Expert Opinion

Microparticles for oral delivery of vaccines

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Nonreplicating antigens are poorly immunogenic when given orally, either due to their degradation in the gastrointestinal tract or because they are not efficiently taken up in the gut. Studies in laboratory animals have clearly demonstrated that microparticles can significantly improve the immunogenicity of orally administered antigens. However, the oral delivery of vaccines using microparticles has not been explored extensively in humans and large animals. In this article the progress in oral microparticle antigen delivery will be reviewed and, where possible, studies in humans and large animals will be highlighted. In addition, possible approaches that have the potential to significantly improve microparticle delivery of oral vaccines will be suggested.

Keywords: microparticles, mucosal immunity, oral vaccines, vaccine delivery

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1. Introduction

Vaccination remains the most cost effective medical intervention in the control of infectious diseases in humans and domestic animals. In fact, it has been reported that the value of vaccination is often grossly underestimated by a factor between 10 and 100, as most risk–benefit analyses of vaccination do not include the 'intangible' value of vaccination, which would include all factors for avoiding disease altogether [1].

Most of the vaccines in use today are injected parenterally and have contributed significantly to the control of many devastating infectious diseases. However, immunisation at mucosal sites is superior to parenteral vaccination in inducing protection against the many infectious agents that invade the body through mucosal surfaces such as the respiratory, gastrointestinal and genital tracts [2,3]. Inducing protective immunity at these mucosal sites should prevent colonisation and even translocation of the pathogens across the mucosal barrier. At present, there is plenty of evidence to suggest that delivering the vaccine to the mucosal sites in the vicinity of organised mucosa-associated lymphoid tissue (MALT) is the most effective way to induce mucosal immunity [4-6]. In addition to the induction of mucosal immunity, mucosal immunisation can also induce systemic immunity [2]. Therefore, effective mucosal immunisation would lead to improved vaccine efficacy by protecting against mucosal and systemic infections. Mucosal vaccination has several other advantages;

- avoids the pain and discomfort associated with injections, which contributes to reduced compliance with immunisation schedules;
- less expensive and easy to administer, as trained personnel are not necessarily required to administer the vaccine;
- eliminates injection site reactions and possible infections from contaminated needles

This is a particular concern to the beef industry where it is estimated to cost ~ $9 for each animal processed in Canada [7]. A fascinating feature of mucosal immunology is that administration of antigen in one mucosal site can lead to generation of immune responses locally and at distant mucosal sites, a phenomenon

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referred to as the common mucosal immune system [8]. Furthermore, the mucosal immune system develops prenatally and is functional early in life, perhaps long before the systemic immune system matures [9]. This makes mucosal immunisation particularly attractive in the neonatal period, a time of increased disease susceptibility when effective vaccination would make a significant impact on disease control.

Mucosal immunisation can be employed via a variety of routes including respiratory (intranasal, intratracheal, intrapulmonary or inhalation), oral, rectal, intravaginal and ocular. Each of these routes has its perceived advantages and disadvantages. For example, delivery to the respiratory tract would circumvent the difficulties presented by the harsh environment of the gastrointestinal tract such as low pH and digestive enzymes that degrade antigen. Indeed, the success of intranasal immunisation has been well documented [10-12], and apparently requires less antigen than oral immunisation [2]. However, it is often difficult to deliver vaccine to the lower respiratory tract without using invasive techniques such as intratracheal and intrapulmonary delivery. Toxicological issues are also a concern, especially in the young. Intravaginal immunisation has been explored for the induction of immunity in the genital tract, based on the notion that delivery of antigen to a mucosal site induces higher levels of immunity locally as compared with distant mucosal sites. Intravaginal immunisation with a variety of antigens was found to induce immune responses in the genital tract of humans, cows and mice [13-16]. Although it is thought that the female genital tract does not respond well to nonreplicating antigens following intravaginal immunisation [17,18], a recent study suggests that this hyporesponsiveness can be reversed with potent adjuvants such as CpG oligodeoxynucleotides (ODNs) [19]. Intranasal immunisation can be effective in inducing immune responses in the female genital tract in mice [12,20]. Of note, immune responses in the female reproductive tract are highly regulated by reproductive hormones [15,18,21], which must be considered in the development of intravaginal vaccination strategies. Rectal immunisation has also been shown to be effective at inducing immune responses in rectal secretions [14]. However, the success of intravaginal and rectal immunisation may be limited by cultural resistance.

Of all the mucosal routes of immunisation, oral vaccination is the most attractive and widely acceptable. Oral vaccination has the advantages of mucosal immunisation noted above. In addition, it uses a natural physiological process (ingestion), is rarely associated with any side effects and, more importantly, oral vaccination induces mucosal as well as systemic immune responses. Microparticles have been investigated as a strategy to improve the delivery of nonreplicating antigens in oral vaccination. Microparticle delivery systems have been previously reviewed [22,23]. In this article the progress in microparticle delivery of antigens will be reviewed with a focus on oral immunisation and, where possible, studies in humans and domestic animals will be highlighted.

2. Oral vaccine delivery

The most effective way to induce active mucosal immunity in the intestine is via oral immunisation; however, investigators have also used experimental procedures such as intragastric, intraduodenal or intraenteric that enhance the delivery of antigens in the immune inductive sites in the small intestine.

Despite its potential advantages, successful oral vaccination remains one of the major challenges in vaccinology and its potential remains largely unexploited. The challenge in developing efficient oral vaccines is largely due to the lack of effective delivery leading to poor availability of vaccine antigens to the gut-associated lymphoid tissue (GALT) where immune responses are initiated. The difficulty in delivering vaccine to GALT is a result of a combination of factors including dilution and degradation of antigen, peristalsis, physical barrier formed by mucus and glycocalyx, and possibly oral tolerance mechanisms, which downregulate systemic immune responses [24]. Consequently, improving delivery of oral vaccines by various approaches, such as protecting them from degradation, improving uptake and availability in the GALT, are areas of considerable interest to investigators working on oral vaccines.

