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Oral Biologic Delivery: Advances Toward Oral Subunit, DNA, and mRNA Vaccines and the Potential for Mass Vaccination During Pandemics

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Abstract

Oral vaccination enables pain-free and self-administrable vaccine delivery for rapid mass vaccination during pandemic outbreaks. Furthermore, it elicits systemic and mucosal immune responses. This protects against infection at mucosal surfaces, which may further enhance protection and minimize the spread of disease. The gastrointestinal (GI) tract presents a number of prospective mucosal inductive sites for vaccine targeting, including the oral cavity, stomach, and small intestine. However, currently available oral vaccines are effectively limited to live-attenuated and inactivated vaccines against enteric diseases. The GI tract poses a number of challenges, including degradative processes that digest biologics and mucosal barriers that limit their absorption. This review summarizes the approaches currently under development and future opportunities for oral vaccine delivery to established (intestinal) and relatively new (oral cavity, stomach) mucosal targets. Special consideration is given to recent advances in oral biologic delivery that offer promise as future platforms for the administration of oral vaccines.

Keywords

oral biologic delivery; oral vaccines; mucosal immunization; protein; DNA; mRNA

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DISCLOSURE STATEMENT

J.W.C. and G.T. are coinventors on multiple patent applications describing systems capable of oral delivery of biologics, including vaccines. G.D.G. has filed a provisional patent application (62/817,094) related to HIV vaccine design. Complete details of all relationships for profit and not for profit for G.T. can be found at the following link: https://www.dropbox.com/sh/szi7vnr4a2ajb56/AABs5N5i0q9AfT1IqIJAE-T5a?dl=0.

INTRODUCTION

Oral delivery is the preferred route for the administration of therapeutics. Compared to parenteral administrations requiring injection, oral delivery is noninvasive, convenient, and can be self-administered (1), which leads to increased compliance and greater potential for improved population coverage and health outcomes. Due to physical barriers and degradative processes in the gastrointestinal (GI) tract, however, efficient oral delivery of fragile macromolecules such as biologics has remained an almost unattainable goal (2). The key exceptions and successes are select types of vaccines (3–5) and, more recently, biologics in combination with permeation enhancers (PEs) (6, 7). The first oral vaccines were developed against poliovirus in the 1950s by Hilary Koprowski and Albert Sabin, with Sabin's trivalent oral polio vaccine (OPV) the most utilized option for much of the twentieth century. Since then, oral vaccines have been developed against cholera, typhoid fever, gastroenteritis (rotavirus), and acute respiratory disease (adenovirus) (3, 4). Most pathogens enter the body through the GI, urogenital, and respiratory mucosal surfaces (4). Oral vaccination elicits both systemic and mucosal immune responses, providing an additional immunological benefit by protecting against infection at mucosal surfaces. While intramuscular (IM) vaccine administration elicits effective systemic immunity, it provides weak mucosal immunity (4, 5) and requires trained personnel to administer, which limits vaccination reach in pandemic outbreaks. Furthermore, mucosal infections may persist in IM-immunized individuals (8, 9), which can contribute to subsequent transmission to unimmunized or immunocompromised individuals. Despite these advantages, however, the proportion of vaccines administered orally remains small.

The GI tract provides significant challenges to the oral delivery of vaccines, including degradation, poor uptake through mucosal barriers, and a bias toward tolerability of antigens (3). A number of regions within the GI tract present prospective inductive sites for vaccine delivery, including the oral cavity (10), stomach (11), and intestines (3, 4), with each presenting unique challenges and opportunities. No comprehensive clinical solution for efficient oral delivery of a broad range of biologics or vaccines has yet been achieved, though this remains a highly active area of research (6, 7, 12–14). Approved oral vaccines are currently limited to diseases that affect the GI tract (3) and whole-virus/cell vaccines (i.e., either attenuated live or inactivated). These structurally intact pathogens are highly immunogenic; can better withstand GI degradative processes; and adhere to or replicate within mucosa, which improves their uptake. Other types of vaccines such as subunit (proteins and polysaccharides), DNA, and messenger RNA (mRNA) vaccines are sensitive to degradation, are considerably more challenging to deliver orally, elicit strong GI immune responses, and have not been successfully translated in the clinic. Furthermore, DNA and mRNA vaccines are of particular interest in response to pandemic outbreaks given their potential for rapid and cost-effective manufacture (15, 16).

