Chapter 9
Mucosal Vaccine Delivery and M Cell Targeting

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Abbreviations

APC	Antigen-presenting cells
BSM	Bovine submaxillary mucin
DODPC	1, 2-bis[(2E,4E)-Octadecadienoyl]-sn-glycero-3-phosphocholine
FITC	Fluorescein isothiocyanate
GALT	Gut-associated lymphoid tissue
HBsAg	Hepatitis B surface antigen
HRP	Horseradish peroxidase
mAb	Monoclonal antibodies
NALT	Nasal associated lymphoid tissue
PLGA	Poly(D,L-lactide-co-glycolide)
PVA	Polyvinyl alcohol
sIgA	Secretory IgA
Tg	Glass transition temperature
UEA-1	*Ulex europaeus* agglutinin 1

9.1 Mucosal Immune System

The first productive interaction between most infectious agents and the host is with mucosal surfaces, specially, the nasal, oropharyngeal, respiratory, genitourinary, and gastrointestinal mucosa. Conventional vaccine strategies that involve parenteral

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immunization with inactivated viruses or bacteria or subunits of relevant virulence determinants of those pathogens do not prevent initial interactions. In-fact, traditional vaccine strategies do not prevent infection but instead resolve infection before disease ensues. Moreover, many bacterial toxins bind to and interact with mucosal epithelial cells, in which case significant damage to the host may ensue before serum antibodies can play a role in protection. The mucosal surfaces of the gastrointestinal and respiratory tracts represent the main portals of entry for most human pathogens. Sexual contact is another mucosal mode of transmission of infection. Direct inoculation of pathogens into the bloodstream is other important route of infection. Most external mucosal surfaces are replete with organized follicles and scattered antigen-reactive or sensitized lymphoid elements, including B cells, T lymphocytes, T-cell subsets, plasma cells, and a variety of other cellular elements involved in the induction and maintenance of immune response. The mucosal surfaces encompass a critical component of the mammalian immunologic repertoire.

Numerous studies have indicated that induction of systemic immunity through parenteral immunization can effectively clear systemic infections, but it usually fails to protect the mucosal surfaces. Mucosal vaccine administration with an appropriate adjuvant, on the other hand, can induce immune responses at both systemic and mucosal sites and as a result, may prevent not only infectious diseases but also colonization at mucosal surfaces [1]. The mucosal immune system differs in several ways from the systemic immune system. Mucosal immunization frequently results in the stimulation of both mucosal and systemic immune responses, while systemic immunization typically only induces systemic responses without activating the mucosal immune system. Induction of mucosal response leads to production of secretory IgA (sIgA) antibodies, which are not usually produced by systemic immunization [2]. The production of sIgA on the mucosal surfaces is result of the local exposure of antigens to the mucosal-associated lymphoid tissues, especially those in the upper respiratory tract, and the gastrointestinal tract. In most cases infectious agents enter the body at mucosal surfaces and therefore the protective immunity at these surfaces can be effectively induced by mucosal immunization through oral, nasal, rectal, or vaginal routes [3].

The stimulation of the mucosal immune system at one mucosal site can lead to sIgA production in the local as well as distal mucosal surfaces. For example, antigen stimulation of the Peyer's patches in the gastrointestinal tract produced sIgA-producing B cells not only in the intestine, but also in the bronchi as well as in the genitourinary tract. This interconnected mucosal system of sIgA induction and production is termed as common mucosal immune system [2, 4]. The immunologic network operating on external mucosal surfaces consists of gut-associated lymphoid tissue (GALT), the lymphoid structures associated with bronchoepithelium and lower respiratory tract (BALT), ocular tissue, upper airway, salivary glands, tonsils, and nasopharynx (nasal associated lymphoid tissue; NALT), larynx (LALT), middle ear cavity, male and female genital tracts, and mammary glands. The organized lymphoid follicles in the GALT and BALT are main inductive sites of mucosal immune response.

9.2 Mucosal Immunization: An Edge Over Parenteral Vaccination

Vaccine delivery via mucosal route has several advantages over parenteral vaccination [5]. The most important reason for using a mucosal route of vaccination instead of a parenteral route is that the vast majority of infections occurs at or takes their departure from mucosal surfaces and in these infections mucosal vaccines are usually required to induce a protective immune response. The parenteral immunization induces poor mucosal immunity; however, mucosal immunization can induce both mucosal and systemic immunity [6]. The immunization at one mucosal site can induce specific responses at distant sites because of the expression of mucosa-specific homing receptors (site-specific integrins) by mucosally primed lymphocytes and complementary mucosal-tissue specific receptors (addressins) on the vascular endothelial cells [7]. This interconnected network is important because protective immunity (for instance against sexually transmitted diseases) could be induced in segregated mucosal sites in a practical way such as by oral or intranasal immunization and without hampering cultural or religious barriers. Mucosal vaccines are potentially useful to overcome the known barrier of parenteral vaccination caused by either preexisting systemic immunity from previous vaccination or in young children from maternal antibodies or selective immunosuppression such as that caused by HIV infection. For example, mucosal antibody response to oral cholera vaccination was observed in AIDS patients even after they had completely lost their ability to respond to an injectable vaccine (tetanus toxoid) [8]. In addition to serum IgG and mucosal IgA antibodies, mucosal immunization can stimulate cell mediated responses including helper CD4+ T cells and CD8+ cytotoxic T lymphocytes, the latter being important to eliminate intracellular pathogens [9]. The mucosal vaccine delivery is crucial for protective efficacy against noninvasive infections at mucosal surfaces that are normally impermeable to serum antibodies transduction, or passive passage across an epithelium, e.g., GIT infection with V. cholerae [7]. The mucosal vaccine delivery is particularly important for pathogens that can infect the host through both systemic and mucosal route because induction of both sIgA and systemic IgG confer protection at both site. This mode of vaccine delivery could be explored for combating pathogens acquired through non-mucosal routes such as blood or skin. Mucosal vaccination is also beneficial to induce peripheral systemic tolerance especially against those T cell mediated immune reaction that are associated with development of delayed type hypersensitivity reactions. This strategy is important to avoid delayed type hypersensitivity reactions and other allergic reactions to many ingested food proteins and other allergens [6]. Mucosal tolerance is a specific systemic hyporesponsiveness that arises after mucosal administration of an antigen. The tolerance is mediated by a combination of suppressor T-cells, inhibitory cytokines and factors which inhibit the inflammatory process. Oral tolerance can be used for the treatment of atopic diseases in human [10]. Oral tolerance varies when the antigen is administered in a soluble form as compared to a particulate state.

However, one major limitation with mucosal immunization is a striking balance between achieving an effective therapeutic response with a particular dose and preventing the induction of tolerance. Therefore, it is important to understand the mechanisms involved in controlling these responses. The key cells that determine the success or demise of a vaccine are antigen-presenting cells (APC), e.g., dendritic cells and T lymphocytes, including sub-populations of T-helper cells, T-cytotoxic cells, and regulatory T cells [7]. Immune interactions at the local level will have a profound effects on the type of immune response generated (e.g., first nuclear factor of activated T cell proteins is involved in both the generation of Th1 or Th2 cells and the maintenance of T-cell tolerance), so a clear understanding of local immune responses at the site of antigen uptake is essential [11].

On the other hand mucosal administration of vaccines also offers a number of practical advantages. Mucosal vaccination, being noninvasive in nature, does not require the use of needles. This would carry less risk of transmitting type of infections still associated with needle reuse [12, 13]. Additionally, mucosal vaccination is relatively easy and does not require expensive trained personnel. The production of mucosal vaccines may be cost effective in comparison to injectable vaccines that require high standards of purity, in addition to sterility. Moreover oral vaccines can also be expected to have much greater acceptability than injectable vaccines by causing no sore arm etc. Further it can enhance vaccine safety and adverse effect by avoiding direct contact between potentially toxic vaccine component and systemic circulation. Finally, mucosal vaccines allow for the easy administration of multiple vaccines [14].

9.3 M Cell as Gateway of Mucosal Immune Systems

The common features of all inductive mucosal sites include epithelial surface containing M cells overlying organized lymphoid follicles (Fig. 9.1). M cells offer functional openings in the epithelial barrier through vesicular transport activity [15]. They are well suited for efficient endocytosis and transcytosis. The M cells lack the rigid brush border cytoskeleton of their enterocyte neighbors, and their apical surfaces have broad membrane, a microdomain from which endocytosis occurs. The M cell basolateral membrane is deeply invaginated to form a large intraepithelial "pocket" containing T lymphocytes, B lymphocytes, and macrophages. This structural specialization brings the basolateral cell surface to within a few microns of the apical surface and greatly shortens the distance that transcytotic vesicles must travel to cross the epithelial barrier. Endocytic or phagocytic uptake of foreign antigens or particles is followed by rapid transcytosis directly to the intraepithelial pocket, with little or no retention in M cell lysosomes. After M cell transport, antigens are processed and presented by macrophages, dendritic cells, and B cells within and below the epithelium, resulting in generation of IgA-committed, antigen-specific B lymphoblast that proliferate locally in the germinal centers of O-MALT and migrate via the bloodstream to distant mucosal and glandular tissues, where