Oral vaccine delivery systems can be broadly categorised into living and nonliving systems. Attenuated live bacterial and viral vectors expressing heterologous antigens have great potential in oral vaccine delivery. Numerous bacteria and viruses survive in the gastrointestinal tract and exploit the harmless M cells in the intestinal epithelium as a mechanism of invading the host [25]. Live vectors have an additional advantage as they can replicate in the host, thereby providing sustained antigen release and may possess immunostimulatory components such as CpG DNA, which have potent mucosal adjuvant activity [26-28]. Indeed, enteric bacteria such as *Salmonella* and *Shigella* are being explored as oral vaccine vectors [29]. A recombinant oral rabies vaccine consisting of vaccinia-rabies glycoprotein is currently used in the control of rabies in wildlife [30,31]. However, research and commercialisation of vaccines based on live vectors has slowed, mainly due to safety concerns. There is the possibility that live vectors may be pathogenic in immunocompromised individuals or may revert to virulence, and the possibility that genetically modified organisms would be continually shed in the environment [29] are a few of these concerns. Thus, primarily for safety reasons, there is a great deal of interest in the development of novel nonliving delivery systems for oral delivery of antigens for important pathogens. This can be achieved with polymeric microparticles. Microparticles can be prepared from a range of polymers designed to protect vaccine against degradation while on transit in the gastrointestinal tract and can also target uptake by the Peyer's patches. As an alternative approach, particulate vaccines can also be designed to simply protect the vaccine from degradation in the gut and release it in the vicinity of the Peyer's patches for subsequent uptake.

3. Induction of immune responses in the intestine

Nonreplicating vaccine antigens are generally poorly immunogenic when given orally, but encapsulation of antigens in microparticles often results in significant enhancement of mucosal and systemic immune responses [5,32,33]. This has been attributed to their ability to withstand degradation and their greater uptake in the gastrointestinal tract [34]; however, other factors are also likely to contribute to the immune enhancement (see below).

Oral administration of antigens has two potential outcomes; active immunity or oral tolerance. Oral tolerance is a state of systemic immunological unresponsiveness resulting from suppression of immune responses to an antigen induced by prior oral administration of the antigen [35]. Oral tolerance presumably evolved to prevent undesirable immune responses to innocuous food proteins and antigens in the intestinal microflora [6,35,36]. There are several excellent reviews on the complexity of oral tolerance [6,35-39] to which the reader is referred for details. In general, soluble antigens given orally in the absence of adjuvants induce systemic tolerance as a consequence of T-cell anergy/deletion or active suppression, depending on antigen dose [37]. Although soluble antigens are more likely to induce oral tolerance than particulate antigens [40], two recent reports suggest that antigen incorporated in microparticles can also enhance the induction of tolerance [41,42], further demonstrating the complexity of the system.

Peyer's patches are the primary sites for the induction of immune responses in the GALT [43]. Initiation of an immune response requires that the antigen be presented to T and B lymphocytes in an organised lymphoid compartment, such as the Peyer's patches and underlying follicles rather than in a diffuse lymphoid compartment such as the lamina propria [44]. In addition, dendritic cells (DCs) which are specialised organised antigen-presenting cells (APCs), are present in larger numbers in Peyer's patches compared with lamina propria [24]. However, it should be noted that while Peyer's patches may play an important role in the induction of active immunity in the gut, it is still controversial as to whether Peyer's patches are absolutely essential for the induction of mucosal immune responses. Conflicting results were obtained from studies in mice devoid of Peyer's patches, with one study reporting a lack of antigen-specific immune responses [45], whereas another showed detectable responses [46].

Lymphocytes primed in Peyer's patches exit through draining mesenteric lymph nodes, where they undergo further differentiation before they migrate through the thoracic duct to the blood. Priming of lymphocytes in Peyer's patches induces a loss of L-selectin and upregulates the expression of $\alpha_4\beta_7$ integrins, which specifically directs the 'homing' of lymphocytes from the blood to various mucosal tissues by interacting with mucosal addressin cell adhesion molecule-1, the ligand for $\alpha_4\beta_7$ expressed on blood vessels in mucosal sites [6]. At the same time there is increased expression of chemokine receptor (CCR) 9 in T cells primed in the intestine, allowing them to respond to the chemokine ligand (CCL) 25, also known as thymus expressed chemokine, which is expressed selectively by small intestinal epithelial cells [36,47]. This pattern of adhesion-molecule and chemokine-receptor expression is specific for mucosally primed T cells and offers a molecular explanation as to why oral immunisation is more efficient at inducing mucosal immunity in the intestine and why systemic immunisation is less effective. This recirculation of lymphocytes between mucosae and blood allows communication within the network of immune apparatus in various mucosal tissues, resulting in the activation of the common mucosal immune system. It should be pointed out that there appears to be compartmentalisation within the common mucosal immune system. For example, oral immunisation elicited more pronounced immune responses in saliva and vaginal secretions, whereas rectal immunisation was more potent in inducing immune responses in nasal secretions, rectum and tears [48]. In general, the amplitude of immune responses are often higher at the site of immunisation or at sites in close proximity but relatively low at distant mucosal sites [49]. Furthermore, the quality of the responses (e.g., antibody isotypes) may depend on the mucosal site of immunisation [50].

