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Delivery strategies to enhance oral vaccination against enteric infections ☆

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ABSTRACT

While the majority of human pathogens infect the body through mucosal sites, most licensed vaccines are injectable. In fact the only mucosal vaccine that has been widely used globally for infant and childhood vaccination programs is the oral polio vaccine (OPV) developed by Albert Sabin in the 1950s. While oral vaccines against Cholera, rotavirus and *Salmonella typhi* have also been licensed, the development of additional non-living oral vaccines against these and other enteric pathogens has been slow and challenging. Mucosal vaccines can elicit protective immunity at the gut mucosa, in part via antigen-specific secretory immunoglobulin A (SIgA). However, despite their advantages over the injectable route, oral vaccines face many hurdles. A key challenge lies in design of delivery strategies that can protect antigens from degradation in the stomach and intestine, incorporate appropriate immune-stimulatory adjuvants and control release at the appropriate gastrointestinal site. A number of systems including micro and nanoparticles, lipid-based *strategies* and enteric capsules have significant potential either alone or in advanced combined formulations to enhance intestinal immune responses. In this review we will outline the opportunities, challenges and potential delivery solutions to facilitate the development of improved oral vaccines for infectious enteric diseases.

Contents

Abbreviations: APC, antigen presenting cell; BCG, Bacillus Calmette–Guérin; cAMP, cyclic adenosine monophosphate; CDCA, chenodeoxycholic acid; CFA, colonisation factor antigen; CMCE, carboxymethylethylcellulose; CSR, class switch recombination; CT, cholera toxin; CTA, A subunit of CT; CTB, B subunit of CT; CTL, cytotoxic lymphocyte; DAMP, damage-associated molecular pattern; DCA, deoxycholic acid; DC, dendritic cell; DC-SIGN, DC-specific intercellular adhesion molecule 3-grabbing nonintegrin; dmLT, double mutant LT; ETEC, Enterotoxogenic *Escherichia coli*; FAE, follicle-associated epithelium; FDC, follicular dendritic cell; GALT, gut associated mucosal tissue; GC, germinal centre; GIT, gastrointestinal tract; GM1, monosialotetrahexosylganglioside; GP, glucan particles; GP2, glycoprotein 2; HepB, Hepatitis B; HIV, human immunodeficiency virus; HPV, human papillomavirus; IBD, inflammatory bowel disease; IEC, intestinal epithelial cell; IEL, intra-epithelial lymphocyte; ILF, isolated lymphoid follicle; Ig, immunoglobulin; IFNγ, interferon gamma; iNKT cell, invariant natural killer T cell; IPV, injectable polio vaccine; IRIV, immunopotentiating reconstituted influenza virosome; ISCOM, immune-stimulating complex; LP, lamina propria; LPS, lipopolysaccharide; LT, heat labile toxin from *Escherichia coli*; M cell, microfold cell; MAIT cell, mucosal-associated invariant T cell; MADCAM-1, mucosal addressin cell adhesion molecule-1; MHC, major histocompatibility complex; MLN, mesenteric lymph node; MNP, mononuclear phagocyte; OMV, outer membrane vesicle; OPV, oral polio vaccine; OVA, ovalbumin; PAMP, pathogen-associated molecular pattern; pIgR, polymeric Ig receptor; PLA, poly(lactic acid); PLGA, poly(lactic-co-glycolic acid); PP, Peyer's patch; PVA, polyvinyl alcohol; SC, stromal cell; SED, subepithelial dome; SIgA, secretory immunoglobulin A; SIV, simian immunodeficiency virus; TB, tuberculosis; TFH, follicular helper T; TLR, toll-like receptor; UEA-1, *Ulex europaeus* agglutinin-1; VLP, virus-like particles; WCK, whole cell killed; UN, United Nations

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1. Introduction

1.1. Enteric infections

Infectious diarrhoea is a significant global health challenge. Most diarrhoeal diseases are spread via the faecal oral route, primarily through contaminated water and food, resulting from poor sanitation. In the developed world, diarrhoeal diseases account for significant morbidity whereas in the developing world, where sanitation systems are often sub-optimal, it is associated with high levels of mortality [1], especially in children under 5 years of age who are most at risk at contracting and succumbing to such diseases [2]. Although oral rehydration therapy has reduced the overall number of fatalities caused by diarrhoeal disease, the long term damage resulting from disease episodes is a significant cause for concern [1]. During the first two years of life, infants undergo a significant period of brain development and physical growth. If, during this critical period, children suffer from diarrhoeal diseases, episodes of which can occur repeatedly and are accompanied by impaired nutrient absorption, there is a significant risk that long term developmental disabilities might occur. Stunted growth (often by up to 8 cm at age 7), lower levels of physical fitness, and impaired cognitive function adversely impacting on academic performance can have serious implications in both adolescent and adult life [3]. Diarrhoeal diseases caused by pathogenic *Escherichia coli*, *Vibrio cholerae* and rotavirus have a particularly large global impact. However, a number of other enteric pathogens including *Shigella dysenteriae*, *Salmonella enterica*, *Helicobacter pylori* and *Clostridium difficile* are major causes of morbidity and mortality globally [4] and the development of effective non-living oral vaccines for these would be very desirable.

Enterotoxogenic *Escherichia coli* (ETEC) infections cause acute watery diarrhoea and are spread through the consumption of contaminated food and water. The global disease burden of ETEC is estimated at over 210 million cases and 380,000 deaths annually, mostly in children. ETEC is also a leading cause of Traveller's Diarrhoea in visitors to endemic regions. Recently, oral vaccine efforts against ETEC have focused on the generation of whole cell killed (WCK) bacteria expressing colonisation factor antigens (CFAs), a family of molecules that mediate the attachment of ETEC bacteria to intestinal epithelial cells (IECs), an essential step in pathogenesis [5,6]. Cholera is a severe diarrhoeal infection that continues to pose a major challenge globally and for which oral vaccination to induce toxin and *lipopolysaccharide* (LPS) specific secretory Immunoglobulin A (*SIgA*) responses is seen as the most appropriate vaccine strategy [5]. The diarrhoea caused by cholera however, is much more severe than ETEC, causing between 3–5 million cases and resulting in over 100,000 deaths annually. Disease outbreaks are frequent in endemic regions and often follow in the wake of natural disasters and conflict. Additionally, changes in global climate patterns are leading to increased disease outbreaks as pathogens find new environmental niches to occupy. Recently a correlation between the rise in the global burden of cholera and global warming due to climate change was proposed, indicating that future outbreaks may be more prevalent and may occur in previously unaffected regions [7]. The devastation resulting from the introduction of a diarrhoeal pathogen is exemplified by Haiti which experienced a cholera outbreak in the wake of the 2010 earthquake that was introduced to the country by *United Nations* (UN) relief soldiers from Tibet [8]. In such situations, oral vaccination is a quickly implementable strategy allowing for the containment and even prevention of such outbreaks [9–11]. Owing to this, oral vaccines have also generated interest as a frontline tool in biodefence against possible terrorist or biological *weapon attack* [12].

1.2. Benefits of oral vaccines

Since the development of the first vaccine against smallpox in 1796 by Edward Jenner [13], vaccines have made an enormous contribution to public health, most notably the global eradication of small pox by 1979 [14]. One of the greatest success stories of modern vaccine discovery was the development of an injectable polio vaccine (IPV) by Jonas Salk. IPV reduced the number of cases of polio in the United States from 35,000 in 1953 to 161 cases in 1961, a mere 6 years after its launch in 1955. Concurrently with Salk's efforts, a group led by Albert Sabin developed a live-attenuated oral polio virus vaccine (OPV). These efforts culminated in large scale clinical trials conducted within the Soviet Union in the 1950s which proved the safety and effectiveness of the OPV concept. These results led to the licensing of Sabin's OPV in 1962. The National Institute of Health launched a large scale OPV campaign in the United States in 1963, which largely replaced Salk's IPV vaccine due to the lower cost and ease of administration of OPV. In fact, up to 20 doses of OPV can be applied to sugar cubes and administered to children in the time it takes to load and administer a single dose of IPV, while the discomfort of receiving the injection is also circumvented. This was the first large scale demonstration of the benefits of oral vaccines versus traditional injectable vaccines. Despite its efficacy, OPV has been largely replaced by IPV in western vaccination schedules and OPV will be phased out globally over coming years in the interest of achieving a polio-free World. Polio virus has been documented in the stool of vaccinated individuals, possibly spreading infectious material [15]. This is likely due to shedding of the virus after non-disease causing

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Table 1
(Adapted from Lycke, N., 2012).

Pathogen	Commercial name (manufacturer)	Components (dosing)
Poliovirus	Generally known as OPV (Many)	Trivalent, Bivalent and monovalent live-attenuated vaccines (3 doses)
Rotavirus	RotaTeq (Merck)	Monovalent, live attenuated (3 doses)
Rotavirus	Rotarix (GSK)	Multivalent reassortment rotavirus (3 doses)
Salmonella typhi	Vivotif (Crucell)	Live attenuated *S. typhi* ; Ty21A (3–4 doses)
Vibrio cholerae	Dukoral (Crucell)	Whole killed O1 and El Tor biotype *V. cholerae* and recombinant CTB (2–3 doses)
Vibrio cholerae	Orochol (Crucell)	Live recombinant V.cholerae mutant lacking CTA (single dose)
Vibrio cholerae	mORC-Vax (VaBiotech)	Whole killed O1, O139 and El Tor biotype *V. cholerae* (2–3 doses)
Vibrio cholerae	Shanchol (Shantha Biotechnics)	Whole killed O1, O139 and El Tor biotype *V. cholerae* (2–3 doses)

colonization of the intestinal epithelium. However, of greater concern, OPV virus can *in rare cases* revert to a virulent form, causing latrogenic (vaccine-induced) polio [16].

Since Sabin's demonstration of the feasibility of the oral route for vaccine delivery, several oral vaccines against intestinal infections including cholera, rotavirus and salmonella have been *licensed* and put into use globally (Table 1). Oral vaccines are regarded as a highly advantageous alternative to *injectable* vaccines from a production, economic and regulatory perspective [17,18]. Regulatory requirements for oral products can be less stringent than for injectables, particularly relating to endotoxins [19]. Improved compliance and a reduced risk of adverse reactions are also commonly cited as additional benefits of oral vaccines. Economically, oral vaccines are best suited for mass-vaccination campaigns in poor regions as no needles are required, potentially enabling the option of self-administration. In addition to reducing the need for highly trained medical personnel to administer vaccines, this also avoids the risks of disease transmission through contaminated equipment [20,21]. *If the oral route is to be an applicable solution for large-scale vaccination with nonliving vaccines in resource-poor settings then addressing the challenge of maintaining the cold chain is a priority. Strategies to increase the temperature stability of vaccine components are required in parallel with innovative delivery strategies. In this context, the process of vitrification [22] to enhance stability is an attractive strategy, possibly combined with approaches such as gastric resistant microcapsules [23]. As with other oral delivery strategies it will be essential that the priorities of thermostabilisation and gastrointestinal delivery challenges are addressed in an integrated manner.*

The vast majority of licenced vaccines are still administered via the parenteral route despite the fact that most pathogens enter the body via a mucosal tissue such as the lung or gastrointestinal tract (GIT) [24]. A significant potential advantage of oral vaccines is the induction of protective responses at the mucosal front line and systemically [25]. While systemic immunity is elicited by injectable vaccines, this strategy generally fails to elicit protective immune responses in the GIT. This is illustrated by the efficacy of both IPV and OPV, with the former protecting against the systemic manifestation of the disease, predominantly via IgG in the serum while OPV *induces a local IgA response and prevents initial viral attachment to the epithelium and subsequent entry* [26]. However a recent study shows that boosting with IPV can enhance mucosal immunity in those previously immunised with OPV [27]. Therefore in future oral prime/parenteral boost vaccine strategies may be considered.