Oral biologic delivery systems for both vaccination (3, 4, 17) and systemic therapies (2, 12, 13) are currently under development. Oral vaccination approaches typically exploit the body's ability to distinguish and process threats at mucosal surfaces, resulting in pathogen-specific mucosal uptake, processing, and the initiation of immune responses. These

approaches have been widely investigated for oral vaccination through intestinal delivery but are unsuitable for the oral cavity or stomach. Systemic delivery of biologics requires physical or chemical mechanisms to pass through mucosal barriers, and in the last few years, several promising platform technologies have been developed for the oral delivery of biologic therapeutics (7, 18–23), which may enable vaccination to a wider array of GI mucosa. This review summarizes the potential and the challenges of oral vaccine delivery to different GI mucosal inductive regions; key design considerations for oral delivery of subunit and nucleic acid vaccines; and the current state of research, including recent advances in oral biologic delivery more broadly, which may ultimately enable new approaches for oral vaccinations.

CHALLENGES OF ORAL VACCINE DELIVERY TO GASTROINTESTINAL MUCOSA

In their simplest form, the GI mucosae consist of a protective mucus membrane and a cellular epithelial layer (Figure 1), which protect the host from unregulated internalization of pathogens and harmful substances. The mucus lining, as the first point of contact, provides a physical barrier that limits molecular diffusion and microorganism intrusion to the epithelial layer. The structure and function of the epithelial layer vary considerably between oral, gastric, and intestinal mucosa and even within the oral cavity, but a consistent function is the prevention of unwanted uptake of pathogens and macromolecules, which is achieved through several tightly packed cellular architectures. Additional protection against microorganisms is imparted by the secretion of antimicrobial compounds within the mucus membrane, as well as by the innate and adaptive mucosal immune system. Regions of low pH (stomach), bile salts (intestines), and digestive enzymes add an extra layer of protection and challenge for oral vaccine delivery. The GI tract is also a dynamic system, with the residence time of contents limited by gastric emptying and intestinal motility, which restrict opportunities for the absorption of macromolecules. In summary, effective delivery of oral vaccines requires overcoming degradative processes, mucosal barriers, transient contact with epithelia, and a relatively tolerant immune system (discussed in the next section). The exact nature of these challenges and opportunities also depends on the target vaccination site within the diverse environment that is the GI tract.

THE GASTROINTESTINAL IMMUNE SYSTEM

The basis of mucosal immunity is the migration of cells that recognize antigens [antigen-presenting cells (APCs)] from mucosal inductor sites through the lymphatics to recruit immune cells that protect against foreign pathogens to mucosal effector sites (24). The GI tract has a well-established immune system that prevents against mucosal infections (though significantly, not all organs/mucosae have been well characterized) (8, 25). Despite this, GI mucosal surfaces are colonized with an abundance of microorganisms, particularly in the intestines, some of which impart significant benefits to the host (26). Colonization is facilitated by complex mechanisms of immune tolerance, with inductor sites distinguishing between commensal and potentially harmful bacteria (27) as well as many benign environmental antigens (e.g., in food). There is thus a substantial bias toward immune

tolerance to avoid unnecessary responses to these bacteria/antigens, which poses a challenge in generating immune responses to vaccination (25, 28). Inductor sites from multiple mucosae (e.g., respiratory, GI, urogenital) form a network known as mucosa-associated lymphoid tissue (MALT). Inductor sites from one mucosa may initiate immune responses at distal mucosae (8, 29–31) in what is referred to as the common mucosal immune system; however, mucosal responses are generally strongest proximal to the site of induction. For example, vaccination to the oral cavity can induce responses in the small intestine and respiratory mucosa (29), while some mucosal compartments [e.g., rectal delivery (32)] induce local rather than broad mucosal responses. Similarly, for optimum oral vaccine delivery, it may be necessary to understand a particular pathogen's route of entry and target a specific delivery site within the GI tract that could offer the best balance of regional mucosal and systemic immune responses.

Several regions of the GI tract offer potential targets for oral vaccination, including the oral cavity, stomach, and small intestine (Figure 1). The immune system of the small intestine is the most extensively studied and the target of all currently approved oral vaccines (3, 4, 8). The oral cavity has received far less attention for vaccination (10), though it has been well studied for allergen immunotherapy (33, 34) and is the most easily accessible GI inductor site. Recently, however, there has been increased development of devices enabling efficient vaccine delivery to the oral cavity mucosa (35) (see Figure 1b). Conversely, the stomach has received little attention as a site for vaccination; however, recent demonstrations of ingestible devices for biologic delivery to the stomach (20) and of gastric tissue being an inductive site (11) suggest that the stomach has potential as a future vaccination site.