The effector arm of the mucosal immune system in the intestine is mediated primarily by lamina propria and intraepithelial lymphocytes. Both humoral- and cell-mediated immune responses can be generated in the intestine, but humoral responses have been investigated in greater detail, perhaps because they are easier to assess. IgA is the most abundantly produced Ig in mammals, and most IgA is secreted across mucous membranes of the intestinal, respiratory, biliary and genital tracts [51]. The translocation of IgA across intestinal epithelial cells into the intestinal lumen occurs following its binding to the polymeric Ig receptor (pIgR) expressed on the basolateral surface of enterocytes [51,52]. The IgA–pIgR complex is endocytosed and then delivered to the luminal surface whereby proteolytic cleavage of the receptor protein results in release of secretory component, free or attached to the IgA [51,52]. In addition to delivering the IgA across the epithelium, the IgA transport system aids in the clearance of antigens that have leaked through the epithelial barrier. In addition, IgA can neutralise viral replication in the epithelial cells during the transit process [51,52]. Although IgA is the predominant Ig in mucosal secretions, IgG has been shown to play a significant role in mucosal protection [53]. Indeed, IgG1 is the predominant Ig isotype in the gastrointestinal tract of ruminants. However, the mechanism by which IgG reaches luminal secretions remained elusive for decades until recently when it was reported that human neonatal Fc receptor is the vehicle that transports IgG across the intestinal epithelial barrier into the lumen where it binds antigen and can recycle antigen back into the lamina propria to DCs [54].

4. Microparticles for oral delivery

4.1 Uptake of microparticles in the intestine

The uptake of microparticles across the intestinal barrier was extensively reviewed recently [55,56]. The particles can cross the intestinal barrier in two ways; between adjacent cells (paracellular uptake) or through cells (transcellular uptake) [56]. The paracellular pathway is not likely to be a significant mechanism by which microparticles are taken up as the paracellular spaces comprise < 1% of the intestinal mucosa surface and are sealed by tight junctions [56]. However, uptake by this route may be increased in inflammatory conditions that compromise the integrity of the tight junctions. Studies in cell culture systems have shown that the paracellular uptake can be enhanced by some formulations such as starch microparticles [57], but the relevance of these observations *in vivo* remain to be seen. Interestingly, DCs can extend their dendrites between adjacent intestinal epithelial cells to sample bacteria in the intestinal lumen [58], but whether this pathway plays any significant role in the uptake of particles is not yet known. Following uptake by M cells, microparticles are phagocytosed by DCs in the subepithelial dome of the Peyer's patch tissues [59].

Transcellular uptake is the major pathway by which microparticles cross the intestinal barrier [60], which can occur at various sites including villus tips, enterocytes and the epithelium overlying the Peyer's patches [55,56]. Overall, a majority of the evidence favour the M cells within the follicle-associated epithelium (FAE) overlying the Peyer's patches as the predominant site for the uptake of microparticles. The M cell is a phagocytic cell type, specialised in the uptake of particulate antigens including microorganisms and microparticles, such as latex beads and macromolecules [61]. Beier and Giebert showed that in the pig intestine, uptake of yeast particles occurred in M cells in Peyer's patches [62].

Using an intestinal loop model in sheep, Kim *et al.* [63] showed that alginate microparticles loaded with colloidal carbon were only associated with the FAE and were not attached to the epithelium in the non-Peyer's patch areas. Furthermore, after a period of 2 h, colloidal carbon in microparticles was visualised within the lymphoid follicles of the Peyer's patches [63]. Particles adhere to M cells and are endocytosed and transported to the basolateral areas where they are exocytosed in the invagination of the M cells where they are then picked up by APCs such as macrophages or DC [64,65]. These APCs then process and present the antigen to the lymphocytes, which are present within the Peyer's patches. However, it is thought that only a small proportion of particles are taken up as M cells are not very abundant, representing < 0.1% of epithelial cells [66]. Indeed, available evidence so far indicates that uptake in the Peyer's patches can vary from < 0.01% of particles to > 0.1% depending on the experimental conditions [56].

Several particle- and animal-related factors affect the extent of uptake of microparticles by M cells and Peyer's patches. Of the particle-related factors, size, charge, hydrophobicity and the presence of specific ligands on the microparticle are important [55,56]. Early studies by Eldridge *et al.*[33] showed that polylactide-co-glycolide (PLG) microparticles with a diameter of 5 – 10 µm were taken up in the Peyer's patches and remained in the dome area, whereas those < 5 µm were detected in the Peyer's patches, mesenteric lymph nodes and spleen. Recent studies in sheep revealed that alginate microparticles < 10 µm were taken up by the Peyer's patches whereas those > 10 µm were not [63], although a study by McLean *et al.* [60] reported that only particles < 4 µm were taken up. Furthermore, 100-nm nanoparticles were taken up at a rate 15 – 250-fold higher than 500 nm and 10 µm microparticles [67]. Kofler *et al.* [68] showed that particles with a size of 0.8 µm were taken up better than those 2.0 µm, and this was consistent with the induction of immune responses. All these studies clearly demonstrate that size is a very important parameter for uptake and that smaller nanoparticles are taken up better than the larger microparticles.

The surface hydrophobicity of microparticles can also be an important determinant of uptake. Hydrophobic polymeric particles, such as polystyrene or PLG, were taken up more efficiently than hydrophilic cellulose and cellulose acetate, or monomers of lactide and glycolide [69]. Apparently, hydrophobicity allows faster diffusion through mucus. The charge of the particle also determines the extent of uptake. As M cells and the mucus layer have a net negative charge [56], positively charged particles are attracted to cells and such an interaction presumably facilitates uptake. Other particle-related factors that influence uptake in the intestine include particle dose and numbers and the volume of particle suspension medium. Immunogenicity of microparticles depends both on the dose of antigen and total number of particles per dose [70].

Of the animal-related factors influencing particle uptake, the intestinal mucus layer is critical. It normally acts as a barrier to particle uptake by entrapping them, thereby reducing their diffusion through the mucus and thereby reducing contact with the cells in the intestinal epithelium. Data reviewed by Shakweh [56] indicate that uptake of particles can vary from one animal species to another. For example, uptake in the ileum of rats was better than in that of rabbits [60]. Other animal factors that may affect uptake include age of the animal as well as its fed state [71].

4.2 Targeting microparticles to M cells

Low uptake of particles remains a significant challenge in the development of microparticle-based oral vaccines [34]. Of the various possible approaches that can increase the uptake of particles, targeting particles to M cells has attracted the most interest.