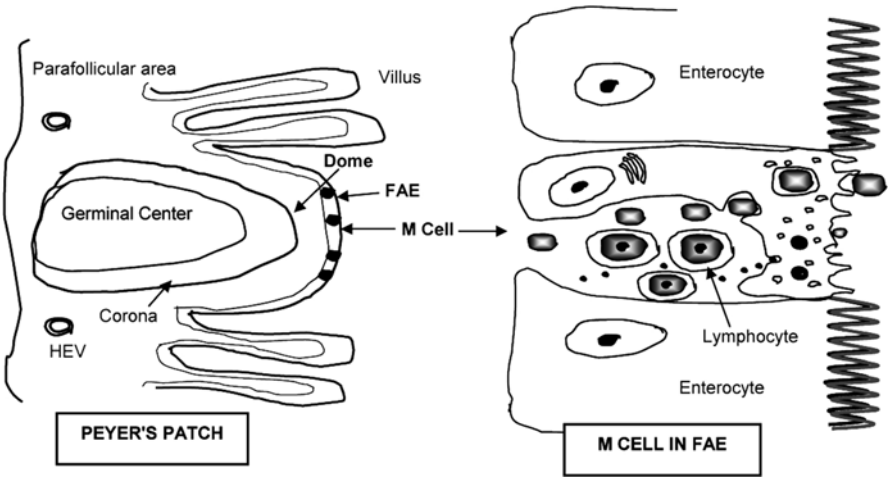

Fig. 9.1 M cells of the Peyer's patches

they differentiate into plasma cells. The dimeric or polymeric IgA antibodies thus produced are selectively bound by epithelial polymeric immunoglobulin receptors, transcytosed across epithelial cells, and released into glandular and mucosal secretions [16]. Thus, M cell plays a pivotal role in the elicitation of secretory immune response.

9.4 Distinguishing Feature of M Cells

The M cell apical surfaces are distinguished from their enterocyte neighbors by the absence of a typical brush border and the presence of variable microvilli [17] and a unique intraepithelial "pocket." This hallmark structural feature of fully differentiated M cells, provides a docking site for lymphocytes and shortens the distance that transcytotic vesicles must travel from the apical to the basolateral surface. The basolateral surface of the M cell includes the two major subdomains typical of all epithelial cells: the lateral subdomain is involved in cell–cell adhesion and contains Na+-K+-ATPase, and the basal subdomain interacts with the extracellular matrix and basal lamina.

The M cell apical surface differs from that of intestinal absorptive cells in other respects as well. Most M cells in Peyer's patches lack the highly organized brush border with uniform, closely packed microvilli typical of enterocytes. The actin-associated protein villin, confined to microvilli in enterocytes, is diffusely distributed in M cells [18], reflecting the modified apical organization and perhaps the ability to rapidly respond to adherence of microorganisms with ruffling and phagocytosis. M cells are also recognized by their lack of certain enterocytes surface glycoproteins. Whereas enterocyte brush borders have abundant hydrolytic enzymes,

these enzymes are usually reduced or absent on M cells [19, 20]. In addition, the thick filamentous glycocalyx typical of enterocytes is often absent from M cells, rendering the M cell surface more accessible to luminal materials. Further, M cell apical surfaces are coated with glycoproteins that display glycosylation patterns different from their enterocyte neighbors, and although the protein backbones have not been identified, the carbohydrate epitopes can be useful M cell identifiers which can be explored for targeted vaccine delivery.

9.5 M-Cell Surface Receptors

On the basis of the observation that M cells are targeted by a variety of soluble tracers and inert particles, it has been suggested that M cell targeting by microorganisms may be mediated, at least in part, by nonspecific, passive mechanisms. Such mechanisms are likely to be influenced by the physical properties of the pathogen, since the interaction of inert particles with M cells is influenced by the physicochemical properties of the particle preparation and by species related variations in M cell surface properties [21–24]. Additionally, specific receptors located in either the M cell apical membranes or the closely associated glycocalyx also contribute to M cell targeting by microorganisms. In the view of the fact that M cells bind and endocytose antibodies located in the lumen, there is interest in ascertaining whether there are specific Ig receptors on M cells and the follicle associated epithelium (FAE). A novel IgA receptor was discovered in mouse M cells with the potential to facilitate transport of sIgA from luminal secretions into GALT [25]. The finding that sialylated Lewis A antigen (SLAA) appeared to be expressed selectively on M cells from a small number of biopsies of human Peyer's and cecal patches was very exciting [26]. Although a "universal" M-cell marker is lacking, some exciting results from targeting experiments have been observed. For example, the coupling of recombinant cholera toxin B subunit to liposomes containing *Streptococcus mutans* antigens generated enhanced mucosal immunization in mice compared to untargeted antigen-loaded particles [27].

Ulex europaeus agglutinin 1 (UEA-1) has high specificity for the carbohydrate moiety, α-L-fucose, located on the apical membranes of mouse M cells [28, 29]. There have been successful efforts made into in vivo targeting to mouse M cells by conjugating the lectin to polymerized liposomes [30] and also to latex particles [31]. There are many success stories of UEA-1based delivery system is animal models; however, it is of limited value in vaccine delivery in human because the lectin is toxic, is subject to intestinal degradation, and its receptor is not expressed in human PP [26]. As another alternative to the lectin UEA-1, the edible orange peel mushroom *Aleuria aurantia* was used to target the α-L-fucose receptor. Coated poly(D,L-lactide-co-glycolide) (PLGA) particles were entrapped with birch pollen antigens and administered to mice as a potential oral allergen immunotherapy [32]. M cells can also be discriminated from enterocytes in the FAE on the basis of altered adherens junction protein expression [33]. Various other receptors and markers on FAE or M cells from various species have been summarized by Brayden et al. 2005 [34].

9.6 Mucosal Vaccine Delivery Systems

The mucosal vaccine delivery systems can be classified into live vectors (e.g., *Salmonella typhi*) and nonliving antigen delivery systems (Liposomes, nanoparticles, immunostimulating complexes, etc.). These mucosal vaccine carriers are summarized in Table 9.1, and in this chapter two potential mucosal vaccine delivery systems, i.e., polymeric particles and liposomes are described.

Table 9.1 Various options for the vaccine delivery by mucosal route

Option for mucosal vaccine delivery	Comments	Ref.
Live bacterial vector, (*Salmonella, E. coli., Mycobacterium*)	The capability of some microorganism to colonize and infect intestinal mucosa and the potential for including genes for unrelated microorganism encoding relevant antigens represent an attractive means for design of novel mucosal vaccines	[35, 36]
Live viral vector, (Vaccinia virus, Canary pox virus, Picornaviruses)	Live recombinant vector vaccines have the advantage that they can stimulate both humoral and cell mediated immune responses and have potential for immunization alone or in combination with a subunit vaccine	[37]
Virosomes	Viral surface glycoproteins possess high affinity for receptors on mucosal surfaces thus providing a mechanism for efficient attachment of antigen to mucosal surfaces	[38]
Liposomes	Liposome vaccine may enhance uptake and processing by enclosing the antigen in the lipid vesicles. Although they are not completely resistant to lipases and bile salts found in the small intestine, cholesterol-containing liposomes can provide at least partial resistance. Polymerized liposomes are considered to be a good candidate for the oral immunization	[30]
Nanoparticles and microparticles	Particles can be taken up by the M cells of the Peyer's patches. Nanoparticles/microparticles have advantage over microbial system in which immune response to the live vector can dominate	[39, 40]
Cochleates	Induce a strong and prolonged immune response manifested by the presence of mucosal and systemic antibody and cytotoxic T cells	[41]
Mucoadhesive polymers	Mucoadhesive polymer avoids the complexity of microencapsulation technology. They have been tested for nasal immunization but had been overlooked for oral vaccine delivery	[42]
Cholera toxin B subunit conjugates	Proteins coupled to CTB acquire its mucosal immunogenic properties due to the high affinity of CTB for cell surface G_{M1} ganglioside and its avid uptake by M cells on intestinal Peyer's patches	[43, 44]
Immune-stimulating complex matrix (ISCOM)	ISCOMs are cage like structures into which antigen can be incorporated resulting in enhanced immune response after their administration. ISCOMs are resistant to solubilization by the bile salts deoxycholate, cholate, and taurocholate	[45]
DNA delivery to mucosal surface	Direct mucosal administration of DNA plasmid expression vector encoding a protein antigen is more efficient than recombinant viral vector for gene transfer to muscle tissue. Mannosylated niosomes are also demonstrated to be a potential adjuvant carrier for oral genetic immunization	[46]
Transgenic plants	This technology represent an important step for the production of inexpensive edible immunogen suitable for immunization of large population	[47, 48]

9.6.1 Polymeric Particles

It has been demonstrated that the incorporation of an antigen into microparticles [49], or its adsorption to the surface of biodegradable microparticles [50], resulted in the induction of enhanced serum and secretory antibody responses, following oral administration. Subsequently, it was shown that oral delivery of an antigen entrapped in PLGA microparticles also resulted in the induction of enhanced immunity [51]. During last two decades biodegradable PLGA based microparticles/nanoparticles have been explored extensively [52–54]. Binding and uptake of the particles was enhanced when particles were conjugated to B subunit of *E. coli* heat labile enterotoxin (LTB), the plant lectin, ConA or vitamin B12 following oral delivery to rats [55]. Covalent attachment of UEA-1 to polystyrene microspheres and oral delivery to mice result in selective binding to and rapid uptake by the Peyer's patch M-cells [56]. Orally administered polystyrene microparticles with attached *Lycopersicon esculentum* agglutinin (LEA) were taken to a greater extent than unconjugated particles in the rats [57]. The linkage of sepharose beads to WGA and *Solanum tuberosum* lectin (STL) enhanced their binding to caco-2 cells [58]. These observations and a body of additional data suggest that lectins are potential tools for the enhanced binding and internalization of orally delivered drugs and drug delivery systems and efficiency of oral vaccines can be improved by M-cell targeting.