Oral Cavity

The oral cavity is the most accessible mucosal inductive site for vaccine delivery. The sublingual (SL) region (underside of the tongue and bottom surface of the mouth) and buccal mucosa (cheeks, gums, and inner surface of the lips) are the most prominent targets for vaccine delivery. Their thinner, nonkeratinized epithelia (Figure 1c) allow for both some adsorption of macromolecules and easier mechanical disruption for rapid macromolecule delivery compared with the keratinized epithelium present elsewhere in the oral cavity (e.g., the hard palate). Furthermore, the lipid lamellae between epithelial cells in nonkeratinized epithelia are comparatively less organized, which allows for increased diffusion and uptake of biomacromolecules (36). APCs that are present in these mucosal areas include Langerhans cells [LCs; suprabasal layer of the epithelium (10)] and several types of dendritic cells (DCs), including myeloid, interstitial [lamina propria (LP)], and plasmacytoid cells (in the submucosal tissue, which infiltrate the mucosa during inflammation). There are key differences between the buccal and SL mucosae, such as epithelial layer thickness and relative abundance of APCs, which play a role in site selection for vaccination. The SL mucosa has a particularly thin and permeable epithelium, which allows for absorption of macromolecules, and has been extensively investigated for sublingual immunotherapy (33, 34). In contrast, the buccal mucosal epithelium is thicker and considered to be less absorptive. Saliva flow may also dilute or wash out vaccine from the oral cavity before it is significantly absorbed, and it contains some potentially degradative enzymes. Thus, perturbing the mucosal barrier for rapid uptake is advantageous for vaccine delivery to both

the buccal and SL mucosae (Figure 1c). Furthermore, interstitial $CD11b^{+}CD11c^{+}$ myeloid DCs appear to be the major DCs responsible for T cell priming in the oral mucosa, which is crucial for the adaptive immune response. These are abundant in the LP of both the buccal mucosa and, to a lesser degree, the SL mucosa. LCs in the epithelium, which are also more abundant in the buccal mucosa, are thought to have a primarily tolerogenic role (37). These mucosae are therefore particularly attractive vaccination sites; however, it is likely beneficial to deliver antigen in sufficient quantities to the depth of these DCs in preference over more superficial LCs, highlighting the need for efficient delivery technologies such as mechanical perforation.

Stomach

The stomach is the next most accessible part of the upper GI tract (Figure 1). Ingested material takes only seconds to minutes to pass through the esophagus. The gastric mucosa contains a relatively thick mucus lining and simple columnar epithelia with tight cell-cell junctions that prevent the absorption of molecules and acidic gastric fluid. Knowledge of the stomach immune system is limited and has been largely characterized in the context of *Helicobacter pylori* infection. The presence of DCs and inductive site capacity in healthy gastric mucosa has been doubted (38–40). However, $HLA^{high}/CD13^{low}$ and $CD13^{high}$ DCs have now been definitively identified in healthy human stomach and implicated in the development of a Th1 response to *H. pylori* (38). Primary and secondary lymphoid follicles have also been described in healthy human gastric mucosae (41) and the mucosae and submucosae of healthy piglets (42). Recently, it was shown that subserous delivery of an *H. pylori* vaccine to the murine stomach wall (via surgical laparotomy) generated new perivascular lymphocyte clusters and $CD4^{+}$ tissue resident memory T cells (11, 43, 44), further confirming inductive potential. Moreover, our group has developed an ingestible self-orienting device that can inject a dose of biologic directly into the gastric wall (20). Thus, while the stomach has been understudied for vaccine delivery, recent demonstrations of its inductive potential and capacity for biologic delivery support its potential as a site for vaccination.

Small Intestine

The small intestine is the target site for all currently approved oral vaccines and for the majority of investigational reports (3, 4, 17). Its mucosal immune system has been extensively characterized, and a review of its key mechanisms elucidates why it has been traditionally considered an ideal target for oral vaccines. Unlike the lining of the oral and gastric cavities, the intestinal mucosa can recognize foreign antigens and transport them through mucosal barriers for presentation to the immune system (25, 45) (Figure 1c). Multiple inductor sites have been identified in the intestinal mucosa, which are collectively referred to as gut-associated lymphoid tissue (GALT). The most prominent of these inductor sites is the Peyer’s patch (PP), consisting of small discrete areas of highly ordered and specialized immune cells beneath a follicle-associated epithelium (FAE). The FAE recognizes and aids the transport of specific pathogens and antigens into the PPs. Microfold cells (M cells) of the FAE act as the primary gateway for antigens into the PPs. These M cells express a diverse variety of receptors to foreign antigens, including pattern recognition receptors (PRRs) (46), on their apical surface through which M cells recognize and