As the M cell is the predominant site for the uptake of microparticles, modifying the surface of the microparticles so that they would target the M cells would be a logical approach to increasing the uptake of particles. Unfortunately, markers that are completely specific for M cells are not yet available [66]. Nonetheless, several approaches are being explored that can significantly improve targeting to M cells.

Various lectins bind specifically to oligosaccharides in intestinal cells and investigators have exploited this specificity to target M cells. In this regard, covalently coating microparticles with the lectin Ulex europaeus 1 (UEA1) resulted in significant selective binding of microparticles to M cells in the intestines of mice, however, the binding to enterocytes was unaffected [72]. Similarly, synthetic compounds that mimic UEA1 mediate M-cell-specific delivery of particles *in vivo* [73]. In addition, UEA1 coated microparticles can target Peyer's patch M cells following intragastric administration in mice [72]. *Yersinia pseudotuberculosis* is a bacterium that targets M cells *in vivo* and this is mediated primarily by the interaction of invasin with cell surface β1 integrins on M cells [74]. Hussain and Florence [75] mimicked this microbial strategy and showed that the *Yersinia* adhesin, invasin, could be used to improve uptake of microparticles.

Pappo *et al.* [76] showed that polystyrene microparticles conjugated to an anti-M-cell-specific antibody (IgM) enhanced their uptake up to 3.5-fold over particles conjugated to IgM of an unrelated specificity in a rabbit intestinal loop model. Similarly, coating latex beads with secretory Ig (sIgA) increased their uptake by M cells in a mouse intestinal loop model [77]. Apparently this is because mouse M cells express an IgA-specific receptor on their apical surface that mediates the transepithelial transport of sIgA from the intestinal lumen to underlying GALT [78]. The IgA is then taken by the DC in the subepithelial dome [79], suggesting that this approach can be used to target both M cells and DCs.

Protein coating can also increase particle binding and uptake by M cells. For example, in mice, binding and uptake of microparticles is enhanced by bovine growth hormone, bovine serum albumin and human IgG [80]. Although these targeting studies are promising, further studies are required to determine whether targeting microparticles to M cells can significantly enhance protective immunity. A potential pitfall with most M-cell-targeting strategies is that the macromolecules used to coat the microparticles can be degraded in the gastrointestinal. Also, this approach may add to the cost of the vaccine. However, this may be counteracted if targeting results in more efficient uptake and, hence, less antigen would be required for optimal results.

4.3 Mechanisms of immune enhancement by microparticles

Microparticles enhance both humoral- and cell-mediated immune responses and can be viewed as adjuvants as well as delivery systems [34]. This enhancement of immune responses is partially due to increased antigen uptake by the immune system as well as improved presentation. Available evidence indicates that antigen associated with microparticles can significantly enhance antigen uptake, presentation and immune responses compared with soluble antigens given systemically [81,82]. *In vitro* studies have confirmed that microencapsulated antigens enter via different antigen-presentation pathways, and both major histocompatibility complex (MHC) class I and class II restricted processing and presentation of microencapsulated antigens have been demonstrated [83]. Indeed, it was shown that encapsulation of protein antigen in a particulate form, which can be phagocytosed by APCs, markedly enhanced antigen presentation and cytotoxic T lymphocyte (CTL) responses [84,85]. This suggests that the cellular compartment where the antigen localises may influence antigen processing and presentation and the resulting immune responses. In this regard, microparticles enter the cell by phagocytosis, ending up in the phagosome and subsequently cross presentation of antigens to the MHC I pathway occurs, whereas soluble antigens enter by pinocytosis [86].

In oral immunisation studies, microparticles protect antigen from degradation and can also increase uptake from the intestinal lumen, factors that certainly contribute to their immune enhancement capacity.

4.4 Microparticles have broad vaccine delivery applications

Microparticles have the potential for a wide range of uses in vaccine delivery applications. Many vaccines used today require multiple booster immunisations to induce protective immunity. Multiple vaccinations reduce compliance with immunisation schedules and also significantly increase the cost of vaccines, making vaccination unaffordable in many developing countries. In the animal health industry, the cost of administering a vaccine contributes significantly to the cost of vaccination and, therefore, reducing the number of vaccinations can dramatically reduce the overall cost of vaccination regimes for producers. Consequently, there is great interest in developing single-dose vaccines in which primary and booster immunisations can be given as a single shot [87-89]. Single-shot vaccines can be achieved by developing microparticles that can provide pulsatile antigen release or sustained release of antigen [87,90]. Singh and colleagues [91] encapsulated hepatitis B surface antigen (HBsAg) in a mixture of microparticles with different degradation and pulsatile release kinetics and demonstrated that a single intramuscular injection of HBsAg in microparticles could maintain the antibody response at a level comparable to a three-injection alum formulation over 1 year. Similarly, intramuscular administration of HIV recombinant glycoprotein 120 (rgp 120) in PLG microparticles with a pulsatile release of rgp 120 in baboons resulted in high, long-lasting neutralising antibody titres that were greater than repeated immunisations with soluble rgp120 plus an adjuvant QS-21 [90]. These studies demonstrate that microparticle formulations may be designed to provide *in vivo* pulses of an antigen eliminating the need for repeated immunisations. However, successful single-shot vaccines delivered orally have not been reported, but investigation into this approach is warranted based on the encouraging results from intramuscular delivery of these particles.

An alternative approach to reducing the number of immunisations would be to use combination vaccines. Many vaccines are efficacious as separate vaccines, but

there is concern that combining several antigens in one preparation may compromise efficacy [92]. Peyre *et al.* [93] showed that a single administration of biodegradable microparticle vaccines provided protective immunity against diphtheria and tetanus, without compromising their immunogenicity. In a subsequent study, the same group reported that strong immune responses could be induced when five antigens were coencapsulated, and microparticle combination vaccine gave better protection than separate injections [94]. Even if there was a reduction in immunogenicity, if a number of antigens are combined in one microparticle, it may be possible to overcome this problem by encapsulating antigens in separate particles and then mixing the particles in a single delivery mode. These studies suggest that microparticles are a promising approach to deliver combination vaccines.