1.3. Challenges of developing new oral vaccines

Despite sustained efforts to develop new oral vaccines, very few have been licensed in comparison to those targeted for injectable routes (Table 1). Many oral vaccines have shown promise in animal models but have later failed in clinical trials. However, live-attenuated vaccines against rotavirus and the causative agent of typhoid fever, *S. enterica* have been successfully used [28]. In the case of cholera, two WCK vaccines, Dukoral® and Shanchol™, have been licensed and are a proof of concept that non-living oral vaccines *are feasible* [28]. One drawback of many prototype WCK and subunit vaccines *is suboptimal immunogenicity*, *requiring delivery strategies and the inclusion of immune-stimulatory adjuvants to drive enhanced innate and adaptive immunity* [19]. A major challenge in this regard is that while a range of adjuvants *have been* included in licensed injectable vaccines, to date, none have been licenced for incorporation in oral vaccines. Despite these challenges, the logistical and immunological advantages of the oral route can out-weigh the challenges and there is renewed interest in the development of such vaccines in order to establish a protective immune response at the site of pathogen entry. Before one can begin to develop a candidate mucosal vaccine one must consider the complexity of the mucosal immune system which differs significantly from the systemic system.

2. The oral route

Oral delivery has long been the preferred means of drug administration as the intestinal epithelium is highly absorptive with an incredibly large surface area, encompassing some 300-400 m^2 [29]. The constituent lymphoid tissue, referred to as the gut associated mucosal tissue (GALT) is categorised in terms of inductive sites where immune responses are initiated and effector sites where adaptive immune responses are manifested and executed (Fig. 1). However, despite the advantages of the oral route and the relative ease with which many drugs can be delivered to intestinal tissues, the delivery of oral vaccines is faced with unique and significant challenges. Among the many obstacles to oral vaccine delivery, particularly for subunit vaccines, are poor stability in the GIT due to low gastric pH, digestive enzymes, dilution, dispersal and the mucus barrier. In order to overcome these challenges, it is necessary to design delivery systems to protect and aid in the delivery of oral vaccine formulations to immune inductive sites.

2.1. Physical and chemical barriers

When developing oral vaccines one must consider the physiochemical barriers which constitute the frontline defences against enteric pathogens. Under normal physiological conditions these can prevent colonisation of the intestinal epithelium by potentially invasive pathogens and maintain symbiotic interactions with the microflora but pose significant challenges for the effective delivery of orally administered vaccines. Comprising a hydrogel with incorporated components including proteins, carbohydrates, lipids, salts, antibodies, cellular debris and bacteria, mucus forms a highly viscous and heterogeneous microenvironment [30]. The viscoelastic properties of mucus are dependent on mucins, a diverse family of heavily glycosylated proteins [31,32]. The relative thickness of mucus varies greatly along the length of the GIT averaging 170 μm in the stomach; providing protection against stomach acids, 10 μm in the ileum and 100 μm in the large intestine which is heavily colonised by commensal bacteria [33,34]. The loosely adherent mucus layer is constantly removed by peristalsis and thus has an extremely high turnover [32] although in the colon an underlying firmly adherent mucus layer is present. Viruses may be trapped by hydrophobic and electrostatic interactions with mucin fibres [35]. Mucus characteristics have significant design implications when it comes to WCK vaccines, for example charged molecules may be repelled or become attached to the mucus, potentially enhancing proteolysis and clearance

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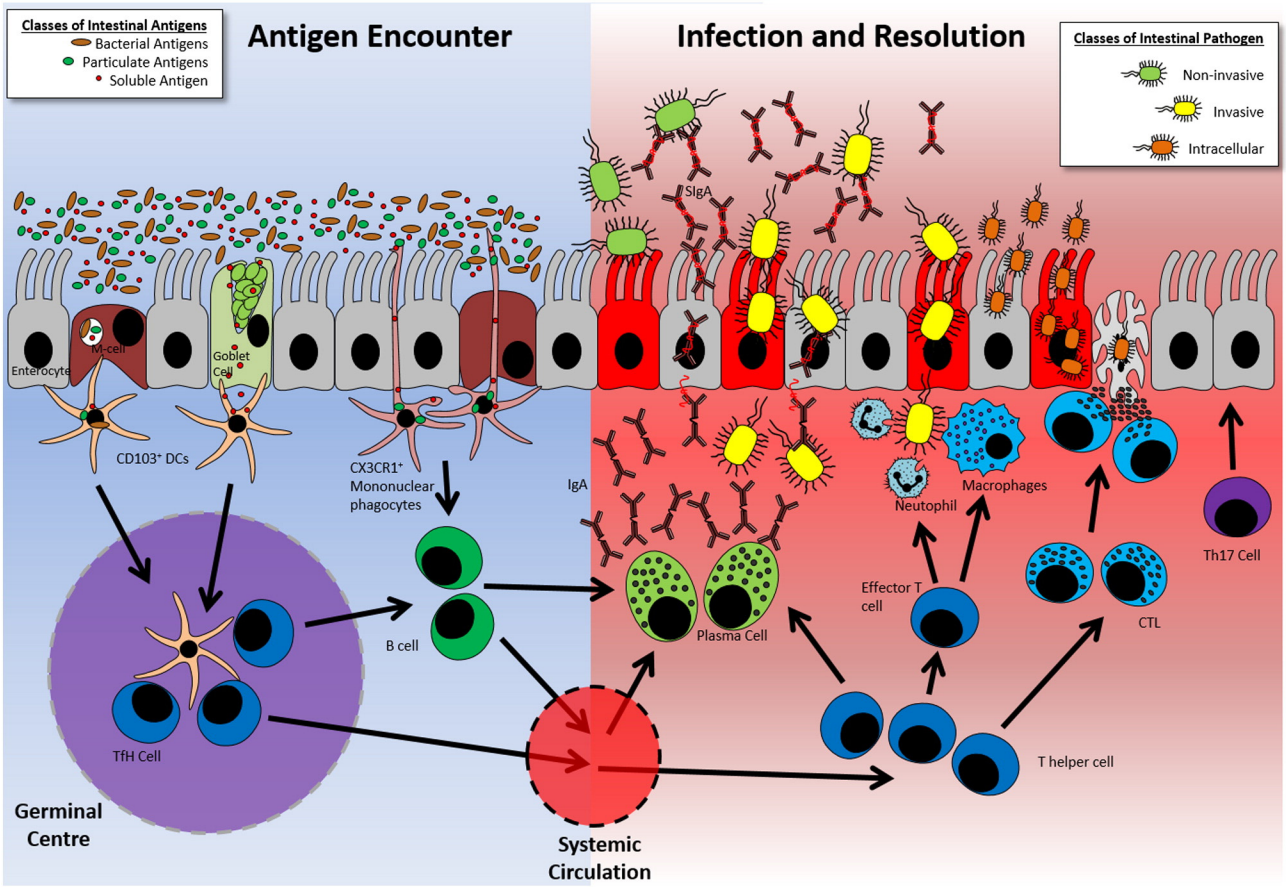

Fig. 1. Antigens are transported across the intestinal barrier by active processes involving sampling and delivery of antigens by microfold (M) cells and goblet cells to dendritic cells (DCs) or by the extension of dendrites by mononuclear phagocytes (MNPs) through tight-junctions in the intestinal epithelium or through pores in M cells. DCs with the help of follicular dendritic cells (FDCs) and T follicular helper (TFH cells) prime T and B cell response in the inductive sites; Peyer's patches (PPs) and the mesenteric lymph nodes (MLNs). This in turn leads to the maturation of B cells into IgA secreting plasma cells and activation of T cells which take up residence at the effector site. Secretory IgA (SIgA) produced by plasma cells can prevent the binding of adherent and invasive pathogens to intestinal epithelial cells (IECs) by neutralizing colonization factors required for adherence. Additionally SIgA can neutralize toxins. Pathogens that cross the intestinal barrier can be trapped and transported back into the intestinal lumen via the pIgR-SIgA transport pathway. In addition helper T cells can recruit and activate neutrophils and macrophages to attack invading organisms. Additionally cytotoxic T lymphocytes (CTL) can produce granzymes and perforins that induce controlled death of cells infected with intracellular pathogens. IL-17 producing cells (including Th17 cells) are also recruited to sites of infection and produce cytokines which repair and reinforce the intestinal epithelial barrier in addition to promoting the production of and secretion of other anti-microbial factors and SIgA.

[36]. Strategies to achieve site-specific vaccine delivery must take into account the significant differences in the nature and thickness of mucus present at specific gastrointestinal sites. Small intestinal mucus was penetrable by polystyrene beads of 0.5-2 μm in size while the inner colonic mucus layer was impenetrable to these particles [37]. Penetration enhancers and mucolytic agents may have potential to address the challenge of the mucus barrier particularly in the large intestine [33]. A further challenge is the large volumes of liquid in the intestine (approximately 10 L of water per day into the adult GIT) which can lead to significant dilution of vaccine components and inefficient delivery to immune inductive sites [36].

Gastrointestinal chemical and enzymatic barriers in the upper digestive tract, stomach and intestine also pose considerable risks to the integrity of delivery systems and labile vaccine components. Lysozyme, phospholipase A and other enzymes found in the saliva can act on peptides or peptidoglycan at the point of entry for vaccines in the oral cavity. The acidic gastric environment (pH 1–2) together with pepsin presents a particularly harsh environment, which is exacerbated by prolonged gastric residency times in humans. Differences in the chemical composition of gastric secretions and stomach residency times varies between species can complicate translation of vaccine findings from small animal models into humans [38]. High concentrations of intestinal proteases can degrade protein and peptide based subunit vaccines, while, bile salts and acids such as deoxycholic acid (DCA) and chenodeoxycholic acid (CDCA) are a further challenge in the case of lipid antigens and lipid based delivery systems.

