. On the other hand, the outer membrane protein OmpH of *Yersinia enterocolitica* recognizes the C5aR [54] and the shock protein Hp-60 of *Brucella abortus* interacts with a cellular prion protein also localized on M cells [55].

In sum, the gut is covered by a mucosal absorptive epithelium that maintains homoeostasis by restricting the transit of macromolecules and foreign particles. However, most of infections occur

along this area. In these circumstances, the obvious approach would be to use the whole natural or recombinant attenuated pathogens as antigen carriers for oral vaccination. However, there are many intrinsic factors that preclude its use: reversion to virulence, immunogenicity to the carrier that neutralizes booster immunizations, and the potential risks associated with the use of recombinant DNA [54,56]. A possible safer solution for the oral delivery of antigens and allergens would be the use of microorganism-like nanocarriers.

5. Microorganism-like nanocarriers

In the last years, efforts have been directed toward the enhancement of mucosal/oral vaccine delivery to the host using a variety of particulate delivery systems such as liposomes, immune-stimulating complexes (ISCOMs) or nanoparticles [57–59]. From a general point of view, these nanocarriers offer some advantages that are of interest for the oral delivery of antigens and allergens for vaccination or immunotherapy purposes, respectively. Thus, the encapsulation of these biomacromolecules in nanocarriers effectively protects them from the harsh conditions of the gastrointestinal tract, minimizing their degradation by hydrolysis or digestive enzymes [60,61]. From a biological point of view, some nanoparticles may also act as adjuvants because they may facilitate both the antigen uptake and internalization by the GALT [62,63] and, further, the antigenic cross-presentation by antigen presenting cells (APCs) via both MHC class I and II pathways [64].

Unfortunately, conventional nanocarriers interact with mucins and can remain immobilized in the mucus layer. Under these circumstances, nanoparticles are cleared as fast as the mucus is removed, following advancing movements by peristaltic forces (Fig. 4A) [65]. Thus, they display a low capability to target specific sites within the gastrointestinal tract (e.g. PP, mucosal dendritic cells [DCs]). As a consequence of this, the elicited immune response with these antigen carriers is usually not as high as necessary to offer the adequate degree of protection to the host, and consequently, high and multiple oral doses are required.

In order to overcome these drawbacks and render nanoparticles more efficient as adjuvants for vaccination, one possible outcome

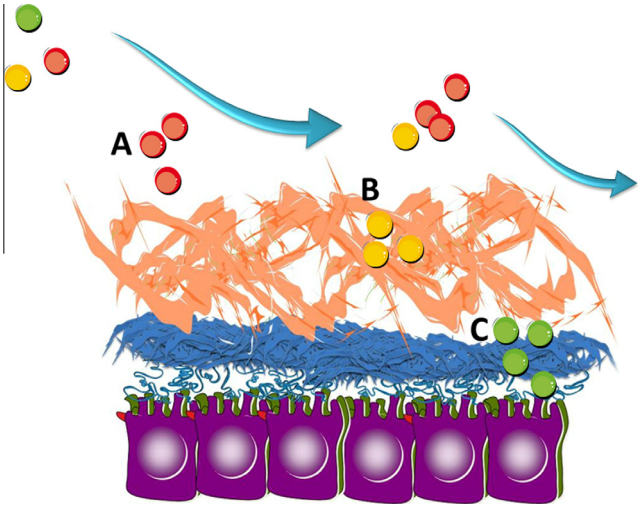

Fig. 4. Interplay between nanoparticles (A) and mucosa in a dynamic environment. If nanoparticles are capable of penetrating the mucus layer and reach the inner mucus layer (IML) the residence would be longer in time, since IML renewal is slower, thus facilitating greater doses of particle cargo to be released nearby the underlaying cells (B). Some of these particles can even reach the glycocalyx, and the cellular membranes as some microorganisms do (C). (For the interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

can be their coating or "decoration" with compounds or molecules involved in the strategies developed by microorganisms to reach the FAE (Fig. 4; Section 4). For this purpose, ligands capable to target and interact with either epithelial glycoconjugates that are specifically activated by pathogens, such as the TLR family or the mannose receptors (MR) have been proposed. It is interesting to note that, in general, these PAMPs are also immunopotentiators and, thus, may improve the intensity and quality of the immune response induced by the antigen-loaded nanoparticles. In parallel, many of these ligands may confer mucus-permeating properties to the resulting nanocarriers by, at least, two different mechanisms. In the former, hydrophilic ligands yield particulates' surfaces less liable to the development of hydrophobic interactions with mucin fibres and other components of the mucus layer. In the later, some ligands (e.g. some types of bacterial LPS or flagellin) may also inhibit the production of mucus glycoproteins (see Section 3.2).