We have described the development of PLGA nanoparticles loaded with HBsAg and the antigen stabilization in the presence of trehalose and $Mg(OH)_2$. Additionally, UEA-1 lectin was anchored to the nanoparticles to target them to M-cells of the Peyer's patches [59]. The results suggest that HBsAg can be successfully stabilized by co-encapsulation of protein stabilizers. The lectinized nanoparticles have demonstrated approximately fourfold increase in the degree of interaction with the bovine submaxillary mucin (BSM) as compared to plain nanoparticles and sugar specificity of the lectinized nanoparticles was also maintained. The serum anti-HBsAg titre obtained after oral immunization with Hepatitis B surface antigen (HBsAg) loaded stabilized lectinized nanoparticles was comparable with the titre recorded after alum-HBsAg given intramuscularly. The stabilized UEA-1 coupled nanoparticles exhibited enhanced immune response as compared to stabilized non-lectinized nanoparticles. Furthermore, the stabilized lectinized nanoparticles elicited sIgA in the mucosal secretion and IL-2 and IFN-γ in the spleen homogenates.

9.6.1.1 Factors Affecting Uptake of Polymeric Particles

The M cell appears to be the primary route of entry into the host for several enteric viral pathogens. The mechanism for the uptake of synthetic and biodegradable microparticles by M cells appears similar to that observed for bacteria. Microparticle uptake initially involves contact with the microvillus projections on the M cell

surface followed by rapid phagocytosis through the extension of apical membrane processes. Various factors affecting the uptake of particles are described below.

Particle Size

In general, smaller microparticles are absorbed to a greater degree than larger microparticles. The smaller particles are distributed more easily to distant sites, and remain detectable for longer periods of time. These conclusions are consistent for microparticles made of different polymers and different size ranges. Jani et al. [60] studied the comparative uptake of 50 nm, 500 nm, and 1 μm polystyrene particles and found that 50 nm particles are absorbed and distributed quicker than 500 nm and 1 μm particles. Florence suggested that decrease in particle diameter may result in increased uptake below 1 μm and particles above 3 μm are taken up by the Peyer's patches but remained there [61].

Hydrophobicity

Hydrophobicity of the particles influences profoundly their uptake behavior. Jung et al. reported that uptake of nanoparticles prepared from hydrophobic polymer was higher than from particles with more hydrophilic surfaces [62]. They further added that hydrophobic polystyrene nanoparticles interact with M cells with more affinity than absorptive epithelia whereas less hydrophobic PLGA nanoparticles interact with both cell types. Other investigators have shown that decreasing surface hydrophobicity, by the adsorption of poloxamers 235, 238, 407, or poloxamines 901, 904, and 908, may decrease the uptake of polystyrene microparticles into cells of the immune system, thereby avoiding elimination [63]. The charge on the particles also determines their uptake by the intestinal epithelia. Although the charged particles are taken up, their uptake was less than the non-ionic hydrophobic particles [61]. The negatively charged and neutral particles exhibited greater affinity to PP in comparison to positively charged particles [64]. This finding was in accordance with previous report that a combination of both, negative charge and increased hydrophobicity of the particles improve the gastrointestinal uptake [62].

Effect of Dose and Vehicle on Uptake

The extent of particle uptake is also influence by the dosing. It was observed that polystyrene particles were identified in Peyer's patches with difficulty after 1 day of feeding, but were readily identified following chronic feeding. Le Ray et al. [65] have shown that changing the vehicle in which the particles were administered could enhance the extent of uptake of polystyrene particles in mice. Further, volume and tonicity of the administered vehicle also have an effect on the extent of uptake [66].

Glass Transition Temperature and Crystallinity of the Polymer

Glass transition temperature (Tg) and crystallinity of the polymer are two important bulk properties of the polymer affecting the release of incorporated components. Without proper release characteristics, drugs or vaccines incorporated into microparticles may be released either prematurely or to insignificant levels before elimination. Tg is the temperature at which a transition occurs from the glassy state to the rubbery state resulting in increase in the molecular motion and free volume of the amorphous polymer, which in turn increases the drug release from the polymer. Above Tg the polymer acquires sufficient thermal energy for isomeric rotational motion or for significant torsional oscillation to occur about most of the bonds in the main chain. This leads to an increase in the free volume of the amorphous polymer, and thus in turn, the release of incorporated bioactive [67].

Migliaresi et al. observed an increase in the degree of crystallinity of polylactic acid with the degradation of the polymer [68]. This could be related to the faster degradation of the amorphous phase of the semicrystalline polymer, resulting in loss of amorphous material and a concomitant increase in crystallinity. A decrease in crystallinity increases the drug release because the diffusion coefficient and solubility of the drug in polymer are inversely proportional to at least the first power of the amorphous content. The structural features, which influence the crystallinity of the polymer, are similar to those, which affect the glass transition temperature.

Bioactive (drug/vaccine) may release from nanoparticles/microparticles by several mechanisms including surface and bulk erosion, disintegration, microparticle hydration, drug diffusion and desorption. These bioactive release mechanisms are in turn controlled by bulk properties such as the molecular weight of the polymer (affecting crystallinity and glass transition temperature), the copolymer composition, polymer matrix density and the extent and nature of the cross-linking. By adjusting the blend ratio of PLGA/polyethyleneglycol (PEG) the release profile of entrapped dextran and rabbit gamma immunoglobulin (IgG) microparticles can be varied [69]. The release rate of entrapped compounds increased with increasing PEG content because of the leaching out of PEG from the polymer blend into the aqueous phase during drug release, resulting in the increased porosity of microparticles. Changing the monomer ratio of lactide/glycolide in PLGA microparticles from 75:25 to 50:50 led to an increase in the release rate of entrapped OVA due to an increase in the degradation rate of the microparticles [70].

Effect of Additives

Various additives are involved in the fabrication of nanoparticles/microparticles. Polyvinyl alcohol (PVA) is the most commonly used emulsifier in the formulation of lactide and poly (D,L-lactide-co-glycolide) nanoparticles/microparticles. A fraction of PVA remains associated with the nanoparticles/microparticles despite repeated washing because PVA forms an interconnected network with the polymer at the interface. The residual PVA affect different pharmaceutical properties of the

particles such as particle size, zeta potential, polydispersity index, surface hydrophobicity, protein loading and also slightly influenced the in-vitro release of encapsulated protein. Importantly, nanoparticles with higher amount of residual PVA had relatively lower cellular uptake despite their smaller particle size [71]. The lower cellular uptake of nanoparticles with higher amount of residual PVA is attributed to the higher hydrophilicity of the nanoparticle surface. Trehalose is well-documented protein stabilizer. We have observed increase in the release of HBsAg with trehalose stabilized PLGA nanoparticles/microparticles when compared with PLGA nanoparticles/microparticles without trehalose [72]. Since the protein stabilizer (trehalose) reduced denaturation at the aqueous-organic interface, the payload of HBsAg was increased and this was reflected in augmented cumulative percent release. Moreover, sugars (e.g., trehalose, sucrose) have appreciable solubility in aqueous media. They dissolve rapidly from the matrix leaving a porous matrix, which in turn releases antigen/bioactive relatively faster.

Effect of Species, Animal Age, and Food Ingestion on Uptake

The species variation can affect uptake extent of the particles. The uptake of polystyrene particles in rabbit was at least an order of magnitude greater than mice because of the greater abundance of the M cells in the Peyer's patches [73]. Le Fevre et al. showed greater uptake of polystyrene particles in older mice [74]. Other investigator reported that age of the animal did not affect the extent of polystyrene particle uptake in rats [75]. The extent of uptake in the mice was enhanced by the presence of food, which may delay the intestinal transit of the particles [75].

Intestinal Mucus Layer Characteristics

The uptake of the nanoparticles/microparticles is preceded by their passage through two barriers, i.e., the mucus gel layer and the mucosa. Intestinal mucus is a high molecular weight glycoprotein secretion, which covers the mucosa with a continuous adherent blanket. The mucus layer protects the gastrointestinal mucosa from potentially harmful bacteria, pathogens, or chemicals [76]. Several investigators have reported diminished diffusion of small and large compounds such as bovine serum albumin (BSA), lysozyme, tertiary amines, and quaternary ammonium compounds [77]. Mucus acts as a barrier by entrapping microparticles, causing agglomeration, which results in an increase in net size and a resultant decrease in diffusion coefficient, and by decreasing the diffusion coefficient through the mucus thereby restricting diffusion to the mucosa layer. Since the high number of sulfate, sialic acid, and sugar moieties in the carbohydrate side chains of the mucin molecule impart a highly negative charge to mucin [76], it may be expected that electrostatic interactions between positively charged drugs and particles would cause binding within the mucin layer. Several mechanisms have been documented in literature for the uptake of the nanoparticles/microparticles (Table 9.2).