Targeting cells involved in active antigen sampling is essential since uptake of macromolecules and particulates by and between IECs is very limited [34] Therefore combined strategies to address the physical and chemical challenges and direct vaccines to the key immune inductive sites are required. Since the main intestinal immune inductive sites, Peyer's patches (PPs) are more abundant in the ileum compared to the duodenum and jejunum [34] targeting vaccines, especially particulate formulations to this area is a priority. Additional GALT inductive sites that offer targets for targeted oral vaccine delivery include caecal and colonic patches [34].

2.2. Poor mucosal immunogenicity (hypo-responsiveness and tolerance)

A key challenge when vaccinating orally is the inherent predisposition towards tolerance and hypo-responsiveness by the immune system in the gut. The immunogenicity of many antigens including heat killed bacteria following oral delivery is lower than after injection. *This may be partially due to the potential to accurately inject the pre-formulated vaccine into a discrete site in the muscle or under the skin compared to the dispersal and degradation of oral vaccine components in the GIT*. Therefore, it is generally necessary to incorporate a mucosal adjuvant into the vaccine formulation in order to overcome these challenges,

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although none have been licensed for oral vaccines to date [36]. A consistent trend has been observed where candidate oral vaccines often perform less effectively in subjects during field trials in endemic areas of developing countries when compared to subjects from developed nations [39]. This phenomenon is known as the "Tropical Barrier" and may have several underlying causes [19]. Preliminary work in rodents *has identified* vitamin A deficiency, which is common in developing countries, likely a result of malnutrition, as a contributing factor in the reduced capacity of such individuals to mount a mucosal immune response [40]. Additional factors such as previous exposure to antigens, microbiota composition and parasite infections can also influence the capacity of the intestinal immune system to mount effector and memory responses against oral vaccines [19,41].

3. Immune Responses to enteric infections

3.1. Antigen uptake in the gut

Multiple mechanisms of intestinal antigen uptake exist to sample and deliver luminal antigens to antigen-presenting cells (APCs) which then migrate to the major inductive sites of the intestine, namely the PPs and the mesenteric lymph nodes (MLNs) (Fig. 1). Several processes of antigen uptake have been identified including direct sampling by mononuclear phagocytes (MNPs) [42,43], specialised IECs called *microfold* (*M*) *cells* [44,45] and mucus secreting goblet cells [46] (Fig. 1). The nature of the antigen strongly influences the mechanism of uptake with M cells playing a decisive role in the case of particulates and large antigens. This is a key issue in vaccine design since the efficiency of M cells at sampling luminal antigen makes them a prime target to enhance oral vaccine efficacy. These cells differ from other IECs which function primarily as absorptive enterocytes, specialized for nutrient uptake [47].

M cells differ significantly from enterocytes in terms of the brush border and surface glycocalyx layer, thus allowing effective internalisation of particulate antigens, bacteria and viruses [48]. *Following uptake*, *antigens can be delivered by transcytosis* to underlying dendritic cells (DCs), via direct contact in the basolateral pocket directly below the M cell [49] (Fig. 1). *The relative abundance of M cells at the* primary inductive sites of mucosal immune responses particularly the PPs, makes these an attractive site for oral vaccine delivery [48]. *However*, *of the specialised follicle associated epithelium* (*FAE*), *M cells only constitute approximately 10% in mice* [48] *while in humans this figure is less than 5%* [50]. PPs are built on a stromal cell (SC) scaffold, rich in APCs including a number of DC subsets. It is also in these tissues and the draining MLNs that isotype switching, differentiation of B cells to plasma cells and imprinting of gut homing on lymphocytes occurs [51, 52] (Fig. 1). *The area of the PP below the M cell is referred to as the basolateral pocket and contains a heterogeneous population of lymphocytes, macrophages and DCs. This microenvironment is highly conducive to antigen translocation and encounter with APCs. Studies have demonstrated that in the absence of M cells, antigen-specific T cell responses are compromised demonstrating their pivotal role in the induction of mucosal adaptive immune responses* [53–55]. *M cell development is thought to occur from the differentiation of epithelial stem cells in the crypts* (*see Mabbott et al., 2013 for an excellent review of M cell development, characterisation and function in the intestine*). *Epithelial cells can produce the chemokine CCL20 in response to flagellated bacterial pathogens including S. typhimurium, S. enteritidis and L. monocytogenes, leading to the recruitment of DCs and lymphocytes* [56]. *In the absence of CCR6, the cognate receptor for CCL20, M cell differentiation was compromised* [57–59]. *A subset of CCR6+, CD11c-expressing B cells have been shown to be recruited to the FAE and promotes M cell differentiation* [57]. *Although it is hypothesized that this may be a bacterial pathogenecity mechanism to enhance uptake via the recruitment of CCR6-expressing cells to upregulate M cell numbers* [48], *the pathway may also serve as a novel strategy to enhance M cell numbers in the GIT prior to oral vaccine delivery. While M cells constitutively sample luminal antigens, the expression of ligand-specific apical receptors suggests their capacity to take up materials selectively, potentially providing novel targets for vaccine delivery. These receptors include glycoprotein 2* (*GP2*) *which binds the FimH component of type 1 pili on the surface of both commensal* (*E. coli*) *and pathogenic bacteria* (*S. typhimurium*) [53]. *Annexin A5, a receptor expressed on the apical side of M cells has been shown to bind to the lipid A domain of the LPS molecule found on gram-negative bacteria* [60]. *LPS antigens have been shown to be protective in the case of the oral cholera vaccine* [5], *and so targeting such receptors may offer a novel approach to enhance the uptake of LPS-based antigens. SIgA is the main Ig isotype associated with gut immune responses and has also been implicated in the transport of antigens from the lumen of the gut across the epithelium* [61,62]. *SIgA containing complexes have been shown to be transcytosed through M cells to APCs and lymphocytes in the subepithelial dome* (*SED*) [61,62]. *Although no specific apical receptor for SIgA complexes has been described, Dectin-1 has been implicated as an important player in this process* [63]. *Therefore exploiting S-IgA specific epithelial receptors may offer opportunities to enhance antigen uptake following oral delivery*.

In addition to PPs, caecal and colonic patches in addition to isolated lymphoid follicles (ILFs) throughout the intestinal tract may constitute inductive sites that can be productively targeted for oral vaccine delivery. While similar in some respects to peripheral lymph nodes, this organized lymphoid tissue contains a higher proportion of *B cells to T cells*. As in the case of PPs, a follicle associated epithelium with M cells is present on the lumen facing side of ILFs. *Uptake of pathogens including S. typhimurium, leads to the formation of germinal centers* (*GCs*) *in mature ILFs* [64–66]. Interestingly, it was found that the presence or absence of GCs in ILFs did not influence the active class switching of naïve B cells to active IgA secreting B cells [67]. *This study also found that unlike in PPs, the ILFs were a major site of T cell independent induction and generation of IgA secreting plasma cells* [67].

3.2. Gut immune responses

The majority of activated lymphocytes in PPs reside in GCs, specialized micro-environments facilitating interactions between T and B cells and *resident follicular dendritic cells* (FDCs). FDCs are assisted by a specialized T helper cell subset called follicular helper T cells (TFH) [68]. Together the FDCs and TFH cells efficiently supply conditions that are conducive to efficient class switching and differentiation of naïve B cells into IgA secreting plasma cells, antigen presentation and T-B cell interactions (Fig. 1). Resident DCs play a determining role in the induction of local adaptive immune responses so resolving which DC subsets regulate specific responses and how is a priority in vaccine research. $CD103^+$ DCs *promote IgA-producing B cell responses in the PP and* can also migrate to the MLNs where they can initiate preferential class switch recombination (CSR) to IgA in B cells [69]. T cells resident in the PPs and MLNs are also educated by gut DCs to produce cytokines that are conducive to B cell class switching [70–72] (Fig. 1).

While both DCs and MNPs have been shown to internalise and present antigen, only $CD103^+$ intestinal DCs have the ability to imprint a gut homing phenotype on lymphocytes [73,74]. *Specific targeting of* $CD103^+$ *DCs was shown to stimulate effective mucosal homing of activated T cells* [75]. This is thought to be the reason why vaccine administration at sites other than the mucosa is generally ineffective at promoting mucosal immunity [36]. The imprinting of gut homing molecules on lymphocytes by DCs is dependent on retinoic acid [76–78]. Upon antigen encounter, $CD103^+$ *DCs* undergo maturation which allows them to migrate to the MLNs, initiating adaptive immune responses [73] (Fig. 1). *Thus specific DC populations and local factors result in preferential generation of IgA-producing B cells in the GALT and MLNs and imprinting of lymphocytes with the specific gut homing receptors* $\alpha4\beta7$ *integrin, CCR9 and CCR10* [66,71]. *In support of the idea of a common mucosal immune system, lung DCs have also been shown to be capable of imprinting a GIT homing phenotype on T cells allowing for their migration to the gut*

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where they facilitated protection against an intestinal Salmonella infection [79].

Once primed, lymphocytes exit the PPs and the MLNs via the thoracic duct and blood and finally migrate back to the gut via homing receptors and cognate ligands expressed only in the gut tissue. *The integrin* α4β7 allows lymphocytes to specifically attach to the mucosal addressin, cell adhesion molecule-1 (MADCAM-1) [74,80] *expressed on endothelial cells of the post capillary venules in the gut lamina propria* (*LP*) *thus guiding lymphocytes toward these key gut effector sites* [66]. *The chemokine receptors* CCR9 and CCR10 allow lymphocyte homing to the small or large intestine where endothelial cells produce CCL25 and CCL28 respectively, adding further gut homing specificity [81,82]. *Effector responses can consequently be manifested in the LP or between epithelial cells in the case of intra-epithelial lymphocytes* (*IELs*) [83–85]. The desired outcome of oral vaccination against diarrhoeal infections is the reinforcement of gut barriers through SIgA antibodies [25,86] and effector/memory T and B cells which enhance frontline defences against pathogen reencounter [83,87]. Other local responses against gut infections include the augmented secretion of host factors such as defensins, cytokines and chemokines [88,89].

3.3. Protective responses against enteric pathogens

IgA is the dominant antibody class present in intestinal secretions and breast milk, *conferring passive* immunity to breast feeding infants. It can be found in monomeric or dimeric form, composed of two IgA monomers linked by a J chain. IgA acts at mucosal membranes to agglutinate and neutralize antigens, trapping them in the mucus, which can then be expelled (Fig. 1). On the other hand, IgG, the most abundant antibody class present in the serum and tissue fluids, promotes opsonisation, neutralization and agglutination of antigens as well as complement activation. It can also cross the placenta, thus inducing passive immunity in the foetus.