As mentioned earlier, nonreplicating antigens given orally rarely induce significant immune responses without adjuvants. Microparticles can be used to codeliver an adjuvant with the antigen, hence increasing the efficacy of the vaccine. Immune response to the rgp120 PLG microparticle formulations was increased by adding the soluble form of the saponin-derived adjuvant QS-21 [90]. Incorporation of the novel adjuvant, CpG ODN with an antigen in microparticles was also shown to enhance immune responses to incorporated antigens [95-97]. Subcutaneous immunisation of mice with tetanus toxoid (TT) and CpG ODN coencapsulated in PLG nanoparticles resulted in enhanced antigen-specific proliferation, IFN-γ production and antibody responses compared with responses to TT and CpG ODN in solution [95]. An interesting report by Hunter *et al.* [97] demonstrated elevated antibody responses in blood and vaginal washes in mice following oral/nasal/vaginal immunisation with *Streptococcal* polysaccharide antigen coencapsulated with CpG ODN in PLG microparticles. This latter study revealed that both strategies (encapsulation in microparticles and the addition of a CpG adjuvant) were required for optimal responses [97]. Encapsulating CpG ODN in microparticles seems a logical approach as the CpG ODN receptor, toll-like receptor (TLR9) is located in the intracellular compartment. Thus, the CpG ODN is protected from degradation until it is inside the cell where it can interact with the TLR9. These exciting preliminary reports should be investigated as a promising new approach to enhancing oral vaccination.

5. Immune responses following oral immunisation with antigens in microparticles

5.1 Polylactide-co-glycolide and related microparticles

PLG particles are made up of biodegradable and biocompatible aliphatic polyesters, which have been safely used in humans for many years as suture materials and controlled-release drug delivery systems [34]. Due to their high safety profile in other applications, PLGs have been extensively investigated for the development of microencapsulated vaccines, and for single-dose vaccine delivery applications [89]. However, PLG microparticles have a number of limitations. The PLG polymers are insoluble in water and are soluble only in some organic solvents. Microencapsulation in PLG microparticles involves emulsification of aqueous solutions of antigens into organic solvents containing polymer, followed by the extraction or evaporation of the solvent to form micropaticles [23]. A major concern with PLGs is that organic solvents may denature antigens and compromise the immunogenicity of critical epitopes in a vaccine. Other factors that may compromise the immunogenicity include high-shear, antigen-organic solvent interfaces, elevated temperatures and slow degradation. The slow degradation may prolong antigen presentation but may reduce the amount of antigen available intracellularly [86,98]. A recent report suggests that this can be circumvented by the use of microparticles made from pH-sensitive material [86], but it is yet to be determined how effective these pH-sensitive microparticles are in oral immunisation.

It is well documented from numerous studies that a variety of antigens can be encapsulated in PLG microparticles with successful induction of protective immune responses (Table 1). Early studies by O'Hagan *et al.* [32] clearly demonstrated that oral immunisation with the model antigen ovalbumin (OVA) encapsulated in PLG microparticles enhanced antibody responses in serum and saliva. Furthermore, subsequent studies using a similar immunisation protocol revealed that anti-OVA secretory IgA was detected in secretions in the nose, gut and lower genital tract, indicating that oral immunisation with microparticle encapsulated OVA could stimulate the common mucosal immune system [99]. A delivery system that can induce both humoral- and cell-mediated immunity would be applicable with vaccines against a broad range of pathogens. In this regard, the capacity of PLG microparticles to induce cell-mediated immune responses was investigated by Maloy and co-workers [100] who showed that PLG microparticle-encapsulated OVA-induced CTL responses in spleen cells following oral immunisation. It was also reported that oral immunisation of mice with OVA encapsulated in PLG microparticles induced responses to a level similar to cholera toxin (CT) [23]. This is significant given that CT is the most potent and most widely investigated mucosal adjuvant.

PLG microparticles have also been shown to enhance immune responses to microbial antigens and in some cases protection against challenge was demonstrated (Table 1). Encapsulation of *Staphylococcal* enterotoxin B in PLG microparticles enhanced serum, salivary and intestinal antibody responses after oral immunisation of mice [101].

Phosphorylcholine (PC), a microbial component present in numerous bacteria and protozoa that colonise mucosae, was encapsulated in PLG microparticles [41,102]. Mice immunised orally with this preparation were protected against oral challenge with *Salmonella typhimurium*, and the level of protection was greater than that achieved by intraperitoneal immunisation with PC in Freund's adjuvant [41,102]. A single

Table 1. Examples of microparticle-based oral immunisation studies.