Table 9.2 Site-specific mechanism for the uptake of the nanoparticles/microparticles

Uptake site	Mechanism	Particle size
Intestinal epithelial cells on villus tip	Paracellular transport	100–200 nm
Villus tips	Persorption	5–150 μm
Intestinal macrophages	Phagocytosis	1 μm
Enterocytes/M cells	Endocytosis	<200 nm
Peyer's patches	Transparacellular	<10 μm

9.6.2 Liposomes

Although some success has been achieved in experimental studies involving oral administration of antigens entrapped in liposomes [78], disappointing results have also been reported by others [79, 80]. Nevertheless, a liposomal vaccine has been shown to induce a salivary IgA response in a small number of human volunteers, following oral immunization [81]. Furthermore, although it has been reported that liposomes are unstable in the gut and are not taken up by epithelial cells [82], the uptake of liposomes into Peyer's patches has been reported [83]. Polymerized liposomes exhibited many characteristics which make them attractive antigen carriers. They are in the nanometer size range suitable to be transported by M cells. Further, liposomes formed by polymerization of 1, 2-bis[(2E,4E)-Octadecadienoyl]-sn-glycero-3-phosphocholine (DODPC) can be manipulated by incorporation of different lipid groups, thus adjusting surface charge and rigidity. DODPC liposomes also have carboxylate groups on their surface which could facilitate cell receptor targeting [84]. A number of studies showed the potential of targeted liposomes in the induction of immune response. Sugimoto et al. [85] and Fukasawa et al. [86] showed that liposomes coated with mannopentose and dipalmitoyl phosphatidylethanolamine (Man5-DPPE) could elicit strong cellular immune responses. It has been shown that UEA1-coated liposomes can be efficiently targeted to murine M cells in vivo [30], thus supporting the hypothesis that decorating liposomes with M cell specific lectins may efficiently target orally delivered antigens to M cells and possibly DCs residing in the FAE.

Liposome offers a number of potential advantages for the mucosal vaccine delivery [87]. While native liposomes may target Peyer's patch M-cells [83], the efficiency of binding and subsequent uptake is thought to be relatively low following oral gavage of mice [88, 89]. Therefore, we have developed lectin conjugated liposomes for M-cell targeted vaccine delivery [90]. The activity of the liposome-conjugated with UEA-1 towards exogenously provided BSM and affinity toward competing sugar were studied to determine targeting efficacy of lectinized liposomes. The lectinized liposomes showed good BSM binding in absence of specific sugar for UEA-1 (α-L-fucose). The same formulations, however, showed significant decrease in the percent BSM binding in the presence of α-L-fucose (Fig. 9.2). M-cell targeting of the liposomes was studied by dual staining by using CLSM. The interaction of liposomes with M-cells may be facilitated by the relatively thin M-cell

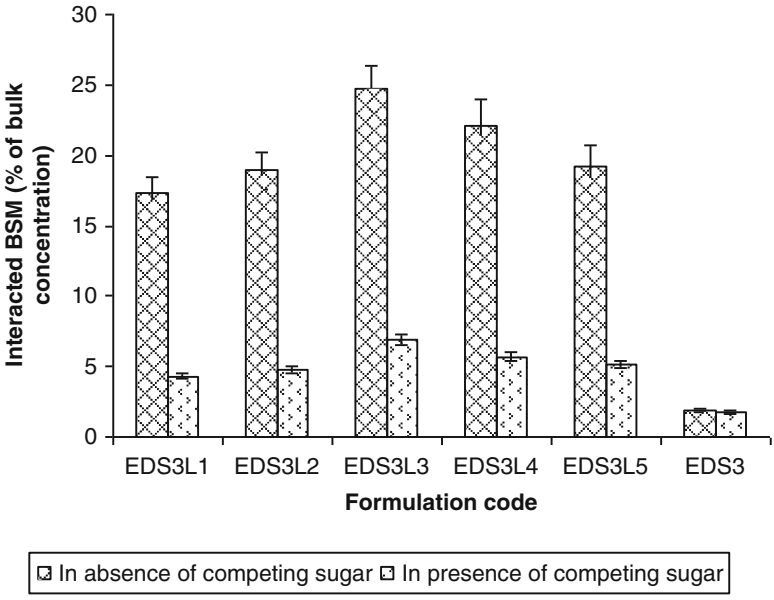

Fig. 9.2 Binding of BSM to lectinized liposomes (EDS3L1-EDS3L5) and optimized non-lectinized liposomes (EDS3) in presence and absence of competing sugar (Reproduced with permission from [90])

glycocalyx which appears to promote the interaction of small particles with the M-cell surface membranes. Also, there are various other factors which may affect the Peyer's patch uptake of particles [5]. Lectinized liposomes showed higher immune response in comparison to non-lectinized formulation (Figs. 9.3 and 9.4). UEA-1 anchored liposomes selectively targeted to M-cells of Peyer's patch, and M-cell adherent liposomes are thought to be rapidly endocytosed. M-cell apical surfaces are coated with glycoproteins that display glycosylation patterns different from their enterocyte neighbors, and although the protein backbones have not been identified, the carbohydrate epitopes can be useful M-cell identifiers [91]. Thus, M-cell targeted liposomes were found to have greater accessibility to M-cell and as a consequence they showed enhanced immune response as compared to non-lectinized liposomes.

9.7 Targeting of Mucosal Vaccine Delivery Systems

Targeting to the specific site of the gastrointestinal tract is an effective means for enhancing the uptake of the particulate systems. Depending on the pharmaceutical application, different targets within the gastrointestinal tract can be exploited (Fig. 9.5)

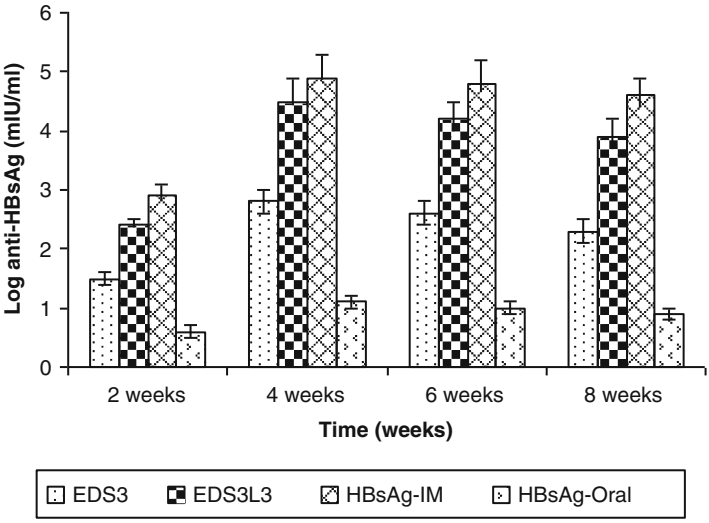

Fig. 9.3 Serum anti-HBsAg antibody levels with lectinized liposomal (EDS3L3) and optimized non-lectinized liposomes (EDS3). The animals were immunized orally with the preparation equivalent to the 10 μg HBsAg for three consecutive days and booster dose was given after third week of first immunization. Single intramuscular immunization with booster dose after third week was given with the alum-HBsAg to serve as standard and HBsAg without liposomes was administered orally followed by a booster dose after third week for the comparison. After 4 weeks there was significant difference ($P<0.001$) among EDS3L3, EDS3, HBsAg-Oral or HBsAg-IM, however, the difference between EDS3L3 and HBsAg-IM was not significant ($P>0.05$). (Reproduced with permission from [90])

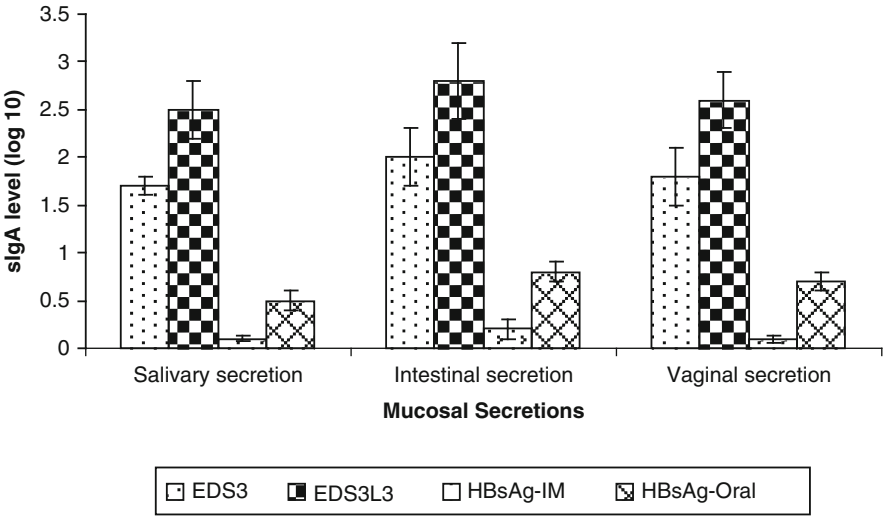

Fig. 9.4 sIgA level in mucosal secretions after oral immunization with HBsAg encapsulating lectinized liposomes (EDS3L3) and optimized non-lectinized liposomes (EDS3). The animals were immunized orally with the preparation equivalent to the 10 μg HBsAg for 3 consecutive days and booster dose was given after third week of first immunization. Single intramuscular immunization with booster dose after third week was given with the alum-HBsAg to serve as standard and HBsAg without liposomes was administered orally followed by a booster dose after third week for the comparison. The salivary, intestinal and vaginal secretions were collected after eight week of first immunization. The differences in the antibody level was significant ($P<0.05$) among EDS3L3, EDS3, HBsAg-Oral or HBsAg-IM. (Reproduced with permission from [90])