The GIT produces IgA in kilogram quantities annually [90]. Produced by plasma cells in the LP of the intestine as a dimer, the IgA binds to the polymeric Ig receptor (pIgR) located on the basal side of the intestinal epithelium which then, via an active mechanism, transports the IgA molecule through the epithelial cell across into the intestinal lumen [91] (Fig. 1). SIgA forms a critical part of the intestinal immune system both in protection from harmful pathogens and in homeostasis [91]. It is especially suited to its role in the intestinal immune system as SIgA is highly protease resistant [92]. SIgA exerts its functions in defence against pathogens by neutralizing *bacterial* toxins, blocking adhesion molecules expressed by pathogens and transporting antigens and pathogens out of the LP back into the gut lumen [93] (Fig. 1). SIgA secreted into the lumen of the intestine functions by limiting direct contact of both commensal and pathogenic bacteria and viruses with the gut epithelium, and together with other anti-microbial factors generates a "no go zone" [94]. *Interestingly commensal bacteria have been shown to stimulate antigen specific IgA production, albeit of lower affinity than pathogen-associated IgA responses* [95]. *Recently a study demonstrated that IgA-coated commensal bacteria isolated from inflammatory bowel disease* (*IBD*) *patients led to the establishment of IBD in germ-free mice suggesting that more pro-inflammatory commensals drive SIgA responses with higher affinities* [96].

Since SIgA has been shown to limit the penetration of colonizing bacteria in the intestine [69,97] *its induction as an anti-toxin or anti-colonisation strategy can be highly effective*. Kiyono *et al.* demonstrated that protection against experimental enteric cholera toxin (CT) challenge following oral vaccination was dependent on SIgA [98]. pIgR was required for vaccine-mediated protection against oral CT and heat labile toxin from *E. coli* (LT). Moreover, intestinal SIgA antibodies against the B subunit of CT (CTB) are known correlates of oral vaccine efficacy in humans and SIgA against bacterial colonisation factors has been established as one of the key mediators of intestinal immunity to ETEC infections [99]. *Thus SigA can act in the exclusion and excretion of antigens in addition to potentially enhancing local immune responses by targeting antigens specifically to M cells* [62].

4. Oral vaccine components

4.1. Classes of mucosal antigen

The current licenced oral vaccines are either composed of live-attenuated organisms or WCK bacteria either alone or in combination with protein subunit components (Table 1). Live-attenuated vaccines can be highly effective as they establish a limited local infection, thus contributing many intrinsic immune-activating signals. However, there can be safety concerns associated with some of these vaccines, particularly in immunocompromised individuals [100]. *While live attenuated vaccines will continue to play a role in global vaccine efforts, there is a move towards non-living, subunit vaccines or enhanced WCK approaches both from a safety and cost perspective* [28].

The over-expression of ETEC antigens by non-pathogenic *E. coli* is one strategy which has delivered *promising* pre-clinical and phase I human trial results [101–103]. Additionally, novel strains of *V. cholerae* expressing multiple surface antigens have been generated and hold promise for future trials [104]. The use of WCK genetically modified non-pathogenic bacteria as antigen vectors may improve safety and ease of production while enhancing antigenicity.

In an alternative approach, outer membrane vesicles (OMVs) derived from *Burkholderia pseudomallei* have been demonstrated to be safe, non-replicating and immunogenic following intra-nasal immunisation [105]. *Early studies in nonhuman primates have also demonstrated the potential of B. pseudomallei OMVs as novel antigens* [106]. *Several other bacteria-derived OMV-based antigens are also in development, while an OMV-based vaccine against Nesseria meningitidis has been licensed for* (*injectable*) *use in humans* [107]. Anthrax spores have also been evaluated by the nasal route and successfully protected against challenge following vaccination [108]. *The potential of these systems for oral vaccination is less clear but their efficacy could be enhanced by capsule based approaches with incorporated adjuvants. Other strategies that may offer potential include the expression of pathogen-derived antigens on live lactic acid bacteria* (*Lactobacillus casei and L. lactis*) [109] *and virus-like particles* (*VLPs*) [110]. *Components of toxins and toxoids have also been used to provide anti-toxic immunity. This is best demonstrated by the CTB component of the oral cholera vaccine Dukoral® which successfully protects against both CT and LT induced diarrhea for up to 6 months* [99]. *It has been suggested that CTB acts in synergy with WCK V. cholerae, enhancing immunity against both components* [99]. *However, Dukoral® is an exception to the rule for many subunit vaccines have been tested and failed* [36]. *This could be due to the high immunogenicity of CTB, its ability to bind to the ganglioside receptor monosialotetrahexosylganglioside* (*GM1*) *and enter nucleated cells and its intrinsic immunomodulatory capacity* [111] *as well as the presence of heat killed bacteria. Regardless of the type of non-living antigen, in most cases immunogenicity is not sufficient when these are administered alone, necessitating delivery systems and adjuvants to enhance adaptive immunity*.

A considerable challenge to developing new mucosal antigens is the requirement for their production under good manufacturing practise (GMP). A Vietnamese oral cholera vaccine ORCVAX® could not be licensed for use by the World Health Organization due to production issues not complying with GMP conditions [112]. These issues were however later addressed by the production of the ORCVAX® formulation in India by Shantha Biotechnics under GMP conditions and it is marketed under the trade name Shanchol®. Therefore the ability to produce a vaccine under GMP is an important early consideration to make and one that is exemplified by the current oral ETEC vaccine candidate undergoing clinical trials where all components are produced under GMP [101].

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4.2. Mucosal adjuvants

While several adjuvants have been developed to improve the immunogenicity of injectable non-living vaccines, these are lacking for the oral route. The most potent category of mucosal adjuvants identified to date are those based on the bacterial enterotoxins, CT and LT. These toxins share significant sequence homology [113] and are complexes composed of an ADP-ribosylating A1 (active) subunit connected to a donut shaped pentamer of B (binding) subunits joined by an A2 linked chain [114]. Following binding to GM1 and internalisation, the A1 subunit of the holotoxin mediates a cascade of intracellular events, culminating in the ADP-ribosylation-mediated increase in the cytoplasmic level of cyclic adenosine monophosphate (cAMP) a feature also associated with osmotic diarrhoea during infection [115]. The adjuvanticity of CT and LT is thought to involve both enhanced permeation of antigens across mucosal epithelial barriers and an increase in antigen presentation and activation of APCs [99,116]. Enhanced M cell targeting is also observed in vaccines adjuvanted with LT and CT [25]. *CTB was shown to promote DC-driven induction of IgA producing B cells by DCs* [117], *which may account for its use as an immunotherapeutic adjuvant* [118] *although the induction of potent antibody responses against orally delivered CTB generally requires the addition of a potent adjuvant*. Due to the high level of structural and sequence homology between CT and LT, it is plausible to speculate that both toxins exert their adjuvant effects by identical or highly similar mechanisms. Unfortunately, *despite their efficacy* in pre-clinical studies these molecules are too toxic in their native form for use in humans. When a nasal VLP-based influenza vaccine adjuvanted with LT was tested in humans, several individuals developed Bell's Palsy (facial paralysis) as a result of LT interacting with GM1 on nerve cells [119–121]. Additionally microgram amounts of CT and LT cause profuse diarrhoea in humans thus making them unsuitable for the oral route [36].

This led to the generation of toxin mutants with attenuated enzymatic activity by site directed mutations in the A1 subunit [122]. Site-directed mutagenesis of LT led to the generation of potent mutant toxin adjuvants which appeared safer than their native counterparts. LTK63 and LTR72, developed by Novartis, contain single amino acid mutations that abolishes or significantly reduces their ADP-ribosylating activity respectively while maintaining their ability to elicit immune responses [123]. In preclinical intranasal studies, whole cell mucosal vaccines adjuvanted with LT mutants led to protection against challenge with the mucosal pathogens *Bordetella pertussis* [124] and *Streptococcus pneumoniae* [125]. Additionally, oral and intranasal administration of a human immunodeficiency virus (HIV)-1 p55 gag subunit vaccine adjuvanted with LT mutants elicited strong cytotoxic T cell responses [126]. However, when tested in clinical trials, despite lacking all enzymatic activity [123], Bell's Palsy was documented in a small number of patients receiving an LTK63 adjuvanted intranasal vaccine [36]. This led to regulatory authorities cautioning against the intranasal use of GM1 binding adjuvants [127] although these molecules may still hold significant promise as oral adjuvants *where they can exhibit unique potency*. Recently a double mutant LT (dmLT) was developed [128], having greater stability in the presence of trypsin, strong oral adjuvant properties [129] and an acceptable enterotoxicity profile, even at high doses, and is currently being assessed as part of a prototype oral ETEC vaccine in clinical trials [130].

Another strategy to overcome the issues associated with toxicity was adopted by Nils Lycke's group to develop a non-toxic CT derivative. This unique adjuvant is a fusion protein composed of the A subunit of CT (CTA) which retains 100% of its enzymatic activity and the dimer of the D-fragment of the *Staphylococcus aureus* protein A to form the adjuvant CTA1-DD [131]. CTA1-DD has been *shown to enhance the immunogenicity of a number of different vaccine antigens* [132]. By exclusively using the CTA portion of CT, CTA1-DD is also unable to bind to GM1 which has greatly enhanced its safety, and no side effects were observed in tests in animal models and non-human primates following nasal administration [133,134] although oral delivery *of this adjuvant has* received less attention.

Recently populations of unconventional T cells have attracted interest as possible adjuvant targets for mucosal vaccination. Their localisation at mucosal sites, immune-modulating capacity and the availability of known ligands for both invariant natural killer (iNKT) cells and mucosal-associated invariant T (MAIT) cells has attracted interest in targeting these cells to potentiate immune responses to orally delivered antigens [135,136]. *Another novel oral adjuvant strategy has been the administration of live bacterial adjuvants such as Bacillus Calmette–Guérin (BCG), Lactobacillus plantarum or L. rhamnosus together with inactivated simian immunodeficiency virus (SIV). This led to the protection of macaques against intra-rectal SIV challenge, albeit by a novel means of tolerance induction rather than antibody or cytotoxic lymphocyte (CTL)-mediated protection* [137].

5. Oral vaccine delivery systems

5.1. Lipid based delivery systems: emulsions, liposomes and ISCOMs

Some of the earliest adjuvants and delivery systems developed took the form of lipid-in-water or water-in-lipid emulsions such as Freund's incomplete adjuvant (Fig. 2C&D). Emulsions have been extensively tested in various human trials, leading to the licensing of systems including MF59; an oil-in-water emulsion containing squalene that drives humoral immunity and CD4+ T memory cells [138]. Micelles are one of the earliest examples of utilizing lipids as delivery vehicles and consist of amphiphilic block copolymers displaying both hydrophobic and hydrophilic segments which allows for differences in solubility. These unique properties drive their self-assembly into core-shell architectures which then allows for a payload to be delivered inside the core or attached on the outside to the shell depending on the electrochemical properties of the vaccine formulation [139]. However, micelles can often dissociate when diluted, leading to loss of the payload. Newer polymers allow for the intentional release of a payload in a controlled-release fashion. Micelles can also be synthesized in the nano-meter range, allowing for the engineering of delivery vehicles that can potentially penetrate mucus and be taken up by mucosal antigen presenting cells.