Polymer	Animal species	Antigen	Observation	Ref.
PLG	Mice	*Staphylococcal enterotoxin B*	Enhanced serum, salivary, intestinal antibody responses	[33]
PLG	Mice	OVA	Serum antibody and mucosal IgA CTL responses Disseminated mucosal IgA in nose, gut, lower genital tract	[99,100,150]
PLG	Mice	*Bordetella pertussis* fimbriae	Protected from intranasal challenge with *Bordetella pertussis*	[106]
PLG	Mice	Phosphorylcholine	Protected against oral challenge with *Salmonella typhimurium*	[102]
PLG	Mice	Anti-iditype	Protected against occular challenge with *Chlamydia trachomatis*	[108]
PLG	Mice	*Helicobacter pylori* lysates	Intestinal IgA, serum IgG	[105]
PLG	Mice	Rotavirus plasmid DNA vaccine	Serum antibodies, intestinal IgA Protection against oral rotavirus challenge	[109,110]
PLG	Mice	Ricin toxoid	Serum IgG, IgA Protection against aerosol ricin challenge	[107]
PELA	Mice	*Helicobacter pylori* lysates	Enhanced IgA in saliva and gut washes	[121]
PELA	Mice	*Vibrio cholera*	Serum IgG, IgA and IgM Protection against challenge	[122]
PLG	Rabbits*	RDEC	Biliary IgA Protection from RDEC disease	[104]
PLG	Rabbits	ETEC fimbriae	Serum IgG	[103]
PLG	Humans‡	ETEC	Jejunal fluid IgA, protection against challenge	[114]
PLG	Humans	ETEC	Serum IgG	[115]
PLG	Chickens	Inactivated *Salmonella enteritidis*	Enhanced intestinal IgA Protection against oral and intramuscular challenge	[117]
Starch	Mice	*Salmonella enteritidis*	Significant DTH, reduction in bacterial CFU in spleen and liver	[129]
Starch	Mice	HSA	DTH	[127]
Starch	Mice	HSA	Mucosal antibody, cellular responses	[128]
Starch	Mice	Diphtheria toxin	IgA, neutralising antibody	[130]
Alginate	Calves	OVA	IgA, IgG in lungs washes	[137]
Alginate	Rabbits	*Pasteurella multocida*	IgA in nasal washes, reduced bacteria lungs	[132]
Alginate	Mice	Rotavirus VP6	Fecal IgA	[63]
Alginate	Mice	*Streptococcus Pneumoniae* capsular antigen	Protection from lethal intranasal challenge	[134]

*Intraduodenal immunisation. ‡Immunisation via intestinal tube.

CFU: Colony-forming units; CTL: Cytotoxic T lymphocytes; DTH: Delayed-type hypersensitivity reactions; ETEC: Enterotoxigenic *Escherichia coli;* HSA: Human serum albumin; Ig: Immunoglobulin; OVA: Ovulbumin; PELA: Poly-D,L-lactide-polyethylene glycol; PLG: Polylactide-co-glycolide; RDEC: Rabbit diarrhoeatogenic *Escherichia coli*.

oral immunisation of rabbits with enterotoxigenic *Escherichia coli* (ETEC) fimbriae adhesin, or colonisation factor antigen (CFA/I) encapsulated in PLG microparticles induced a vigorous serum IgG antibody responses, whereas unencapsulated antigen induced little or no circulating antibody [103]. Encapsulation in microparticles presumably protected the CFA/I antigen from degradation in the gut and effectively delivered it to the immune inductive sites in the intestine [103]. McQueen *et al.* [104] immunised rabbits intraduodenally with rabbit diarrhoeagenic *E. coli* (RDEC) pilus protein in PLG microparticles and subsequently challenged the rabbits with RDEC. Vaccinated rabbits were protected against RDEC disease and protection was presumably mediated by IgA as the levels of this antibody in the bile was

elevated [104]. It was recently demonstrated that multiple oral immunisations of mice with *Helicobacter pylori* antigens in PLG microparticles in the nanoparticle range (0.5 – 0.86 µm) elicited significant intestinal IgA and serum IgG responses compared with antibodies induced in mice injected with soluble antigens, indicating an induction of both mucosal and systemic immune responses [105].

Interestingly, oral immunisation with PLG-microparticle formulated antigens effectively stimulates the common mucosal immune system. A single oral immunisation with fimbriae from *Bordetella pertussis* encapsulated in PLG microparticles protected mice from intranasal challenge with bacteria [106]. Similarly, significant antibody production and protection against aerosol challenge with ricin toxin was demonstrated in mice immunised orally with ricin toxoid in PLG microparticles but not in mice immunised with toxoid in aqueous preparation [107]. In another study, oral immunisation of mice with an anti-idiotype vaccine encapsulated in microparticles protected against occular challenge with *Chlamydia trachomatis* [108].

Oral immunisation of mice with DNA vaccines encapsulated in PLG microparticles have also been evaluated. Mice orally immunised with rotavirus VP4, VP6 and VP7 DNA vaccines encapsulated in PLG microparticles elicited both rotavirus-specific serum antibodies, intestinal IgA and protection against challenge with rotavirus [109,110]. It was recently reported that mice orally immunised with plasmid DNA encoding HBsAg encapsulated in PLG exhibited antigen-specific IFN-γ and CTL responses in the spleen and GALT, whereas naked DNA vaccines given intramuscularly induced only systemic cellular and humoral responses, which were lower than responses induced by oral DNA in PLG microparticles at equivalent doses [111]. PLG encapsulation presumably protects plasmid DNA against degradation in the gastrointestinal tract, and facilitates its cellular uptake and subsequent expression and antigen presentation to elicit both systemic and mucosal antibody responses [112]. However, it is thought that encapsulation of plasmid DNA causes significant damage to the DNA as a result of shear. Adsorption of DNA on the surface of PLG microparticles can overcome this problem and the success of this approach in intranasal DNA immunisation has been reported [113]. It is not yet clear whether adsorption of DNA is an effective method in oral immunisation.

PLG microparticles have also been evaluated in other species. In one study, adult human volunteers were immunised via an intestinal tube with ETEC CFA/II encapsulated in PLG microparticles [114]. Of the 10 volunteers, 5 developed secretory IgA in duodenal fluid and, following challenge with ETEC, 3 of the individuals were protected whereas all 10 unimmunised controls developed clinical disease [114]. Recently Katz *et al* [115] orally immunised adult volunteers with CS6 (polymeric antigen of ETEC) in PLG microparticles. Microencapsulated CS6 antigen induced the best immune responses as indicated by the number of IgA antibody secreting cells in the blood and by serum IgG antibody responses. Results from these small-scale clinical trials suggest that PLG microparticles have potential for oral delivery of vaccines in humans.