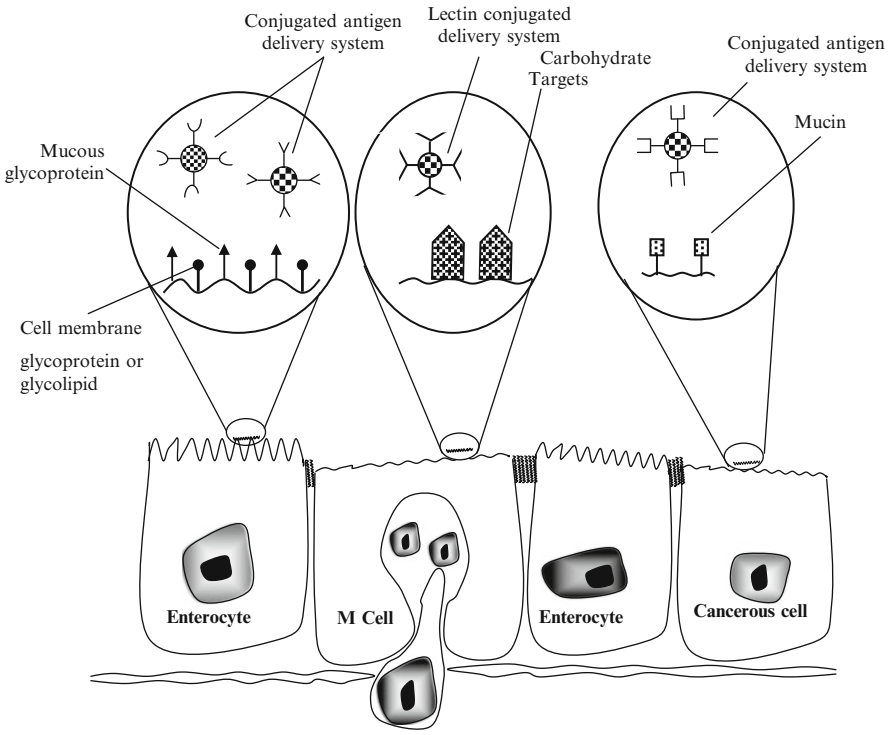

Fig. 9.5 Various options for the targeted delivery. Mucus glycoprotein, M-cells and abnormal glycoprotein secreted by the cancerous cells can serve as receptor for binding with various ligands conjugated to drug/vaccine delivery system

including mucus glycoproteins (mucins), epithelial cells, M-cells, Peyer's patches or GALT, and abnormal glycoproteins secreted by cancerous cells (local tumors). Brayden et al. reviewed novel M-cell surface receptors that could be used to target orally delivered antigens [34]. Gene expression technology has provided evidence that coculture model has many characteristics of Peyer's patches. It has been demonstrated that epithelial genes that were unregulated in coculture corresponds to genes expressed selectively in mouse FAE [92]. These include claudin 4, laminin β3, tetraspanTM4SF3 and a matrix metalloproteinase. Claudin 4 appears to have dual location at tight junctions (M cell–enterocyte), and as an M cell and enterocyte cytoplasmic receptor, it is involved in the trafficking of pathogens across M cells to lymphocyte or dendritic cells. Peptidoglycan recognition protein (PGRP)-S and PGRP-L are other potential targets co-localized with UEA-positive cells in microdissected mouse Peyer's patches and in the FAE respectively [93]. Other targeting agents like lectins, invasins, antibodies, etc. can be used as a means of enhancing targeting and thus in turn particle uptake. Various ligands for the targeted immunization are summarized in Table 9.3.

Table 9.3 Various ligands for targeted mucosal immunization

Targeting ligand	Targeting site	Conjugated material	Ref.
Ulex europaeus 1	Mouse Peyer's patch M cells	FITC, HRP	[94]
		Polystyrene microparticles	[56]
		Liposomes	[89]
mAb 5B11	Brush border of both M cells	Polystyrene latex microparticles	[95]
Lycopersicon esculentum	Rat intestine	Polystyrene microparticles	[96]
Secretory IgA	Mouse Peyer's patch M cells	Polystyrene microparticles	[97]
		Liposomes	[98]
Triticum vulgaris	Mouse intestine	Liposomes	[89]
Bandeiraea simplicifolia I isolectin B 4	Hamster NALT	Biotin, HRP	[99]
	M cells		
Invasin-C192	Intestinal M cells	Polystyrene nanoparticles	[100]
O-palmitoyl mannan	Peyer's patch	Niosomes	[101]
Cholera toxin B subunit	M cells of Peyer's patches	Liposomes	[102]
		Bilosomes	[103]

9.7.1 Lectin Mediated Targeting

Lectins are proteins or glycoproteins capable of specific recognition of and reversible binding to carbohydrate determinants of complex glycoconjugates, without altering the covalent structure of any of the recognized glycosyl ligands. Lectin receptors are expressed on various cells such as endothelial cells, hepatocytes, macrophages, monocytes and lymphocytes. They are efficient in recognizing the complex oligosaccharide epitopes, which are also present on the cell surface or could be exogenous glycoconjugate ligands mimics of endogenous carbohydrate epitopes [104]. Lectins are potential tools for the targeting of particulate vaccines to the M cell of the Peyer's patches, which are the sampling site of the mucosal immune system. Nanoparticles/microparticles may also be targeted to mouse Peyer's patch M cells by coating with the lectin UEA1 for the development of an effective mucosally targeted vaccines. In studies reported by Foster et al. [56], polystyrene microparticles (0.5-mm diameter) were covalently coated with the lectin UEA1 and administered to mice both by injection into ligated gut loops of anaesthetized animals and by oral gavage. In contrast to other proteins, lectin UEA1 coating selectively targeted the microparticles to mouse Peyer's patch M cells, and M cell adherent microparticles were rapidly endocytosed. Although the lectins specific for the human intestinal M cells await identification, human M cells preferentially display the sialyl Lewis A antigen [26] and this could be envisaged for targeting vaccines to the mucosal immune system. Future studies should determine whether lectins may similarly be used to target vaccine candidate in PLA/PLGA based delivery construct to intestinal M cells, and whether such targeting enhances the immune response to antigens delivered by these carrier systems. Recently our group has developed biodegradable polymer based stabilized microparticles and nanoparticles for the mucosal vaccination [39] and also envisaged lectin for the targeted

mucosal immunization [40]. Additionally we have also explored cholera toxin B subunit conjugated bilosomes [43] and mannosylated niosomes [101] as potential carrier-adjuvants for the targeted oral mucosal immunization.

9.7.2 Invasin Mediated Uptake

Invasins are virulent factor usually associated with the bacterial cell wall, and have the capacity to stimulate cytokine synthesis and to interact with mammalian cells by distinct mechanisms [105]. Young et al. demonstrate the potential of invasins for the internalization process [106]. For this purpose, microparticles were coated with Yersinia enterocolitica invasin and the resulting conjugates put in contact with human laryngeal epithelial cells (Hep-2 cells). The presence or absence of internalised conjugates was monitored by transmission electron microscopy and light microscopy. It was clear that conjugates not only bound, but also were internalised by the Hep-2 cells. In contrast, control conjugates were rarely associated with these cells. *Salmonella typhimurium* selectively bind to, invade and destroy murine M cells and have been studied as live oral vaccine delivery vehicles. The M cell targeting by *S. typhimurium* is mediated by a specific adhesin (long polar fimbria; LPF) [107]. Reovirus type 1 is another ligand, which selectively adhere to, and endocytosed by intestinal M cells. It was demonstrated that proteolytic processing of native reovirus type 1 is required for adhesion to murin M cells and this is dependent on retention of modified σ1 and/or product of μ1 outer capsid protein. It has been suggested that σ1 protein has potential for targeted delivery [108].

9.7.3 Antibody Directed Targeting

The use of antibodies and monoclonal antibodies has been proposed for specific targeting within the gastrointestinal tract. It was observed that binding of the 5B11 monoclonal antibody, with specificity for rabbit M cells, to polystyrene particles, enhanced uptake by rabbit M-cells 3–3.5-times when compared to controls (plain latex and IgM of unrelated specificity-conjugates) [109]. The ability of different conjugates, obtained by coating latex microparticles with albumin, bovine growth hormone (bGH), human IgG, secretory IgA (hIgA), and bGH complexed with an IgG antibody raised against bGH (bGH-Ab), to be taken up by M cells was studied. It was found that the selectivity in binding to and entry into M-cells was improved by the use of IgG or bGH-Ab. Moreover, the appearance of conjugates in rat mesenteric lymph showed a similar selectivity to that found for binding and entry into M-cells. Ferritin-loaded liposomes conjugated to IgA were investigated for mucosal immunization via the rectum [109]. It was observed that the presence of IgA on the liposome surface increased the uptake of conjugates by Peyer's patches, and the local rectal/colon immune response to ferritin about fivefold over uncoated liposomes.