5.1.1. Liposomes

While micelles are composed of lipid monolayers, liposomes are characterized by a single or collection of multiple phospholipid bilayers surrounding an aqueous core [140]. This structure grants liposomes the ability to be tailored to suit a large variety of different applications (Fig. 2F). While currently no liposome-based systems are licensed for human use, several are in various stages of clinical trials. AS01 developed by GlaxoSmithKline, is liposome-based and includes the toll-like receptor (TLR)-4 agonist MPL and the purified QS21 fraction of Quil A saponin. This adjuvant is a vaccine component in an ongoing phase III malaria trial [141,142]. While most clinical trials involving liposomes have focused on the intra muscular route, there *is some* evidence to support their use as mucosal delivery systems [143].

An important determinant of the adjuvanticity of liposomes has been shown to be the surface charge on the vesicle. This is determined by the chemical properties of different phospholipids with charged head groups (Fig. 2F). Positively charged (cationic) liposomes have been demonstrated to possess the strongest adjuvanticity compared to neutral and negatively charged liposomes [144]. This is likely due to the overall negative charge (anionic) of most cell membranes resulting in *interactions with the* cationic liposomes, which may explain the enhancement of antigen uptake [145]. *However, a concern is the greater toxicity of cationic than anionic liposomes. Recently a study was conducted into the mechanisms of toxicity of cationic liposomes and carriers, reporting that cationic delivery vehicles induce necrosis, the release of damage-associated molecular patterns (DAMPs) and inflammation in vivo* [146]. *However these effects are likely to be highly dose*

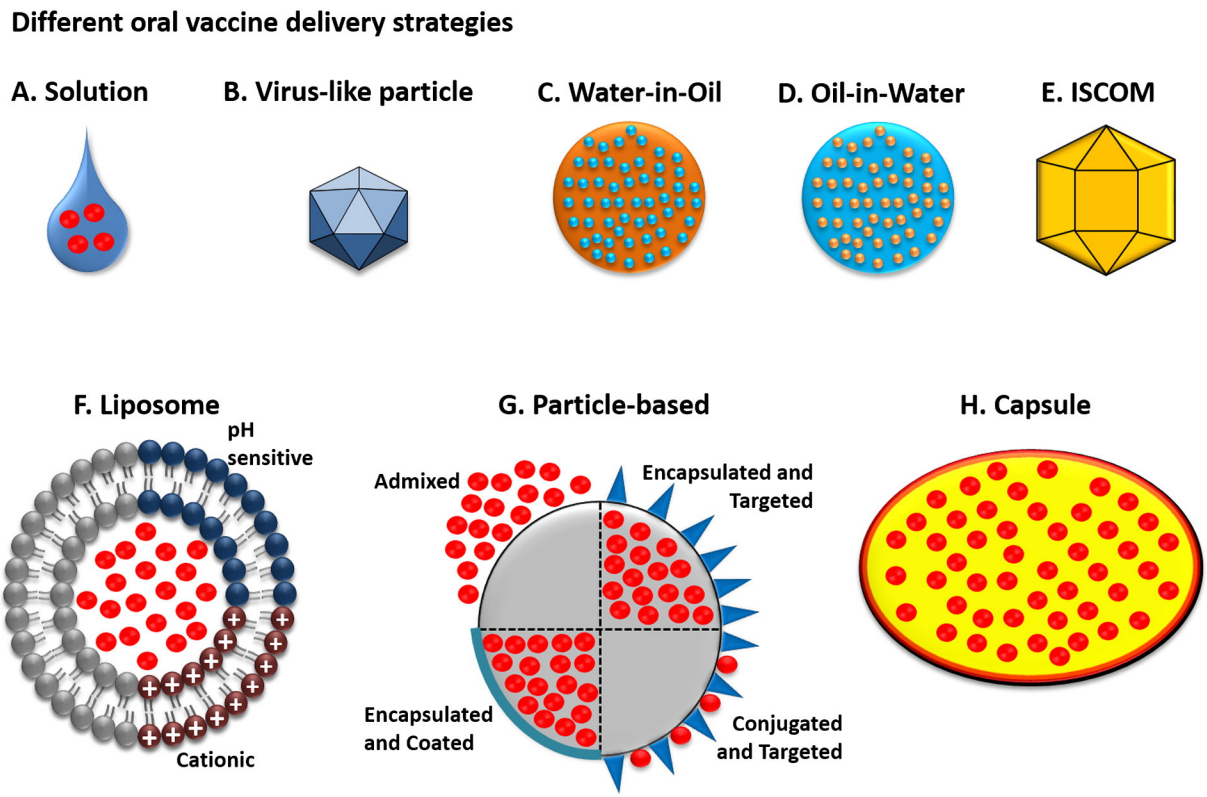

Fig. 2. Many different delivery technologies have been developed for the oral route for a diverse range of applications. A) Delivery of oral vaccines dissolved or suspended in solution has to date been the preferred route due to simplicity. Often a bicarbonate buffer is added to neutralise stomach acids. (B) Virus-like particles and virosomes are composed of non-replicating viral capsid components that self-assemble and preserve both antigen sequences and 3D structure. Lipid based delivery strategies have been used for many routes of vaccination. Emulsions composed of (C) Water-in-oil or (D) Oil-in-water can help to delivery poorly soluble components while also displaying potent adjuvant properties. (E) Immune-stimulating complexes are related to (F) liposomes but form icosahedral pentagons due to the inclusion of cholesterol and saponins in the membrane leading to intrinsic adjuvant properties. (F) Liposomes are composed of one or more lipid bilayers encasing an aqueous core. By varying the charge in the head groups of the lipids in the membrane it is possible to generate pH sensitive and positively charged cationic liposomes. (G) Particles varying in size from the nano to the micro range have been successfully used as delivery vehicles and adjuvants or oral vaccine delivery. Antigens can be admixed together with particles, encapsulated or conjugated to particles. The addition of targeting molecules and coatings can add further tissue/cell specificity to particles and allow for the protection against harsh environments such as in the stomach. (H) Capsules and pills have been used as oral delivery vehicles, with established manufacturing processes, accurate dosing and ease of administration. Vaccine components can be incorporated into a matrix or held in reservoirs in the structure of the capsule. Furthermore, the capsule approach allows for the incorporation of the other delivery systems outlined above (A-G) into a large delivery vehicle.

and route dependent. Many peptide and protein antigens are anionic, thus allowing for the exploitation of electrostatic associations in the association and loading of antigens. CAF01 is an example of a cationic liposome that has been shown to enhance cellular immunity against Tuberculosis (TB), HIV, chlamydia and malaria antigens [147–150]. Interestingly, CAF01 was shown to be a highly effective adjuvant/delivery system for intra-nasal administration of vaccines. Co-delivery of CAF01 with the influenza vaccine Vaxigrip® led to enhanced systemic IgG and Th1 responses in mice [151]. Furthermore, intra-nasal vaccination with cationic liposomes enhanced mucosal IgA and systemic IgG responses together with potent cell-mediated immune responses but most importantly protection against lethal infection following the delivery of a DNA vaccine against *B. pseudomallei* [152]. The natural hydrophobicity of liposomes endows them with the ability to fuse with target cell membranes, allowing for direct delivery of antigens into the cytosol and trafficking to the major histocompatibility complex (MHC) class I presentation machinery. Such fusogenic liposomes have been shown to stimulate cell mediated immunity and are capable of augmenting $CD8^+$ T cell responses to vaccine antigens [153,154].

While the efficacy of liposomes has been extensively demonstrated for the injectable and more recently the intra-nasal route, their use orally is more limited. As with emulsions and micelles, liposomes are susceptible to degradation in the GIT through acidic pH, lipases and bile salts. Bile salts such as CDCA and DCA are secreted into the GIT to digest lipids making it unlikely that liposomes would retain their structural integrity in such an environment, thus compromising their ability to protect antigens and potentiate immune responses long enough to reach inductive sites in the gut. *One strategy to enhance oral efficacy was the use of lectin bearing polymerized liposomes* [155] *with increased gastrointestinal stability. Additionally, attempts to coat liposomes with polymers to protect them from degradation in the GIT has yielded some success in mouse models* [156] *but the potential of this technology remains to be extensively evaluated.*

5.1.2. Immune-stimulating complexes

The addition of cholesterol and saponins; most commonly Quil A to liposomes results in the generation of self-assembling pentagonal dodecahedrons. The incorporation of these additional components confers intrinsic adjuvant properties earning their name as immune-stimulating complexes (ISCOMs) (Fig. 2E). ISCOMs were developed in the 1980s and can be considered a second generation liposome in many regards [157]. Two variants of ISCOMs exist with the first "classical" variety containing entrapped protein antigens of either viral or bacterial origin allowing them to act as both a delivery and adjuvant system. The second variety, referred to as ISCOM matrices, contain no entrapped antigens and are utilized as a co-delivered adjuvant more so than a delivery system. Early ISCOM formulations could not be evaluated in humans due to the reactogenicity of Quil A. *QS21 is now commonly tested alone in vaccine formulations and within ISCOMs due to* its improved safety profile while still remaining active as an adjuvant [158,159]. One major challenge when working with ISCOMs is the

difficulty associated with antigen incorporation. ISCOMs are negatively charged and so cannot interact with negatively charged protein subunit antigens. The development of PLUSCOMS and Posintro™ has been successful in altering the surface charge of the particle [160,161]. The incorporation of positively charged cholesterol derivatives led to the induction of potent immune responses following vaccination *with* hepatitis B (HepB) surface antigen [161]. These modifications allow the incorporation of hydrophilic antigens with greater ease in contrast to hydrophobic antigens as was the case with classical ISCOMs. However, several trials have suggested that ISCOM matrices used purely as adjuvants may be just as beneficial at eliciting immune responses as classical or charge-modified ISCOMs [162]. The use of unmodified ISCOMs as adjuvants would significantly simplify production, however the benefit of antigen encapsulation would also be lost, which for the oral route is an important key to success. ISCOMs containing diphtheria toxin were capable of priming both humoral and systemic responses following injection [163] but neutralizing antibody responses in the serum and intestinal IgA responses were limited following oral administration [163]. These results suggest that to improve the oral efficacy of ISCOMs a modification of the immunization protocol such as including an oral/injectable prime-boost regimen or the use of additional coatings or adjuvants may improve efficacy. The inclusion of the mucosal adjuvant CTA1-DD did enhance the ability of nasally delivered ISCOMs to enhance immune responses [164], suggesting that the addition of an additional immune stimulatory component may serve to potentiate the oral efficacy of ISCOMs.