5.1. Functionalization with microbial ligands

In this biomimetic approach different ligands have been proposed including the use of flagellin [66,67] and LPS or its derivatives [68]. Apart from their capabilities to modulate the production of mucins and their specificity for TLRs, these ligands have also an important effect as immunomodulators (adjuvants). In fact, these compounds alert the APCs to the presence of "pathogenic" material and, thus, facilitate the induction of the adequate immune response [69].

5.1.1. Lipopolysaccharide and derivatives coated nanocarriers

The lipopolysaccharides are globally recognized as one of the main PAMPs [50,51]. These macromolecules are located on the outer membrane of Gram negative bacteria and show the capability of activating APCs, through receptors on their membrane such as the TLR4 [70], potentiating Th1 (cellular) responses [71]. However, LPS, which is also known as endotoxin, shows a potent biological activity with deleterious side effects. These effects are related to the presence of Lipid A. Nonetheless, natural LPS from different bacteria may exhibit different biological properties, including their capability as TLR agonist or their effect as pyrogenic material [72,73]. On the other hand, nontoxic alternatives have been developed including the synthesis of modified products structurally related to LPS but devoid of its toxicity. In this way, monophosphoryl lipid A (MPL, derivative of LPS from *Salmonella enterica* serovar. Minnesota) was the first TLR ligand and biological adjuvant approved for human use for its safety and effectiveness [74,75]. Moreover, it has been tested in numerous human trials against different infectious diseases (hepatitis B, malaria or herpes simplex virus) and allergen immunotherapy [76,77].

Regarding its use to improve the immune response of antigens encapsulated in nanocarriers, MPL was incorporated into the external bilayers of liposomes containing the glucosyltransferase antigen from *Streptococcus mutans*. When administered orally, the liposomes induced high levels of salivary, plasma, and vaginal IgA, demonstrating the capability of the combination between nanocarriers and the LPS derivative to induce strong mucosal immune responses [78]. In a similar way, Sarti and co-workers, using PLGA nanoparticles associated to MPL, demonstrated an important improvement of the immune response against ovalbumin (OVA) only when the LPS derivative was present [79]. Interestingly, these results were obtained with the administration of one single oral dose. In another study, PLGA-lipid nanocarriers functionalized in surface with MPL and a M-cell specific lectin stimulated effective mucosal and serum antibodies against the model antigen in mice [80]. Again, the presence of MPL appeared to be the key factor to elicit the immune response.

Nowadays, new generations of TLR4 agonists are being developed such as glucopyranosyl lipid adjuvant and aminoalkyl glucosaminide 4-phosphates [81,82]. However, to the best of our knowledge, researches about their use as an oral vaccine delivery system are limited. Another approach to take advantage of the adjuvant potential of LPS is the use of molecules with low toxicity. In this context, it has been proposed the use of the rough LPS from *Brucella ovis*, which shows a very low endotoxicity [73]. This LPS was used to decorate poly(anhydride) nanoparticles carrying OVA as model allergen. Orally administered to mice, LPS-coated nanoparticles were capable to reach in a large extent the surface of the intestinal epithelium, including PP [68]. More important, this capability to reach the epithelium was in line with the very high degree of protection offered by LPS-nanoparticles (close to 90%) against an anaphylactic shock in OVA-sensitized animals [68]. In a similar approach, but using a *Lolium perenne* protein extract, the coating of poly(anhydride) nanoparticles with LPS from *B. ovis* shifted the immune response from a Th2 (observed with naked nanoparticles) to a Th1 profile in a sensitized murine model to this allergen [83]. This cellular response induced with LPS-coated nanoparticles was identified as the key aspect responsible for the efficacy of the nanoparticles. In fact, in the challenge experiment with sensitized mice, LPS-nanocarriers decreased both the levels of mMCP-1 (mouse mast cell protease 1) and the severity of the anaphylactic symptoms, increasing the survival rate of animals compared with the controls [83].