9.8 Link Between M Cell Uptake of Particles and Induction of Mucosal Immunity

Generally, macromolecules that adhere to mucosal surfaces tend to induce vigorous mucosal immune responses, whereas soluble, non-adherent proteins do not [110]. Pathogens and vaccines that can bind selectively to M cells appear to be most effective in mucosal invasion and induction of mucosal immune responses, and this assumption underlies many of the current approaches to vaccine design. Presently, there have been few successful oral vaccine trials in man using non-live antigens in particles. Mixed results were obtained in a limited number of human subjects dosed with untargeted PLG microspheres containing the highly potent *E. coli* colonization factor antigen II as potential vaccine for enterotoxigenic *E. coli* [111]. These studies suggest indirectly that there is likely to be some particle uptake in man; however, a quantifiable relationship between enhanced M-cell targeting of vaccine loaded particles and an enhanced immune outcome remains illusive. Apart from the immunology issues, pharmaceutical factors are critical in the design of antigen-loaded particles. These include antigen stability issues, premature antigen release from particles in the intestine and incomplete antigen release within Peyer's patch at the right time. There are many reports of induction of immune response using M cell targeted particles in animal models; however, more clinical studies are required to establish a correlation between M cell targeting and elicitation of immune response. A Phase I trial of a single shot tetanus toxoid (TT) and diphtheria toxoid in PLGA microspheres is still some way off, even though outcomes in mice and guinea-pigs showed positive and durable immune responses using antigen-loaded microparticles [54]. It is possible that particulate antigens, when targeted to inductive immune sites, might perform even better in man than in laboratory animals because the pathways of antigen presentation by human dendritic cells are relatively well established and might even be superior [112]. Advancement in the non-living vaccine delivery system coupled with the suitable targeting strategy may lead to a successful clinical trial.

9.9 Future Perspectives

Recent discoveries in both mucosal vaccine delivery and mucosal adjuvant research have significantly improved the effectiveness of mucosal immunization in animal models. The mucosal immune system is a complex system that generate large amount of s-IgA as well as cell mediated immunity at mucosal surfaces to prevent pathogen infiltration and inflammation. The mucosal immune system should be most efficient in providing protection against pathogens and generating longer lasting protection through using attenuated pathogen for vaccine. The only mucosal vaccines approved for humans are attenuated pathogens. Future mucosal vaccines will also involve vaccines strategies other than attenuated pathogens. New delivery strategies such as immunization of live recombinant vectors, DNA plasmids, and

transgenic plants to deliver antigens present promises to improve the efficiency of mucosal antigen delivery. Further, DNA vaccines and subunit vaccines such as bacterial adhesion in combination with potent mucosal adjuvants (such as QS21; a saponin, unmethylated CpG motifs or cytokines such as IL-12) or mucosal delivery systems based on nanoparticles/microparticles will have the potential to be the next generation of vaccines.

Mucosal delivery of vaccines offers a number of significant advantages over systemic delivery. There are many alternative approaches to the mucosal delivery of vaccines and our group has explored various versions of delivery systems [39, 40, 43, 90, 101, 113]. One potential approach to the mucosal delivery of vaccines is the encapsulation or entrapment of antigens into polymer based nanoparticles or microparticles. Polymeric delivery systems can be manipulated to enhance the efficacy of mucosally administered vaccines in a number of ways; they can protect antigens from degradation, concentrate them in one area of the mucosal tissue for better absorption, extend their residence time in the body, or target them to sites of antigen uptake (e.g., Peyer's patches in the gut).

Immunization does not always stimulate immunity because of the insufficient elicitation of immune responses. Such limitations have spurred the development of new adjuvant and antigen-delivery systems. Adjuvant plays an important role in enhancing the efficacy of vaccines. Recombinant proteins or synthetic peptides are safer than crude inactivated microorganism, but less immunogenic. This limitation can be overcome by using specific adjuvant. The adjuvant selection depends on several criteria, like the target species, the antigens, the type of desired immune response, the route of administration, or the duration of immunity. So far, biodegradable polymers particularly of PLGA have been used, considerably because of their well-known degradation properties. An area requiring additional efforts is analytical characterization of protein-encapsulated nanoparticles/microparticles. Advanced methods for protein characterization is in demand to approach problem of protein stabilization in polymer based delivery systems. Development of in vitro–in vivo correlation for protein release from protein nanoparticles/microparticles is another issue. More intensive interactions between immunologists and drug delivery specialists are required to understand protein release and its presentation to the immune system. Significant progress has been made recently with biodegradable polymer, mainly PLGA and various approaches are being considered for the effective stabilization of proteins in microparticles during the preparation process. Among them we have focused on the basic additives mediated stabilization of therapeutic protein within carrier construct [39, 40]. Nevertheless, all the approaches involving encapsulation of antigens into nanoparticles/microparticles are likely to suffer from the some significant drawback; the extent of uptake of the particles across the gut appears to be limited. Whether or not the extent of uptake in humans is sufficient to allow the development of an effective oral vaccine is currently unknown. However, it is clear that in rodents, the extent of uptake of nanoparticles/microparticles can be enhanced using targeting ligands.

Numerous studies suggest that the efficiency of particle absorption can be improved through modification of particle surfaces with targeting molecules such as

antibodies or lectins. Albeit the results are promising, it is not known if any of the strategies will be effective in humans because it is currently unclear if particulates are taken up in human GIT. Additionally the uptake mechanisms and absorption efficiencies are not known. Thus, current knowledge obtained from animal models may or may not be extendible to human beings. Continued research to understand the interconnection and sub-compartmentalization of the common mucosal system will certainly guide the rational selection for routes of mucosal administration. An efficient delivery vehicle, combined with an effective adjuvant given through an optimal route of administration, will ultimately allow for the development of a successful needle-free (mucosal) vaccine in humans.

References

1. Davis SS (2001) Nasal vaccines. Adv Drug Deliv Rev 51:21–42
2. Nugent J, Po AL, Scott EM (1998) Design and delivery of non-parenteral vaccines. J Clin Pharm Ther 23:257–285
3. Neutra MR, Kozlowski PA (2006) Mucosal vaccines: the promise and the challenge. Nat Rev Immunol 6:148–158
4. Mestecky J, Michalek SM, Moldoveanu Z, Russell MW (1997) Routes of immunization and antigen delivery systems for optimal mucosal immune responses in humans. Behring Inst Mitt 98:33–43
5. Vyas SP, Gupta PN (2007) Implication of nanoparticles/microparticles in mucosal vaccine delivery. Expert Rev Vaccines 6:401–418
6. Holmgren J, Czerkinsky C, Eriksson K, Mharandi A (2003) Mucosal immunization and adjuvants: a brief overview of recent advances and challenges. Vaccine 21:S2/89–S2/95
7. Holmgren J, Czerkinsky C (2005) Mucosal immunity and vaccines. Nat Med 11(Suppl 4): S45–S53
8. Eriksson K, Kilander A, Hagberg L, Norkrans G, Holmgren J, Czerkinsky C (1998) Induction and expression of intestinal homoral immunity in HIV-infected individuals: prospects for vaccination against secondary enteric infections. Pathobiology 66:176–182
9. Magistris MTD (2006) Mucosal delivery of vaccine antigens and its advantages in pediatrics. Adv Drug Deliv Rev 58:52–67
10. Ogra PL, Faden H, Welliver RC (2001) Vaccination strategies for mucosal immune responses. Clin Microbiol Rev 14:430–445
11. Macian F (2005) NFAT proteins: key regulators of T-cell development and function. Nat Rev Immunol 5:472–484
12. Levine MM (2003) Can needle-free administration of vaccines becomes the norm in global immunization? Nat Med 9:99–103
13. Ryan EJ, Daly LM, Mills KHG (2001) Immunomodulators and delivery systems for vaccination by mucosal routes. Trends Biotechnol 19:293–304
14. Chen H (2000) Recent advances in mucosal vaccine development. J Control Release 67:117–128
15. Neutra MR, Pringault E, Kraehenbuhl JP (1996) Antigen sampling across epithelial barriers and induction of mucosal immune responses. Annu Rev Immunol 14:275–300
16. Apodaca G, Bomsel M, Arden J, Breitfeld PP, Tang KC, Mostov KE (1991) The polymeric immunoglobulin receptor: A model protein to study transcytosis. J Clin Invest 87: 1877–1882
17. Owen RL (1977) Sequential uptake of horseradish peroxidase by lymphoid follicle epithelium of Peyer's patches in the normal unobstructed mouse intestine: an ultrastructural study. Gastroenterology 72:440–451