However, oral immunisation of pigs with ETEC antigens encapsulated in PLG microparticles did not induce any significant serum antibody responses or reduction in *E. coli* shedding [116]. This is in stark contrast to the studies in rabbits in which PLG-encapsulated ETEC antigens enhanced immune responses and even protected animals against challenge with ETEC [103,114,115]. However, despite the failure in the pig study, results from other animal and human studies warrant further investigations.

A single oral immunisation in 2-week old chickens with formalin-inactivated *Salmonella enteritidis* in PLG microparticles elicited significant intestinal IgA antibody response and protected birds against oral and intramuscular challenge [117]. Systemic priming can significantly improve the success of subsequent oral vaccination. In a study involving nonhuman primates, systemic priming followed by oral boosting was required to induce protection against SIV whereas multiple oral immunisations were ineffective [118].

Modification on the polymer composition of PLG has been explored as an approach to improve particle characteristics. To overcome the hydrophobicity of PLG microparticles, a second hydrophilic polymer, polyethylene glycol (PEG) was introduced to form block copolymers poly-D,L-lactide-polyethylene glycol (PELA) [119]. Microparticles made from PELA had improved protein encapsulation and stability of protein, and reduced the initial burst release of proteins [119]. In addition, *in vitro* studies showed that these copolymer microspheres have high DNA loading efficiency and that the DNA in microparticles had good transfection efficiency and gene expression [120]. *In vivo* studies by Ren *et al.* [121] revealed that oral immunisation of mice with *Helicobacter pylori* antigens encapsulated in PELA microparticles enhanced sIgA in saliva and gut washes compared with animals immunised with soluble antigens. Yeh and Chiang [122] recently reported that oral immunisation of mice with *Vibrio cholera* antigens in a similar blend of copolymer microparticles induced significant serum IgG, IgA and IgM antibodies and conferred protection against challenge as indicated by survival rate of mice [122]. Although these results are encouraging, more work is required with a variety of antigens and additional animal studies to confirm whether PELA microparticles are indeed superior to PLG microparticles.

5.2 Poly-ε-caprolactone

Like PLG, poly-ε-caprolactone (PEC) microparticles are biodegradable polyesters that are biocompatible and hydrophobic, but unlike PLG they do not generate an acidic environment in the intracellular compartment [123]. This suggests that PEC may preserve the antigenic epitopes better than PLG. Oral (or subcutaneous) immunisation of mice with a single dose of an antigenic extract from *Brucella ovis* encapsulated in PEC microparticles was as effective in protecting mice

against intraperitoneal challenge with live *B. ovis* [124]. In contrast, encapsulation of antigens in PLG did not confer significant protection [124]. Subsequent studies by the same group showed that encapsulation of the brucella antigens in PEC elicited a T helper cell type 1 (T_H1) response characterised by high IFN-γ and IL-2 production whereas PLG was associated with a T_H2 response [125]. A study by Baras and colleagues showed that a single oral immunisation of mice with a recombinant glutathione *S*-transferase of *Schistosoma mansoni* encapsulated in PLG or PEC induced serum antibodies [126]. Although more studies are required, so far the data suggests that, perhaps depending on the nature of antigens, PEC can have an impact on the quality of immune response.

5.3 Starch-based microparticles

Oral immunisation of mice with a model antigen, human serum albumin (HSA), in polyacryl starch microparticles induced strong cellular responses as indicated by a delayed-type hypersensitivity reaction [127]. High levels of sIgA were also detected in mice immunised with HSA in microparticles although no antibodies were detected in mice immunised with soluble HSA [127].

In another study, oral immunisation of mice with HSA in starch microparticles induced strong mucosal and cellular immune responses, and oral priming tended to skew the responses towards a T_H2 type immune response as opposed to intramuscular priming [128]. The role of starch in inducing these responses is not known. The situation is likely to be more complex and may be influenced by other factors such as the nature of the antigen.

Strindelius *et al.* [129] showed that *S. enteridis* extracellular antigens conjugated or mixed with starch microparticles protected mice against challenge and interestingly, the subclass profile of antibodies was more of a T_H1 response [129]. This suggests that the nature of the antigen may influence the type of response (T_H1 or T_H2) that is elicited. Rydell and Sjoholm [130] used diphtheria toxin and found that only formulations given orally induced a strong IgA antibody response that neutralised the toxin *in vitro* [130].

5.4 Alginate microparticles

Alginate is a naturally occurring carbohydrate produced by kelp. Alginate polymerises into particles when mixed with divalent cations. With the hypothesis that such particles would be a good way to deliver a variety of antigens orally to a wide range of animal species, Bowersock *et al.* [131] conducted a series of studies in cattle (Ova), rabbits (*Pasteurella multocida*) and chickens (*S. enteritidis*) with alginate encapsulated antigens. *P. multocida* is a pathogen of rabbits causing chronic rhinitis, otitis media and systemic internal abscesses. Rabbits were orally immunised with an outer membrance extract of *P. multocida* encapsulated in alginate microparticles with and without cholera toxin as an adjuvant. The group immunised with the encapsulated antigen had higher nasal IgA responses compared with animals given unencapsulated antigen [132]. Furthermore, addition of cholera toxin to the microparticle formulation further enhanced the IgA responses, although the increase was not statistically significant [132]. Increased protection against *P. haemolytica* challenge was demonstrated by encapsulating antigens in alginate microspheres and delivering them both orally and subcutaneously in mice [133]. Mice immunised orally with the capsular polysaccharide antigen of *Streptococcus pneumoniae* encapsulated in alginate microspheres induced significant protection against lethal intranasal challenge with *S. pneumoniae* [134]. Adenoviruses are an effective vector for delivering heterologous antigens to mucosal sites. However, they are not very stable when administered orally. Encapsulation of bovine adenovirus 3 (Bad3) in alginate microspheres enhanced Bad3-specific IgA response in mice immunised orally [135]. Furthermore, co-encapsulation of Bad3 and a plamid DNA appeared to further enhance the antibody response [135]. A modification of alginate microcapsules has been developed that provides even better stability in gastric simulated fluids and could potentially provide a better means of oral delivery of antigens. Alginate microcapsules were coated with poly-L-lysine, pectin, poly-L-lysine and then alginate. These stable microcapsules now require testing in animals in order to demonstrate their potential use in vaccine delivery [136].