5.1.2. Flagellin-coated nanocarriers

Flagellin is the monomeric protein that conforms the bacterial flagellum, which is a key virulence factor in some pathogens by providing motility and increasing adhesion [84]. Some examples of flagellated bacteria include *H. pylori*, *Vibrio*, *Salmonella* and *Pseudomonas* species. Flagellin has been extensively investigated as a PAMP, since it binds TLR5 [85,86]. Furthermore, flagellin induces the maturation of intestinal DCs, activates CD4+ T cells in vivo and promotes the development of mixed effector Th cell responses [87,88]. As a mucosal adjuvant, flagellin is almost as potent as *V. cholerae* and *E. coli* heat-labile toxins but much safer than these two compounds [86].

In order to evaluate the advantages offered by the combination between this PAMP and nanocarriers as antigen oral delivery systems, flagellin from the flagella of *Salmonella* Enteritidis was used to functionalize poly(anhydride nanoparticles [89]. When administered orally, these nanocarriers displayed an important capability to reach the surface of the epithelium, mainly in the ileum of laboratory animals. Interestingly, the distribution profile of these nanoparticles within the gut correlated well with the described colonization profile for *Salmonella* Enteritidis [90,91], including a broad concentration in PP. Using ovalbumin as model antigen, these flagellin-coated nanoparticles elicited a strong and balanced secretion of both IgG2a (Th1) and IgG1 (Th2) specific antibodies. Furthermore, these nanoparticles were able to induce a much more strong mucosal IgA response than naked nanoparticles [92].

Flagellin and other related compounds have been also used to decorate other type of nanocarriers including liposomes [93], virus like particles [94] and polypropylene sulphide nanoparticles [95]. More recently, flagellin-functionalized calcium phosphate nanoparticles induced a significantly higher immunostimulatory effect, mainly related with high levels of proinflammatory cytokines (IL-8, IL-1β and IL-6) than controls [96].

5.2. Functionalization with mannose and glycoconjugates

Glycoconjugates enriched in mannose residues promote the interaction of a number of microorganisms (e.g. *C. albicans*, *L. monocytogenes*, *Leishmania donovani*, HIV, *Enterobacteriaceae* or *Bifidobacterium*) with different tissues and substrates, including lymphoid and non-lymphoid cells of different mucosal surfaces [97,98]. This binding is mediated by the high affinity between either mannose or glycoconjugates ending in mannose and the so-called mannose-binding lectins (or MR). In immune cells (i.e. DCs and macrophages), the MR mediate endocytosis, function as antigen capture receptors and are involved in antigen capture and presentation [99–101].

In this context, mannosylated nanocarriers obtained by the decoration of particulates with mannose or its derivatives have been considered as promising non-live vectors for mucosal vaccination. Thus, mannosylated niosomes loaded with tetanus toxoid (TT) were evaluated as oral vaccines against tetanus [102]. The coating of these vesicles with a linear polymer of mannose (o-palmitoyl mannan) improved their stability in the presence of bile salts and digestive enzymes. Furthermore, the functionalized nanocarriers were capable to target PP and to elicit important humoral and cellular responses as measured of the IgG2a/IgG1 sera levels. Similarly, the IgA levels in mucosal secretions were also high against TT [102]. In a similar work, the same mannosylated niosomes were evaluated as oral vaccine carrier of a plasmid designed for the expression of hepatitis B virus proteins. Only animals immunized with these mannosylated niosomes offered adequate antibody levels to get seroprotection against hepatitis B virus infection [103]. Mannosylated liposomes have also been proposed for oral vaccination. Thus, liposomes functionalized with a mannose derivative (mannose-PEG-cholesterol conjugate) induced potent immune responses against a model antigen (bovine serum albumin, BSA) when orally administered. These immune responses were characterized by high levels of both sera IgG and sIgA in different mucosal secretions [104].