18. Kerneis S, Bogdanova A, Colucci-Guyon E, Kraehenbuhl JP, Pringault E (1996) Cytosolic distribution of villin in M cells from mouse Peyer's patches correlates with the absence of a brush border. Gastroenterology 110:515–521
19. Owen RL, Bhalla DK (1983) Cytochemical analysis of alkaline phosphatase and esterase activities and of lectinbinding and anionic sites in rat and mouse Peyer's patch M cells. Am J Anat 168:199–212
20. Savidge TC, Smith MW (1995) Evidence that membranous (M) cell genesis is immunoregulated. In: Mestecky J (ed) Advances in mucosal immunology. Plenum, New York
21. Jepson MA, Clark MA, Foster N, Mason CM, Bennett MK, Simmons NL, Hirst BH (1996) Targeting to intestinal M cells. J Anat 189:507–516
22. Jepson MA, Simmons NL, O'Hagan DT, Hirst BH (1993) Comparison of poly(DL-lactide-co-glycolide) and polystyrene microsphere targeting to intestinal M cells. J Drug Target 1:245–249
23. Ermak TH, Giannasca PJ (1998) Microparticle targeting to M cells. Adv Drug Deliv Rev 34:261–283
24. Clark MA, Jepson MA, Hirst BH (2001) Exploiting M cells for drug and vaccine delivery. Adv Drug Deliv Rev 50:81–106
25. Mantis NJ, Cheung MC, Chintalacharuvu KR, Rey J, Corthesy B, Neutra MR (2002) Selective adherence of IgA to murine Peyer's patch M cells: evidence for a novel IgA receptor. J Immunol 169:1844–1851
26. Giannasca PJ, Giannasca KT, Leichtner AM, Neutra MR (1999) Human intestinal M cells display the sialyl Lewis A antigen. Infect Immun 67:946–953
27. Harokopakis E, Hajishengallis G, Michalek SM (1998) Effectiveness of liposomes possessing surface-linked recombinant B subunit of cholera toxin as an oral antigen delivery system. Infect Immun 66:4299–4304
28. Clark MA, Jepson MA, Simmons NL, Booth TA, Hirst BH (1993) Differential expression of lectin-binding sites defines mouse intestinal M-cells. J Histochem Cytochem 41:1679–1687
29. Giannasca PJ, Giannasca KT, Falk P, Gordon JI, Neutra MR (1994) Regional differences in glycoconjugates of intestinal M cells in mice: potential targets for mucosal vaccines. Am J Physiol 267:G1108–G1121
30. Clark MA, Blair H, Liang L, Brey RN, Brayden D (2001) Targeting polymerized liposome vaccine carriers to intestinal M cells. Vaccine 20:208–217
31. Lambkin I, Pinilla C, Hamashin C, Spindler L, Russell S, Schink A, Moya-Castro R, Allicotti G, Higgins L, Smith M, Dee J, Wilson C, Houghten R, O'Mahony D (2003) Toward targeted oral vaccine delivery systems: selection of lectin mimetics from combinatorial libraries. Pharm Res 20:1258–1266
32. Roth-Walter F, Scholl I, Untersmayr E, Fuchs R, Boltz-Nitulescu G, Weissenbock A, Scheiner O, Gabor F, Jensen-Jarolim E (2004) M cell targeting with Aleuria aurantia lectin as a novel approach for oral allergen immunotherapy. J Allergy Clin Immunol 114:1362–1368
33. Clark MA, Hirst BH (2002) Expression of junction-associated proteins differentiates mouse intestinal M cells from enterocytes. Histochem Cell Biol 118:137–147
34. Brayden DJ, Jepson MA, Baird AW (2005) Keynote review: intestinal Peyer's patch M cells and oral vaccine targeting. Drug Discov Today 10:1145–1157
35. Banerjee S, Medina-Fatimi A, Nichols R (2002) Safety and efficacy of low-dose Escherichia coli enterotoxin adjuvant for urease-based oral immunization against Helicobacter pylori in healthy volunteers. Gut 51:634–640
36. Metzger WG, Mansouri E, Kronawitter M (2004) Impact of vector-priming on the immunogenicity of a live recombinant Salmonella enterica serovar typhi Ty21a vaccine expressing urease A and B from Helicobacter pylori in human volunteers. Vaccine 22:2273–2277
37. Smythies LE, Novak MJ, Waites KB, Lindsey JR, Morrow CD, Smith PD (2005) Poliovirus replicons encoding the B subunit of Helicobacter pylori urease protect mice against H. pylori infection. Vaccine 23:901–909
38. Durrer P, Gluck U, Spyr C (2003) Mucosal antibody response induced with a nasal virosome-based influenza vaccine. Vaccine 21:4328–4334

39. Jaganathan KS, Vyas SP (2006) Strong systemic and mucosal immune responses to surface-modified PLGA microspheres containing recombinant Hepatitis B antigen administered intranasally. Vaccine 24:4201–4211
40. Gupta PN, Mahor S, Rawat A, Khatri K, Goyal A, Vyas SP (2006) Lectin anchored stabilized biodegradable nanoparticles for oral immunization: development and in-vitro evaluation. Int J Pharm 318:163–173
41. Perez O, Bracho G, Lastre M (2006) Proteoliposome-derived Cochleate as an immunomodulator for nasal vaccine. Vaccine 24:S2/52–S2/53
42. Alpar HO, Somavarapu S, Atuah KN, Bramwell VW (2005) Biodegradable mucoadhesive particulates for nasal and pulmonary antigen and DNA delivery. Adv Drug Deliv Rev 57:411–430
43. Singh P, Prabakaran D, Jain S, Mishra V, Jaganathan KS, Vyas SP (2004) Cholera toxin B subunit conjugated bile salt stabilized vesicles (bilosomes) for oral immunization. Int J Pharm 278:379–390
44. Kozlowski PA, Williams SB, Lynch RM (2002) Differential induction of mucosal and systemic antibody responses in women after nasal, rectal or vaginal immunization: influence of the menstrual cycle. J Immunol 169:566–574
45. Aguila A, Donachie AM, Peyre M, McSharry CP, Sesardic D, Mowat AM (2006) Induction of protective and mucosal immunity against diphtheria by an immune stimulating complex (ISCOMS) based vaccine. Vaccine 24:5201–5210
46. Jain S, Singh P, Mishra V, Vyas SP (2005) Mannosylated niosomes as adjuvant–carrier system for oral genetic immunization against Hepatitis B. Immunol Lett 101:41–49
47. Rice J, Ainlev WM, Shewen P (2005) Plant-made vaccines: biotechnology and immunology in animal health. Anim Health Res Rev 6:199–209
48. Thanavala Y, Mahoney M, Pal S (2005) Immunogenicity in humans of an edible vaccine for hepatitis B. Proc Natl Acad Sci U S A 102:3378–3382
49. O'Hagan DT, Palin NK, Davis SS (1989) Poly(butyl-2-cyanoacrylate) particles as adjuvants for oral immunisation. Vaccine 7:213–216
50. O'Hagan DT, Palin NK, Davis SS, Artursson E, Sjoholm I (1989) Microparticles as potentially orally active immunological adjuvants. Vaccine 7:421–424
51. Eldridge JH, Hammond CJ, Meulbroek JA, Stass JK, Gilley RM, Tice TR (1990) Controlled vaccine release in the gut-associated lymphoid tissues. I. Orally administered biodegradable microspheres target the Peyer's patches. J Control Release 11:205–214
52. Gutierro I, Hernandez RM, Igartua M, Gascon AR, Pedraz JL (2002) Size dependent immune response after subcutaneous, oraland intranasal administration of BSA loaded microspheres. Vaccine 21:67–77
53. Yeh MK, Liu YT, Chen JL, Chiang CH (2002) Oral immunogenicity of the inactivated Vibrio cholerae whole cell vaccine encapsulated in biodegradable microparticles. J Control Release 82:237–247
54. Katz DE, Delorimier AJ, Wolf MK, Hall ER, Cassels FJ, van Hamont JE, Newcomer RL, Davachi MA, Taylor DN, McQueen CE (2003) Oral immunization of adult volunteers with microencapsulated enterotoxigenic Escherichia coli (ETEC) C56 antigen. Vaccine 21:341–346
55. Russell-Jones GJ (2001) The potential of receptor mediated endocytosis for oral drug delivery. Adv Drug Deliv Rev 1:59–73
56. Foster N, Clark MA, Jepson MA, Hirst BH (1998) Ulex europaeus 1 lectin targets microspheres to mouse Peyer's patch M-cells in vivo. Vaccine 16:536–541
57. Florence AT, Hillery A, Hussain N, Jani PU (1995) Factors affecting the oral uptake and translocation of polystyrene nanoparticles: Histological and analytical evidence. J Drug Target 3:65–70
58. Gabor F, Stangl M, Wirth M (1995) Lectin mediated bioadhesion: binding characteristics of plant lectins on the enterocyte like cell lines Caco-2, HT-29 and HCT-8. J Control Release 55:131–142
59. Gupta PN, Khatri K, Goyal AK, Mishra N, Vyas SP (2007) M-cell targeted biodegradable PLGA nanoparticles for oral immunization against hepatitis B. J Drug Target 15:701–713