5.2. Virus-like particles

VLPs have delivered more licensed vaccines with less expenditure than adjuvanted subunit antigens, begging the question as to whether *particulate antigen presentation should be the key focus for* non-living vaccines [165]. Their intrinsic antigenicity coupled with the ease of their manufacture has attracted a significant degree of interest [165–167]. VLPs make for a very practical alternative to traditional live-attenuated viral antigens in that they consist of a self-assembling 3D viral envelope complex similar to their native virus [165,167]. These 3D structures maintain not only the unique peptide sequence of the antigen, but also the antigenic structure (Fig. 2B). Additionally, this multimeric complex lacks any viral RNA/DNA and is therefore incapable of replication [168]. Moreover, VLPs can be expressed with additional antigens on or, in the case of polysaccharide or lipid molecules, conjugated to the surface to create a multivalent vaccine [166, 169–171]. VLPs contain one or more viral capsid proteins which make up the protective outer shell of a virion and are usually expressed in a eukaryotic cell [110]. An example of a clinically and commercially viable VLP vaccine is the HepB vaccine [110], a product of the self-assembly of HepB surface antigen expressed in recombinant yeast cells [165]. Another licensed VLP vaccine is the human papillomavirus (HPV) vaccine which was also the first prophylactic vaccine against a carcinogen [165]. Produced by Merk & Co, Gardasil® is a self-assembling VLP providing quadrivalent protection against genitournial HPV infection. It is composed of the L1 capsid protein of HPV-6, −11, −16 and −18 types expressed by recombinant yeast cells [172]. Interestingly, this vaccine, which is administered in adolescents via injection, elicits strong protection at genital mucosal surfaces via neutralizing serum IgG production which transudes across the epithelium [173–175]. VLPs against rotavirus have also been developed and have been shown to be both stable and protective against rotavirus challenge in animal models following injection [109]. Virosomes differ from VLPs as they are composed of both viral proteins and lipids, either viral or otherwise, and resemble liposomes [176]. Immunopotentiating reconstituted influenza virosomes (IRIVs) include influenza membrane proteins hemagglutinin and neuraminidase in the construct [177] and in addition to their application for influenza vaccines [178,179] have been shown to be efficacious delivery vehicles for vaccines against hepatitis A [180] and HIV [181].

Although the landscape for the mucosal application of VLP and virosome-based vaccines is currently sparse, both technologies represent potential platforms to support the development of novel, non-living oral vaccines against enteric viruses [182]. The advantages of VLPs and virosomes for oral delivery are their small size and the variable composition of their surface chemistry, which allows for the selection of appropriate electrochemical properties to ensure desirable interactions with membranes and mucus. VLPs and virosomes can also be engineered to incorporate proteins with a variety of beneficial functions including targeting and immunostimulatory activity by inserting the corresponding genes into the recombinant vector's genome. However, VLPs and virosomes both suffer from challenges in formulation, scaling up and both require extensive purification and quality control which adds to manufacturing costs compared to synthetic polymer-based systems. In addition, VLPs require the addition of adjuvants in order to elicit enhanced immune responses. However, virosomes may not require the addition of adjuvants and so may be the more attractive of the two options as oral delivery vehicles [183]. *Results in mice following intra-nasal application of Noravirus-based VLPs have been encouraging and will perhaps in time lend themselves to the oral route* [184,185] *although it may be essential to incorporate these lipid based systems in enteric capsule based formulations to enhance their oral effectiveness and protect them from the lipid digesting gastrointestinal environment.*

5.3. Synthetic particle-based strategies

Synthetic particles are one of the most versatile oral delivery systems where a vaccine payload is either attached, adsorbed or dispersed and encapsulated within a polymer matrix (Fig. 2G). Their size can be controlled, ranging from 30 nm up to millimeters in size. Leveraging these technologies *potentially* enables the circumvention of many of the barriers imposed on mucosal vaccination allowing antigens and adjuvants to be delivered to the desired mucosa. *Particles* can be designed to deliver low molecular weight drugs or biological molecules upon degradation, swelling and diffusion from the polymer or change in electrostatic interactions. These delivery systems have attractive versatility due in part to the large number of potential components and formulation methods. Such factors facilitate the production of particles of defined sizes and various architectures with distinct chemical properties in order to target specific cell types or specific mucosal sites and to suit specific payloads based on desired surface charge and release kinetics. Furthermore, polymer selection can influence intrinsic adjuvant effects, biocompatibility and biodegradability. The most widely studied particles so far are those produced from poly(α-hydroxyl acids), poly(amino acids) and polysaccharides with the most common polymers being poly(lactic-*co*-glycolic acid) (PLGA), poly(lactic acid) (PLA), β-glucans, alginate and chitosan [186–188]. While biodegradable particles have been extensively investigated for vaccine delivery, the potential of non-biodegradable alternatives such as carbon, silica and gold has also been suggested. The advantage of using such particles is the impressively small size at which these can be manufactured (2 nm-50 nm). Small nanoparticles have been shown to greatly aid uptake of payloads across challenging physiological barriers such as the intestine and blood brain barrier. The porous nature of carbon and silica particles also provides cavities in which to load antigens and adjuvants.

Many of these particulates have already seen clinical application for drug delivery and their role in improving the efficacy of mucosal vaccination is the topic of much investigation [186]. Some of the *suggested merits* which make them applicable for mucosal vaccine delivery include their ability to protect the payload from degradation, penetrate the mucus barriers and to control and target the release of both antigens and adjuvants. In principle these properties can be optimised by altering particle size, surface chemistry and 3D architecture. The size of a particle plays a critical role in determining the dose of both antigen and adjuvant

that can be loaded. When vaccinating at mucosal surfaces, it has been demonstrated that particulate antigens are often more immunogenic than antigens in solution [19]. It is thought that particulate antigens are more likely to be trafficked across the mucosa and be taken up by APCs [189]. Uptake of particles primarily occurs via M-cells (Fig. 1). *Although, few studies have conclusively addressed the role of size in dictating mucosal immunity following oral delivery, there is a consensus that particles with a diameter smaller than 1 μm are more readily taken up by M cells* [190]. In addition to the role of size in regulating the potential for trans-epithelial uptake, the ability of particles to migrate through the pores in the mucus is dependent on both size and surface electrochemical properties [33,191]. *While M cells remain a key target for particulate oral vaccines, it was recently shown that particles of 1-5 μm in size were taken up across the jejunum and ileum via non phagocytic processes, suggesting that particle uptake is not solely via M cells* [192]. *In the latter study, particle uptake was greater for 0.5 μm and 1 μm particles than for those of 2 μm or 5 μm in size.*

Nanoparticles formulated using the copolymer PLGA are most widely used as a result of its approval [193] for applications including biodegradable sutures and controlled release properties [194]. PLGA nanoparticles have been shown to penetrate and distribute through mucosal tissue after local administration, most likely via cellular trafficking [195]. *Although challenging, the surface chemistry of PLG particles* can be altered to increase diffusion through mucus and uptake by M cells [193,196,197]. The architecture of PLGA nanoparticles also allows for the encapsulation of antigens and adjuvants, thus providing a protective function [196]. *However, the formulation process, generation of a low pH during polymer hydrolyisis and exposure of surface antigens to gastrointestinal proteolysis are major challenges for PLG based oral vaccine approaches.*

Nevertheless, association with PLG particles was shown to result in enhanced systemic and mucosal immune responses following vaccination by the oral [198,199] and nasal [200–202] route in the case of antigens from *B. pertussis*, *V. cholerae*, *S. aureus*, SIV as well as the toxin ricin [198–202]. The mechanisms underlying the adjuvanticity of PLG particles are currently unclear but are the subject of ongoing research. PLG nano- and microparticles enhanced IL-1β production by dendritic cells via a NLRP-3 inflammasome dependent process [203]. The crucial role of the IL-1 family of cytokines in both innate and adaptive immunity is well documented [204,205] although the role of the inflammasome in the induction of adaptive immunity following mucosal vaccination with particulate adjuvants is currently unclear.

Despite the promising results with PLG nano and microparticles in rodent models, progress towards clinical application has been disappointing. Oral immunization with a PLG encapsulated SIV antigen enhanced protective immunity against a subsequent intravaginal challenge with SIV [198], however, this effect was dependent on a systemic prime vaccination [198]. A phase 1 trial with orally delivered PLG-encapsulated CS6, a prominent ETEC antigen, delivered poor results in human volunteers. While well tolerated, the total number of subjects responding to the vaccine was less than 50% [206]. While these results should not condemn the use of a particle-based approach to oral vaccines it is likely that these original formulations will need to be improved upon for example by targeting and that particles may need to be included as one component of an advanced delivery strategy.

Both synthetic and naturally occurring polymeric particulate delivery systems can be engineered to have a specific surface chemistry and composition in order to overcome mucosal barriers, interact with specific cell types and exert desired immunomodulatory functions. Altering the electrochemical properties of the particle surface can not only determine its interactions with mucus, but hydrophobic particles have been shown to engage DAMP receptors leading to activation of innate cells via this intrinsic adjuvant property [207]. Complement mediated immune activation by particles can also be achieved by adding hydroxyl groups to the surface to enhance interaction with the complement activating protein C3b [208]. Pathogen-associated molecular patterns (PAMPs) can also be attached to the surface of particles to enhance innate activation via pathogen recognition receptors including TLRs [193].

Glucan particles (GPs) are derived from *Saccharomyces cerevisiae*, and can enhance intestinal SIgA responses and Th1/Th17 mediated cellular responses to associated antigens following oral vaccination [209]. GPs can be efficiently loaded with antigen (>90%), target M cells for uptake and also exhibit an intrinsic immunomodulatory capacity [210,211]. β-glucans are fungal PAMPs, signalling through receptors such as dectin-1 and complement receptor 3 expressed on DCs, monocytes and neutrophils [209,212]. Furthermore GPs have been generally recognized as safe by the Food and Drug Administration [213]. However, GP manufacture is currently limited to liquid formulations, which require cold-chain storage and thus not optimal for use in poorer settings. In addition there may be a requirement to use muco-lytic agents such as N-acetylcysteine when evaluating the oral efficacy of GPs suggesting that are susceptible to becoming trapped in the mucus layers [214].

While particle-based delivery systems offer many advantages for both antigen uptake and enhancement of mucosal immune responses, antigens can be exposed to degradative steps during manufacture. Particles are often formulated using emulsification techniques requiring organic solvents [215] although there are other approaches include gelation of polymers suspended in emulsion droplets when the suspension is exposed to a change in temperature, pH or addition of cross-linking agents [216]. By tailoring polymers and formulation conditions, it is possible to exert control over intrinsic triggers for antigen release (pH, the presence of certain enzymes etc.) as well as the rate of degradation [217]. Depending on their specific nature, labile antigens can be damaged by high shear rates, use of organic solvents and elevated temperatures. In order to overcome these challenges, some groups have adsorbed or conjugated antigens to the surface of particles, thereby sparing antigens the harsher manufacturing steps that may result in their destruction (Fig. 2G). However, in the context of oral vaccination, as antigens are now on the surface of particles, this exposes them to the degradative environments of the GIT. This poses additional delivery challenges, which need to be overcome to ensure protection of the payload.