In a more recent study, mannosamine-coated polymeric nanoparticles were used to load a hot saline extract from *B. ovis* (HS). The vaccination of mice with a single oral dose of these nanocarriers offered an important protection against an experimental infection with the bacteria. In fact, the degree of protection (measured as reduction of *B. ovis* CFU in the spleen) obtained with mannosylated nanoparticles was about 10-times higher than for naked nanoparticles and 100-times higher than for the control [105]. However, when the animals were conjunctivally vaccinated with mannosylated nanoparticles the degree of protection against the challenge was the highest, even than that observed for the commercial vaccine intramuscularly administered. This degree of protection was related with the fact that mannosylated nanoparticles, after their instillation in the eyes, were distributed (via the nasolacrimal duct) to both the nose and the gastrointestinal tract. In fact, 4 h after instillation, nanoparticles were visualized in the cornea, nose and intestinal mucosa, including PP [105]. It is important to highlight that in all of these areas, nanoparticles can encounter APCs and, thus, induce and potentiate the immune response.

Glucomannan (a water soluble polysaccharide comprised of glucose and mannose) has also been proposed to decorate different nanocarriers including bilosomes [106] and chitosan nanoparticles [107]. In both cases, using TT, it was demonstrated that these functionalized nanocarriers elicited significantly higher systemic and mucosal immune responses than controls. In addition, these TT-loaded in glucomannan nanocarriers also induced a cell mediated immune response (IL-2 and interpheron-gamma), which was not induced by the conventional vaccine based on alum intramuscularly injected.

5.3. Functionalization with lectins

The intestinal epithelial cells possess a cell surface glycocalyx composed of membrane anchored glycoconjugates. It may, therefore, be possible to exploit these surface exposed carbohydrate res-

Table 1
Examples of oral immunizations using lectin-functionalized nanocarriers.

Lectin	Antigen	Carrier	Results	Refs.
Asparagus pea lectin	Hepatitis B surface antigen (HBsAg)	PLGA nanoparticles	Induction of significantly higher Th1/Th2 responses as compared to Alum based vaccine	[120]
Ulex europaeus 1 agglutinin	HBsAg	PLGA nanoparticles	Lectin coated nanoparticles elicited secretion of IgA and high levels of Il-2 and IFN-γ	[121]
Aleuria aurantia lectin or wheat germ agglutinin	*Mycobacterium tuberculosis* cell lysates	Albumin microspheres	Both lectin-coupled microspheres displayed an affinity for M-cells and showed preferential binding to PP	[122]
Ulex europaeus 1 agglutinin	BSA	Liposomes	UEA1-functionalized liposomes induced simultaneously both systemic and mucosal immune responses in mice	[123]
Aleuria aurantia lectin	Birch pollen proteins	PLGA microspheres	Only allergic mice treated with lectin-functionalized microparticles induced important levels of IgG2a and Il-10 and IFN-γ	[119,124]

idues as targets for lectin-mediated delivery to specific regions and cell-types within the gastrointestinal tract. Several studies have revealed that, in many species and at many MALT sites, the M cell surface glycocalyx differs in carbohydrate composition from that of enterocytes [108,109]. One of the first attempt to evaluate the capability of lectins to specifically target M-cells was the coating of liposomes [110] and nanoparticles [111] with *Ulex europaeus* I agglutinin (UEA1), a lectin specific for α-L-fucose residues [112]. Using model antigens (e.g. OVA or BSA), UEA1 coated particulates were capable to reach and target M cells in PP [113] and induced systemic humoral responses significantly higher than those elicited with non-targeted antigen [114,115]. More recently, Malik and coworkers have demonstrated that the coating of BSA-loaded chitosan nanoparticles with UEA1 conjugated alginate produced nanocarriers capable to induce superior systemic responses in laboratory animals along with a mucosal immunity significantly higher than that induced by a conventional aluminium-based vaccine [116]. All of these immunity effects would be consequence of a rapid endocytosis process of these nanocarriers after adhesion to M cells that would facilitate their capture by mucosal DCs and other immunocompetent cells in the subepithelial dome of the intestinal PP tissue [117,80].