60. Jani P, McCarthy DE, Florence AT (1992) Nanosphere and microsphere uptake via Peyer's patches: observation of the rate of uptake in the rat after a single oral dose. Int J Pharm 86:239–246
61. Florence AT (2005) Nanoparticle uptake by oral route: fulfilling its potential? Drug Discov Today Technol 2:75–81
62. Jung T, Kamm W, Breitenbach A, Kaiserling E, Xiao JX, Kissel T (2000) Biodegradable nanoparticles for oral delivery of peptides: is there is a role for polymers to affect mucosal uptake? Eur J Pharm Biopharm 50:147–160
63. Moghimi SM, Hawley AE, Christy NM, Gray T, Illum L, Davis SS (1994) Surface engineered nanospheres with enhanced drainage into lymphatics and uptake by macrophages of the regional lymph nodes. FEBS Lett 344:25–30
64. Shakweh M, Besnard M, Nicolas V, Fattal E (2005) Poly (lactide-co-glycolide) particles of different physicochemical properties and their uptake by Peyer's paches in mice. Eur J Pharm Biopharm 61:1–13
65. Le Ray AM, Vert M, Gautier JC, Benoit JP (1994) Fate of poly (DL-lactide-co-glycolide) nanoparticles after intravenous and oral administration to mice. Int J Pharm 106:201–211
66. Eyles J, Alpar HO, Field WN, Lewis DA, Keswick M (1995) The transfer of polystyrene microspheres from the gastrointestinal tract to the circulation after oral administration in the rat. J Pharm Pharmacol 47:561–565
67. Norris DA, Puri N, Sinko PJ (1998) The effect of physical barriers and properties on the oral absorption of particulates. Adv Drug Deliv Rev 34:135–154
68. Migliaresi C, Fambri L, Cohn D (1994) A study on the in-vitro degradation of poly (lactic acid). J Biomater Sci Polym Ed 5:591–606
69. Cleek RL, Ting KC, Eskin SG, Mikos AG (1997) Microparticles of poly (DL-lactic-co-glycolic acid)/poly (ethylene glycol) blends for controlled drug delivery. J Control Release 48:259–268
70. O'Hagan DT, Jeffery H, Davis SS (1994) The preparation and microcharacterization of poly (lactide-co-glycolide) microparticles: III microparticle/polymer degradation rates and the in vitro release of model proteins. Int J Pharm 103:37–45
71. Sahoo SK, Panyam J, Prabha S, Labhasetwar V (2002) Residual polyvinyl alcohol associated with poly (D, L-lactide-co-glycolide) nanoparticles affects their physical properties and cellular uptake. J Control Release 82:105–114
72. Jaganathan KS, Singh P, Prabakaran D, Mishra V, Vyas SP (2004) Development of a single-dose stabilized poly (D, L-lactic-co-glycolic acid) microsphere-based vaccine against hepatitis B. J Pharm Pharmacol 56:1243–1250
73. Pappo J, Ermak TH (1989) Uptake and translocation of fluorescent latex particle by rabbit Peyer's patch follicle epithalium: a quantitative model for M cell uptake. Clin Exp Immunol 76:227–280
74. Le Fever ME, Boccio AM, Jeol DD (1989) Intestinal uptake of fluorescent microsphere in young and aged mice. Proc Soc Exp Biol Med 190:23–27
75. Simon L, Shine G, Dayan AD (1994) Effect of animal age on the uptake of large particulates across the epithelium of rat small intestine. Int J Exp Pathol 75:369–373
76. Strous GJ, Dekker J (1992) Mucin-type glycoproteins. Crit Rev Biochem Mol Biol 27:57–92
77. Desai MA, Mutlu M, Vadgama P (1992) A study of macro-molecular diffusion through native porcine mucus. Experientia 48:22–26
78. Michalek SM, Childers NK, Katz J, Denys FR, Berry AK, Eldrigge JH, McGee JR, Curtiss R (1989) Liposomes as oral adjuvants. Curr Top Microbiol Immunol 146:51–58
79. Clarke CJ, Strokes CR (1992) The intestinal and serum humoral immune responses of mice to systemically and orally administered antigens in liposomes: I. The response to liposome entrapped soluble proteins. Vet lmmunol Immunopathol 32:125–138
80. Clarke CJ, Strokes CR (1992) The intestinal and serum humoral immune responses of mice to systemically and orally administered antigens in liposomes: II. The response to liposome entrapped bacterial proteins. Vet lmmunol Immunopathol 32:139–148

81. Childers NK, Michalek SM, Pritchagd DG, McGhee JR (1991) Mucosal and systemic responses to an oral liposome Streptococcus mutans carbohydrate vaccine in humans. Reg Immunol 3:289–296
82. Chiang CM, Weiner N (1987) Gastrointestinal uptake of liposomes. I In vitro and in situ studies. Int J Pharm 37:75–85
83. Childers NK, Denys FR, McGhee NF, Michalek SM (1990) Ultrastructural study of liposome uptake by M cells of rat Peyer's patches: an oral vaccine system for delivery of purified antigen. Reg Immunol 3:8–16
84. Foster N, Hirst BH (2005) Exploiting receptor biology for oral vaccination with biodegradable particulates. Adv Drug Deliv Rev 57:431–450
85. Sugimoto M, Ohishi K, Fukasawa M, Shikata M, Kawai H, Itakura H, Hatanaka M, Sakakibara R, Ishiguro M, Nakata M (1995) Oligomannose-coated liposomes as an adjuvant for the induction of cell-mediated immunity. FEBS Lett 363:53–56
86. Fukasawa M, Shimizu Y, Shikata K, Nakata M, Sakakibara R, Yamamoto N, Hatanaka M, Mizuochi T (1998) Liposome oligomannose-coated with neoglycolipid, a new candidate for a safe adjuvant for induction of CD8+ cytotoxic T lymphocytes. FEBS Lett 441:353–356
87. Childers NK, Michalek SM (1994) Liposomes. In: O'Hagan DT (ed) Novel delivery systems for oral vaccines. CRC, Boca Raton, FL
88. Chen H, Torchilin V, Langer R (1996) Polymerized liposomes as potential oral vaccine carriers: stability and bioavailability. J Control Release 42:263–272
89. Chen H, Torchilin V, Langer R (1996) Lectin-bearing polymerized liposomes as potential oral vaccine carriers. Pharm Res 13:1378–1383
90. Gupta PN, Vyas SP (2011) Investigation of lectinized liposomes as M-cell targeted carrier-adjuvant for mucosal immunization. Colloids Surf B Biointerfaces 82:118–125
91. Neuta MR, Mantis NJ, Frey A, Giannasca PJ (1999) The composition and function of M cell apical membranes: implications for microbial pathogenesis. Semin Immunol 11:171–181
92. Lo D, Tynan W, Dickerson J (2004) Cell culture modeling of specialized tissue: identification of genes expressed specifically by follicle associated epithelium of Peyer's patch by expression profiling of caco-2/Raji co-cultures. Int Immunol 16:91–99
93. Lo D, Tynan W, Dickerson J (2003) Peptidoglycan recognition protein expression in mouse Peyer's patch follicle associated epithelium suggests functional specialization. Cell Immunol 224:8–16
94. Clark MA, Jepson MA, Simmons NL, Hirst BH (1995) Selective binding and transcytosis of Ulex europaeus 1 lectin by mouse Peyer's patch M-cells in vivo. Cell Tissue Res 282:455–461
95. Kabok Z, Ermak TH, Pappo J (1994) Microheterogeneity of follicle epithelium and M cells in rabbit gut-associated lymphoid tissues defined by monoclonal antibodies. FASEB J 8: A1008
96. Carreno-Gomez B, Woodley JF, Florence AT (1999) Studies on the uptake of tomato lectin nanoparticles in everted gut sacs. Int J Pharm 183:7–11
97. Porta C, James PS, Phillips AD, Savidge TC, Smith MW, Cremaschi D (1992) Confocal analysis of fluorescent bead uptake by mouse Peyer's patch follicle-associated M cells. Exp Physiol 77:929–932
98. Zhou F, Kraehenbuhl JP, Neutra MR (1995) Mucosal IgA response to rectally administered antigen formulated in IgA-coated liposomes. Vaccine 13:637–644
99. Giannasca PJ, Boden JA, Monath TP (1997) Targeted delivery of antigen to hamster nasal lymphoid tissue with M-cell- directed lectins. Infect Immun 65:4288–4298
100. Hussain N, Florence AT (1998) Utilizing bacterial mechanisms of epithelial cell entry: Invasin-induced oral uptake of latex nanoparticles. Pharm Res 15:153–156
101. Jain S, Vyas SP (2006) Mannosylated niosomes as adjuvant-carrier system for oral mucosal immunization. J Liposome Res 16:331–345
102. Harokopakis E, Childers NK, Michalek SM, Zhang SS, Tomasi M (1995) Conjugation of cholera toxin or its B subunit to liposomes for targeted delivery of antigens. J Immunol Methods 185:31–42

103. Conacher M, Alexander J, Brewer JM (2001) Oral immunization with peptide and protein antigens by formulations in lipid vesicles incorporating bile salts (bilosomes). Vaccine 19:2965–2974
104. Vyas SP, Singh A, Sihorkar V (2001) Ligand-receptor-mediated drug delivery: an emerging paradigm in cellular drug targeting. Crit Rev Ther Drug Carrier Syst 18:1–76
105. Henderson B, Poole S, Wilson M (1996) Bacterial modulins: novel class of virulence factors which cause host tissue pathology by inducing cytokine synthesis. Microbiol Rev 60:316–341
106. Young VB, Falkow S, Schoolnik GK (1992) The invasin proteinof Yersinia enterocolitica: internalization of invasin bearing bacteria by eukaryotic cells is associated with reorganization of the cytoskeleton. J Cell Biol 116:197–207
107. Jepson MA, Clark MA (1998) Studying M cells and their role in infection. Trends Microbiol 6:359–365
108. Clark MA, Hirst BH, Jepson MA (2000) Lectin-mediated mucosal delivery of drugs and microparticles. Adv Drug Deliv Rev 43:207–223
109. Durrer C, Irache JM, Duchene D, Ponchel G (1994) Study of the interactions between nanoparticles and intestinal mucosal. Prog Colloid Polym Sci 97:275–280
110. De Aizpurua HJ, Russell-Jones GJ (1988) Oral vaccination: identification of classes of proteins that provoke an immune response upon oral feeding. J Exp Med 167:440–451
111. Tacket CO, Reid RH, Boedeker EC, Losonsky G, Nataro JP, Bhagat H, Edelman R (1994) Enteral immunization and challenge of volunteers given enterotoxigenic E. coli CFA/II encapsulated in biodegradable microspheres. Vaccine 12:1270–1274
112. Peyre M, Sesardic D, Merkle HP, Gander B, Johansen P (2003) An experimental divalent vaccine based on biodegradable microspheres induces protective immunity against tetanus and diphtheria. J Pharm Sci 92:957–966
113. Jain S, Sharma RK, Vyas SP (2006) Chitosan nanoparticles encapsulated vesicular systems for oral immunization: preparation, in-vitro and in-vivo characterization. J Pharm Pharmacol 58:303–310