5.4. Tablets and capsules

In contrast to micro and nanoparticles, capsules are significantly larger in size and contain one or multiple reservoirs of a vaccine formulation usually suspended or dissolved in an oil- based or aqueous solution, encapsulated in a solid or semi-solid shell. *While no subunit or WCK oral vaccine is currently delivered by capsule or tablet, the live attenuated Salmonella vaccine Vivotif® and many drugs and supplements are routinely delivered using this strategy*. Capsular approaches exploit an appropriate physical size (average capsules/tablets range from 5 mm to 20 mm in size) and these systems can be highly versatile. In principle, capsules allow for the incorporation of many, if not all of the previously introduced delivery technologies in a primary delivery vehicle that can be loaded with an accurate dose during manufacture. *In fact, this may be an essential requirement in most cases to protect and increase the effectiveness of these systems*. It is suggested that some capsules and tablets can be designed to enhance the stability of vaccines to environmental conditions such as high humidity and temperature, which may reduce the need for specific storage conditions and can greatly reduce the cost of implementing such a strategy in the field. Recently a tablet-based oral influenza *vaccine was shown to elicit* strong anti-viral antibody and cellular responses against avian flu antigens in humans [218]. This approach utilized a non-replicating adenovirus vector expressing avian flu antigens together with a TLR3 agonist [218]. This approach may be adaptable to an oral vaccine against adenoviral diarrheal infections, which have significant incidence in children, especially in developing countries [219]. Using capsules, it may be possible to adapt delivery technologies designed for other mucosal tissues

Table 2
Common oral delivery systems.

Delivery System	Application	Advantages	Disadvantages
Solution	Live attenuated, WCK, proteins, peptides, conjugates other delivery systems	Inexpensive buffer, flexible	Often requires the use of bicarbonate salts to neutralise stomach acid, dilution of formulation, lack of clean water in poor nations
Emulsions	WCK, proteins, peptides, conjugates	Potential for different formulations to facilitate specific immunomodulation e.g. Th1 (Water-in-oil) and Th2 (Oil-in-water)	Does not protect vaccine from GIT environment, efficacy by the oral route uncertain
Virus-like Particles	Plasmid DNA, proteins, peptides, conjugates	Non-replicating, high uptake, self-assembling, can conjugate additional molecules to provide targeted tissue/cell specificity	Recombinant technology is expensive and difficult to scale up, requires purification and often incorporation of additional adjuvants
Liposomes	Proteins, DNA, peptides	Surface can easily be modified, suits a wide variety of antigen types, controlled release	Poor antigen loading efficiency, low stability, non-specific interactions, toxicity of cationic liposomes, degradation by bile sales and lipases
ISCOMs	Proteins, peptides	Intrinsic adjuvant capabilities, ease of antigen loading, can modify surface, efficient induction of CTLs	Loading hydrophobic antigens is difficult
Synthetic Particles	Proteins, peptides, Conjugates	High adaptable, can protect contents from both environmental and physiological effects, controlled release, modifiable surface chemistry	Low loading efficiency, manufacturing process may degrade antigens, surface antigen exposed to proteolysis, can become trapped in mucus
Pills and Capsules	Live attenuated, WCK, proteins, peptides, conjugates other delivery systems	High adaptable, can protect contents from both environmental and physiological challenges, controlled release, easy to administer	Formulation process may damage components, loading complications

towards improved oral vaccines. For example, enteric coatings can be applied to tablets and capsules, imparting protection from gastric pH and controlled release properties, which can facilitate delivery to discrete locations. Furthermore, the composition of the vehicle can be tailored in such a way that it aids in the release of poorly-soluble components, facilitates sustained release over time or allows sequential release of various components allowing for a considerable degree of flexibility.

An interesting recent development has been the expression of *recombinant antigens* in plants with a view to producing edible vaccines, especially those utilizing rice as a vector due to the natural resistance of rice grains to degradation in the stomach [220]. MucoRice is a prophylactic anti-toxic cholera vaccine that has shown promise in both murine and non-human primate preclinical studies [98,221,222]. *The CTB content of each grain of MucoRice was found to be approximately 30 μg* [220]. *The consistency of these results is important when measuring the dose of antigen delivered to each vaccinee, however considering the total percentage of protein in each grain represented by CTB is 2.1%, the total weight of CTB per grain can be calculated at 0.15% allowing for dosing based on the weight of grains* [220]. If successful, such a system could provide a rapidly scalable and economically viable solution to mass oral vaccination [221]. CTB found in MucoRice is localized inside protein bodies within the rice grains, therefore providing protection from the harsh environment of the GIT and thus MucoRice acts in a similar fashion to a capsule for the delivery of CTB to gut inductive sites [221]. A similar rice-based oral vaccine against rotavirus has also shown promise in murine studies [223]. Human trials will in time reveal if such a system can indeed provide a sustainable solution to eradicating enteric diarrhea.

6. Technical adaptations to oral delivery vehicles

While next generation antigens, powerful adjuvants and a suitable delivery system have been demonstrated to greatly enhance the efficacy of many mucosal and parenteral vaccines, there remain many oral route-specific challenges that must be overcome. Various technological adaptations can be made to augment oral delivery systems in order to adapt them for purpose and enhance their existing capabilities.

6.1. Protection against degradation

An effective oral vaccine formulation can only elicit a protective immune response when delivered intact and to the appropriate anatomical location in the GIT. The vaccine must also be formulated in such a way as to maximize its immunogenicity. Selecting the appropriate formulation method and delivery vehicle can promote mucosal antigen uptake and immune activation. *While oral vaccines delivered in solution can be given with a buffered solution to neutralise gastric acidity, significant dilution of vaccine contents and enzymatic degradation can still occur so efficacy may be improved using a suitable delivery* system (Table 2).

The tri-polymer Eudragit® FS30D is known to remain intact at a pH below 7 allowing transport of a coated particle through the stomach and duodenum. Recently, particles coated in this polymer were shown to selectively release their payload in the terminal ileum [224]. Similarly, Eudragit® FS30D was shown to remain stable at low pH and a subsequent increase in pH led to a sustained release of WCK *V. cholerae* antigen from alginate micro-particles in *in vitro* models [225]. Coating a particle in this way can increase its size, thereby potentially compromising its uptake at the mucosal interface. However, premature uptake of the particle may be prevented until the coating completely degrades thus facilitating tissue specific release. Enteric coatings can also be applied to lipid-based delivery systems such as liposomes. Such coatings can protect liposomes from the degradative actions of bile salts and lipases. The use of a fungal derived polymer coating allowed for enhanced mucosal responses in the GIT following oral vaccination with liposomes [156]. Furthermore, altering the composition of lipids in these membranes can add stability in acidic environments. The use of dipalmitoyl phosphatidyserine, dipalmitoyl-phosphatidylcholine and cholesterol can enhance the resistance of liposomes to acidic pH [226,227].

The use of coatings on particles is an attractive means to protect exposed antigens and to achieve controlled release. PLG micro-particles *stabilised using* either EUDRAGIT® L100-55 or carboxymethylethylcellulose (CMCE) were rendered resistant to gastric degradation thus protecting encapsulated ovalbumin (*OVA*) [228]. *CMCE*-stabilized micro-particles were also capable of eliciting greater systemic and mucosal immune responses compared to EUDRAGIT® L100-55 following oral immunisation in mice [228]. *This approach was adopted as encapsulation in PLG particles stabilised using the conventional poly (vinyl alcohol) stabiliser led to surface exposure of the antigen which was highly sensitive to gastric proteolysis. While there remain issues with the oral use of PLG particles including the well-documented burst effect and challenges relating to damage to antigens during encapsulation and as a result of polymer hydrolysis, surface adsorption or attachment of antigens and incorporation in a protective enteric capsule may provide a productive avenue for further studies. This could be further enhanced by*

the use of specific targeting ligands. EUDRAGIT® L 30 D-55 coating of an oral cholera micro-particle based vaccine composed of WCK *V. cholerae* incorporated into micro-particles resulted in enhanced immunogenicity in a rat model [225]. *The polymer did not compromise* antigen stability and led to minimal antigen release under low pH conditions [225] but release increased rapidly at an elevated pH [225]. In addition to protecting antigens from low pH, coatings can be used to facilitate controlled antigen release in the intestine which may contribute to enhanced immunogenicity. A number of studies have suggested that the combination of initial high doses followed by a sustained release of antigen could help overcome the predisposition towards tolerance [86, 229,230]. Sustained antigen exposure may contribute to the efficacy of the oral polio vaccine as the attenuated virus establishes a limited local infection, providing both innate stimuli via PAMPs and a reservoir of antigen to establish a *memory response* [26].

6.2. Targeted delivery to specific tissues and cells

To induce effective mucosal immune responses in the gut it is desirable to direct a vaccine towards the main sites of antigen entry and immune activation (Fig. 1). The pivotal role played by M cells in the uptake of intestinal antigens has made them a key target for oral vaccine delivery. The unique glycosylation pattern displayed by these cells suggested the potential for specific targeting with lectins [231]. Targeting of particulates to murine intestinal M cells with the α-L-fucose specific lectin, *Ulex europaeus* agglutinin-1 (UEA- 1) resulted in enhanced uptake [232,233]. Furthermore, a low molecular weight UEA-1 peptidomimetic has also been shown to enhance particle uptake after oral delivery [234]. Nasal delivery of a UEA-1 mimetic targeted micro-particulate antigen resulted in enhanced immune responses following intranasal delivery [235]. Furthermore, both nasal and oral delivery of lectin-targeted nanoparticles (300-390 nm) promoted antigen specific cellular immunity in the absence of mucosal or systemic antibody responses [201]. Liposomes can also be targeted to M cells following oral administration utilizing UEA-1 [155]. Recent findings have also demonstrated that fucosylation patterns on IECs can be influenced by TLR agonists [236], opening up new possibilities for vaccine targeting. *However*, *since M cells in the upper respiratory tract differ significantly from those in Peyer's patches* [83–85], *one cannot infer that all targeting strategies that are effective by the nasal route will be effective orally*.

An alternative targeting approach involves the use of M-cell specific antibodies conjugated to particulates in order to enhance uptake in the GIT [237]. Antibody coated delivery systems such as liposomes and micro-particles may target mechanisms that sample antibody coated bacteria from the intestinal lumen and delivery these to APCs [238, 239]. Additionally it may be possible to exploit the same mechanisms used by enteric pathogens for invasion to enhance the uptake of oral vaccines. Particles coated in *Yersinia*-derived invasins displayed enhanced uptake via β1 integrins expressed on the apical side of M cells [240]. *Mannose-binding fimbriae* expressed on *Salmonella* and invasive *E. coli* could also be used to enhance antigen uptake by M cells in a similar manner to GP2 binding antibodies [53] and the fimbriae have the additional benefit of enhancing mucosal innate immunity via TLR5 and NLRC4. Enhanced mucosal immune responses were reported when antigens were coupled the M cell targeting σ1 reovirus protein [241]. However, while targeting of antigens attached to micro and nanoparticle surfaces is a promising approach, the exposure of the antigens to gastrointestinal secretions is a major obstacle. To increase efficacy and enhance the potential for translation, coatings or capsule strategies will be required to facilitate delivery of the targeted particles to immune inductive sites intact.