Interestingly, oral immunisation of cattle with the model antigen Ova encapsulated in alginate microspheres enhanced immune responses in the respiratory tract, providing evidence that oral immunisation can stimulate the common mucosal immune system in a large animal [137]. Together, these studies clearly show that alginate microparticles are effective for the oral administration of vaccines in small and large animals. However, so far, there are no published reports on the use of alginate microparticles for oral immunisation in humans.

5.5 Polyphosphazenes

Polyphosphazenes are a relatively new class of synthetic biodegradable polymers with great potential for dual application as vaccine adjuvants and delivery systems. Several reports have documented the adjuvant activity of the polyphosphazene polydi([carboxylatophenoxy]phosphazene) (PCPP), which in some cases performed better than the conventional adjuvants; complete Freunds adjuvant, Quil A and alum [138-140]. PCPP is a water-soluble polymer and microparticles can be generated in an aqueous environment, with no organic solvents are needed [141]. The procedure for the preparation of PCPP microparticles has been described [142]. Briefly, an aqueous solution of polymer (and antigen) forms microdroplets when sodium chloride is added and the microdroplets are then stabilised as microparticles with calcium ions [142]. This is apparently a simple, highly reproducible procedure that can generate microparticles with a narrow size range. The size of the microparticles can be controlled by manipulating the conditions of the encapsulation procedure [142]. There are only a few reports on the *in vivo* testing of polyphosphazene microparticles. Encapsulation of TT in PCPP microparticles induced a significant increase in anti-TT serum IgG titres in

mice after a single intranasal immunisation [143]. In addition, the immunogenicity of influenza antigens was enhanced when the antigen was encapsulated in PCPP microparticles [141]. Further work is required to establish whether polyphosphazene microparticles are an effective oral delivery vehicle. Considering that the procedures for making PCPP microparticles are simple, does not require expensive equipment, and the polymer also has adjuvant activity, more investigations are warranted to explore the full potential of polyphosphazene microparticles for the oral delivery of vaccines.

6. Other forms of oral vaccines

Enteric coating to protect the vaccine from low pH in the stomach and release it in an unencapsulated form in the intestine has been explored as another strategy for delivering oral vaccines. Cellulose acetate phthalate and methacrylic acid are acrylic resins used to coat tablets or granules that protect drugs from the enzymes and the low pH of the stomach. They are also useful to coat vaccines for oral delivery. Vaccine preparations of *Mycoplasma hyopneumoniae* have been prepared using different coatings to stabilise the antigens and to protect them from the acidic environment of the stomach. The antigens are released into the intestines where they can be taken up. Another respiratory pathogen of pigs, *Actinobacillus pleuropneumoniae*, has also been encapsulated in a similar manner. Both vaccines have been tested and shown efficacy when given orally to swine [144-146]. *E. coli* antigens have also been successfully encapsulated using ethylcellulose. Mice vaccinated orally with such microparticles had reduced infection [147]. Fimbriae of ETEC were encapsulated into a cellulose-based enteric delivery system and were also able to reduce disease in pigs in a challenge model [148,149].

7. Conclusions

The current review has attempted to demonstrate that microparticles have great potential as adjuvants and delivery systems for improving the success of oral vaccination. From the evidence of many studies in laboratory animals, it is clear that oral immunisation with antigens in microparticles enhances immune responses and protection against infection. However, there are very few studies in humans and large animals demonstrating the potential of oral immunisation with microparticle formulated antigens. There are a few reports on the use of PLG microparticles in humans, but a clearly successful oral vaccination with microparticles is yet to be demonstrated and marketed. Alginate microparticles have been successfully used in a variety of animal species; however, certainly more work is required in large animals. Alternative approaches such as enteric coating have yielded positive results in pigs, suggesting that various approaches may be useful for different species.

8. Expert opinion

An ideal microparticle delivery system for oral immunisation should protect the antigen from degradation while in transit in the gastrointestinal tract, diffuse through the mucus gel and selectively adhere to M cells, be readily phagocytosed and induce significant mucosal and systemic immune responses. These are very stringent criteria that few microparticles used so far have been able to achieve in humans and large animals. However, advances in our understanding of the factors that influence microparticle and antigen uptake provide opportunities for improving oral microparticle-based vaccines. In the authors' opinion, efforts in several of the areas outlined below have the potential to contribute significantly in the development of highly efficacious microparticle-based oral vaccines;

- Approaches to improve uptake through targeting by linking microparticles with ligand for receptors on M cells (or dual targeting of M cells and DCs) remains one of the most promising approaches. Identification of novel receptors specific to M cells or to the FAE would provide additional targets. Identification of these novel targets could be achieved through a combination of genomic and proteomic approaches.
- Coadministration of adjuvants and antigens in the same microparticle formulation should lead to, not only improved uptake, but also enhanced immune responses. For example, CpG ODN encapsulated in microparticles has potential as the safety and potency of CpG as a mucosal adjuvant is well established. In addition, studies have revealed that optimal adjuvant effects of CpG are achieved when antigen and the ODN are administered in close vicinity. Encapsulation would protect CpG ODN from degradation by nucleases in the gut, and release it in to the intracellular compartment where the ODN receptor TLR9 is localised.
- Further investigations with smaller nanoparticles are warranted, as smaller particles are taken up more easily and presumably enhance immune responses better than the larger microparticles.
- Although studies in small laboratory animals have provided insight on oral microparticle delivery system, they have not translated well to humans and large animals. This raises the issue of relevance or appropriateness of the animal models used. The use of large animal models such as pigs and cattle in the development of the oral vaccines has a distinct advantage in that they would directly benefit the animal health industry and also yield information useful in oral vaccination of humans.

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