Other lectins that have demonstrated an important ability to both target and enhance PP uptake when associated to nanocarriers are the following: wheat germ agglutinin [110], peanut agglutinin [118], asparagus pea lectin [118] and *Aleuria aurantia* lectin [119]. Table 1 summarizes some examples related with the functionalization of nanocarriers with lectins for oral vaccination purposes.

6. Concluding remarks and perspectives

The oral administration of antigens or allergens, for vaccination or immunotherapy, is very attractive for patient compliance (needle free systems), logistical (no cold-chain requirements) and it is supported by immunological foundations. In fact, gut mucosal surfaces are the major portal of entry for the majority of known pathogens and allergens, acting mucosae as the first line of the immune response (GALT). Nevertheless, the arrival of antigens or allergens to the GALT has to face to a number of barriers. First, these compounds are highly sensitive to the harsh conditions of the gut and, in general, they are rapidly degraded by extreme pH conditions and/or digestive enzymes. Second, the mucus layer constitutes a formidable hurdle that greatly hampers the encounter and interaction of these antigens and/or allergens with the antigen presenting cells. Third, the antigens have to elicit a strong, long-lasting and adequate (protective) immune response.

In order to solve these barriers, different strategies have been proposed including the use of nanocarriers. Nanocarriers (e.g. polymeric nanoparticles, liposomes, and ISCOMs) are a good option to protect the cargo against its early degradation within hostile environmental conditions (e.g. acidic pH, enzymes). However, when these nanocarriers are orally administered, they interact with the mucin fibres and, then, an important fraction of the given dose remains trapped in the protective mucus layer. As a consequence, these nanocarriers are rapidly eliminated by the physiological mucus turn-over and the gut peristaltism.

One possible solution to minimize this problem would be the "decoration" of nanocarriers with ligands capable of mimicking the ability of some microorganisms to cross the protective mucus layer and reach the epithelium. Basically, bacteria and virus use two types of mucus-permeating strategies. The first set includes particular physico-chemical properties to minimize the interaction with components of the mucus layer (e.g. size, surface charge and a hydrophilic character). The second set encompasses biological solutions such as the release of proteolytic/glycosidic enzymes, the use of propeller systems, and the presence of compounds capable of specifically interact with receptors of the host. However, it is interesting to note that, in general, microorganisms do not use a simple and unique strategy but a combination of them to cross the mucus layer.

From our point of view, the association of nanocarriers with compounds with a particular specificity for certain receptors localized at the GALT such as TLR, MR or particular glycoconjugates, can be a good option to induce the adequate immune response. For this purpose, the ligands (e.g. glycoconjugates, flagellin, LPS, lectins) should be covalently bound to the surface of nanocarriers loaded with the biologically active molecules. Important advantages can be obtained from this combination.

First, the hydrophilic nature and neutral character of these ligands attached on the surface of nanocarriers would decrease both the electrostatic and hydrophobic interactions with mucins, increasing the possibilities of these nanocarriers of reaching the epithelial surface to deliver their cargo. In case of flagellin and LPS, and due to their capability to decrease the synthesis of mucins, it can be hypothesized that they could also decrease the viscosity of the mucus layer, favouring the arrival of the nanocarriers to the epithelium. Second, the capability of these ligands to specifically interact with PRR on the cell surface (e.g. TLR, MR, glycoconjugates) would improve the possibilities of the resulting nanocarriers to reach the GALT, including M cells in PP. Thus, these targeting properties would be in line with the colonization pattern observed for microorganisms in their colonization process of the host gut mucosa, facilitating the antigen presentation and the activation of the immune system. Last but not least, the immunoadjuvant properties of these ligands would boost the protective immune response.

Another interesting alternative to this biomimetic approach would be the incorporation of proteases or glycosidases (specific to mucins) to these PAMPs-coated devices. This combination should increase the fraction of such nanocarriers capable of reaching the surface of the epithelium and, thus, the efficacy of the antigen/allergen delivery system. However the binding of a second compound to the surface of nanocarriers may negatively affect to their targeting properties and, indeed, their efficacy. Further

research is necessary in order to implement adequate methodologies to "decorate" nanocarriers with different compounds without loss of their efficacy as mucosal delivery systems as well as to select the most adequate ligand to boost the more appropriate immunity to a pathogen or allergy.

Conflict of interest

The authors certify that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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