In addition to the clear benefits of targeting intestinal M cells, because of the key role they play in eliciting mucosal immune responses and their position at the frontline of the mucosa, many strategies to target mucosal DCs have been developed. Specific targeting of vaccines to DC specific receptors was pioneered by the late Ralph Steinman [242, 243]. Examples of such DC-specific receptors include CD205/DEC205 and DC-specific intercellular adhesion molecule 3-grabbing nonintegrin (DC-SIGN) [244]. DC-SIGN has also been shown to recognize SIgA which may present a novel targeting strategy [245]. Targeting of DCs can also be coupled to the delivery of TLR ligands as adjuvants which potently enhanced the ability of this approach to enhance Th1-mediated responses following subcutaneous vaccination [246].

Expression patterns of different DC-specific receptors varies between different mucosal DC subsets, and between mature and immature DCs [247]. One must also consider the intracellular routing following uptake by specific receptors as these may influence the consequent antigen presentation pathways that the antigens enter. This avenue has not yet been explored in depth in the context of oral vaccination but in parallel with M cell targeting, optimal targeting of particulates to gut DC and other mononuclear cell populations should be considered.

Other factors that influence the optimal choice of delivery system include the type and numbers of inductive sites, particularly PPs which are more numerous in the ileum, and competition from dietary and commensal antigen, the former of which are most abundant in the duodenum and the latter in the terminal ileum and caecum [34]. The relative abundance of cell populations such as iNKT and MAIT cells, activators of which may present novel adjuvant opportunities, also vary along the GIT but seem to be predominantly located in the jejunum [34,135]. Additionally specific DC subsets vary in abundance in different regions of the GIT, making the targeting of individual subsets reliant on the correct anatomical localisation of the delivery system [34].

6.3. Muco-adhesion and delivery

Considering the benefits of antigen persistence at immune inductive sites for enhancing uptake in the intestine, muco-adhesion and muco-penetration *strategies* may offer *benefits* for enhancing immunogenicity. A vaccine administered to a site of rapid mucus clearance may benefit from enhanced muco-penetration, whereas *at* a site with lower rates of mucus clearance, muco-adhesion may facilitate longer residence. To this end it is thought that more hydrophilic carriers with net-neutral surfaces *exhibit* enhanced muco-penetration, while muco-adhesion can be achieved by highly hydrophobic or positively charged surfaces which interact with the largely negatively charged mucus, thus prolonging the presence of the carrier in the mucus layer [33]. In addition to the potential for vaccine muco-adhesion to prolong the exposure of the vaccine to GALT tissues, this approach may also limit the dilution of vaccine material in gastrointestinal secretions. Work on naturally occurring biomaterials such chitosan (a compound formed by the deacetylation of chitin) has revealed that its strong muco-adhesive properties stem from the presence of numerous hydrogen-bond forming groups [248]. *Other research has shown that altering the ionic strength of the buffer can increase particle diffusion through the mucus and uptake by M cells* [197]. *A novel nanogel technology* developed by Kiyono and colleagues enables the persistence of vaccine material in the nasal passage after administration, ensuring adequate antigen exposure [249,250]. *This system has recently been evaluated in a non-human primate model where intra-nasal administration of pneumococcal surface protein A in nanogel led to robust mucosal and systemic immune responses and provided protection from Streptococcus pneumoniae challenge* [251]. *While*, *there are no published data on the efficacy of the nanogel by the oral route*, *its application as an oral delivery strategy has been patented* [252].

Plant-based muco-adhesives such as those derived from aloe also have potential to enhance gut residence through promotion of an antigen depot effect [253]. Muco-adhesives such as carboxy vinyl polymers *have also been* shown to prolong antigen persistence in the intestine, increasing the potential for antigen uptake [254]. Formulating particulates directly from or coating them with muco-adhesive molecules [255] combines the benefits of both particulate antigen presentation and enhanced mucosal residence times. In addition to its muco-adhesive

properties, chitosan may enhance uptake by opening tight junctions [256] *as well as displaying intrinsic adjuvant effects* [257]. Depending on the target site in *the GIT*, *the choice* of muco-penetrating agent or muco-adhesive agent may play a decisive role in navigating the system through thicker mucus layers or allowing persistence of the formulation at areas of more rapid clearance.

7. Conclusions and future perspectives

The efficacy of OPV and other oral vaccines against Cholera, Traveller's Diarrhoea, Noravirus, Rotavirus and *S. typhi* (Table 1) [19] demonstrates the great potential of oral vaccination. In depth studies into the effectiveness of oral cholera and polio vaccines has served as not only a strong proof-of-concept but also a testament to the benefit of wide scale oral vaccination campaigns [15,258,259]. However, the development of new oral WCK and subunit vaccines is hampered by the many physical, chemical and immunological challenges posed by delivery to the gastrointestinal tract. Despite this, many prototype vaccines against enteric infections including ETEC, other pathogenic *E. coli* strains and *H. pylori* and improvements to existing oral vaccines have emerged, demonstrating the significant degree of interest in this field [19]. *The potential for triggering* protective intestinal humoral and cellular immunity to block infection remains very attractive and justifies interest in developing advanced delivery solutions (Table 2). While many candidate and prototype oral vaccine delivery *systems for nonliving vaccines* have been investigated to date, none have been licensed for clinical application in humans, with most remaining largely in the early development or pre-clinical phase. *Given the many challenges*, approaches that combine solutions to multiple hurdles in a single formulation may be required (Fig. 3). Integrated systems that can address the challenges of low gastric pH, dilution effects, targeting of immune inductive sites and the need to include potent orally active adjuvants should be considered. Following safe passage through the gastric environment (Fig. 3A) and controlled and sustained release, the payload would be delivered to the target site by either a lipid-based or nano/micro-particulate sub-delivery system (Fig. 3B), thereby ensuring adequate uptake of vaccine components, activation of innate immune cells and the induction of mucosal and systemic adaptive immune responses (Fig. 3C). In this context,

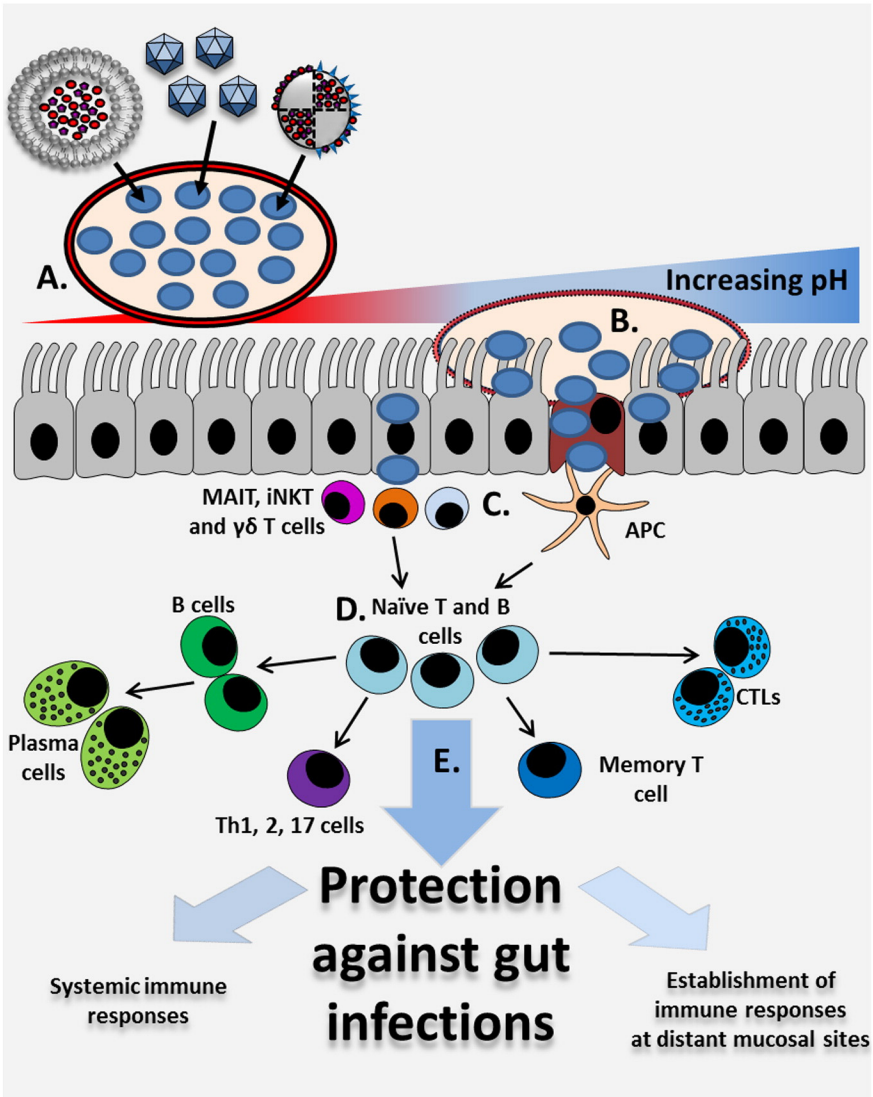

Fig. 3. The "Ideal" oral vaccine delivery strategy should integrate the advantages of different technologies in an attempt to overcome all the challenges of the oral route via a multifunctional system. An enterically coated capsule into which a lipid, virus-like particles (VLP) or particle based sub-delivery system is loaded would allow for transit through the GIT without exposure to (A) stomach acids, degradative enzymes and bile salts in the small intestine while also preventing the dilution of vaccine contents by intestinal secretions. Additional benefits such as protection from environmental factors during storage, accurate control over dosing and ease of delivery and administration can also accompany capsular delivery systems. The capsule should be designed in such a way that (B) controlled release of the sub-delivery system is achieved at a distinct site to maximise exposure to (C) mechanisms of antigen uptake and immune enhancement. Furthermore this system should incorporate next generation antigens such as VLPs and engineered bacterial strains in addition to one or more adjuvants to polarise and enhance the desired (E) effector and memory response.

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next-generation antigens such as bacterial vectors over-expressing antigens or VLPs that improve immunogenicity but also reduce cost through simplified manufacture and dose sparing could be considered. A key and pressing challenge is the identification of potent orally active and safe adjuvants for vaccination in humans. Understanding of intestinal innate immunity has greatly advanced over recent years offering valuable new insights for the design of optimal mucosal adjuvants. *Although human trials of many oral delivery systems have delivered disappointing results, these have principally focused on the efficacy of specific adjuvants or delivery systems in isolation. We propose that the potential of many oral vaccine delivery systems could be enhanced if used as a subcomponent of a larger delivery strategy. Such a system would likely compose a larger pill or capsule based "Super" delivery system containing a particle or liposome-based sub-delivery system as part of a controlled release mechanism.*

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