transcytose foreign pathogens to the basolateral membrane and deliver them to underlying APCs (45). Furthermore, M cells may also transcytose particles based on size ranges comparable to those of pathogens (47–51). The surface of M cells also has a thinner or absent mucus lining that lacks or contains shorter microvilli than neighboring epithelial cells, which facilitates pathogen contact with the cell (52). Beneath the FAE is the subepithelial dome, which contains DCs, the major APC of the immune system. Pathogens and antigens are transferred by M cells to these DCs, which process antigens and present fragments to downstream cells (naïve $CD4^+$ T cells) in the immune system (45). Additionally, the PP also contains germinal centers that are rich in both B and T cells.

Other discrete inductor sites in the intestinal mucosa include mesenteric lymph nodes (MLNs) and the lesser studied lymphoid follicles. DCs may also be dispersed in smaller numbers throughout the mucosa and, unique to GI DCs, possess dendrites that extend through the epithelia into the lumen and may directly recognize and uptake antigens (53, 54). Enterocytes—the most ubiquitous cells of intestinal epithelia—are also capable of taking up and presenting some antigens via the neonatal fragment crystallizable (Fc) receptor (55). Finally, some studies suggest synthetic particles may be transported by enterocytes through non-Fc receptor–mediated pathways or by paracellular transport between enterocyte cell junctions, presenting possible opportunities for vaccine design (56). In contrast, goblet cells have been shown to transport low molecular weight–soluble antigens to DC subsets that promote tolerance (57). In summary, a range of pathogenic features that are recognized by the intestinal mucosa enable internalization and direct transfer to APCs for presentation to the immune system, including particle size, antigenic epitopes, molecular patterns, and other targeting ligands, which have the potential to be leveraged as mechanisms for vaccine uptake.

Effector Sites and the Mucosal Immune Response

Following the initial exposure to antigen in the GI tract, mucosal lymphocytes migrate through the lymphatics, priming and activating immune cells that then home to effector sites. The major effector sites of the GI tract are the LP (24); the surface of the epithelium (24); and, in the oral cavity, the secretory saliva glands (58, 59). DCs at mucosal inductor sites typically internalize and process antigens into small fragments for presentation to naïve T cells via one of two routes. A portion of DCs migrate via draining lymphatics to germinal centers in the MLNs, where they prime naïve $CD4^+$ T cells (which are crucial in initiating an immune response). Primed $CD4^+$ T cells then activate neighboring antigen-specific B cells in the germinal center, which class-switch to become IgA^+ B cells. The IgA^+ B cells then enter the bloodstream before homing to tissue effector sites. Alternatively, DCs may prime local naïve $CD4^+$ T cells within mucosal germinal centers, e.g., within the PPs. These T cells in turn activate antigen-specific B cells, which class-switch to IgA^+ B cells and then leave the PP through the lymphatics and MLN and reach the circulation for homing to effector sites.

DCs in the GALT also produce retinoic acid, which interacts with activated T cells to release homing receptors. These homing receptors direct the migration of circulating IgA^+ B cells to mucosal effector sites in the LP. There, they differentiate and mature to become IgA-

producing plasma cells, which produce a high-affinity version of antigen-specific IgA. Enterocytes in the epithelium express the polymeric Ig receptor (pIgR) on their basal surface, which binds to IgA (and also IgM antibodies). Thus, IgA from the LP binds to the basolateral pIgR, which is internalized and transported across the epithelial cell. The IgA is then internalized and translocated to the luminal surface where it is released as secretory IgA (sIgA), the primary molecule responsible for mucosal immunity. This sIgA can then neutralize pathogens (24), conferring the epithelial surface as a major effector site of the GI immune system. In the oral cavity, sIgA may also be produced in salivary glands (58, 59).

CLINICALLY APPROVED ORAL VACCINES

Currently, all approved oral vaccines (see Table 1) protect against diseases that affect the GI tract or against pathogens that have a crucial life cycle stage within the GI tract. These include cholera, gastroenteritis (rotavirus), polio, typhoid fever, and acute respiratory disease (adenovirus), which although not pathogenic in the GI tract, establishes an asymptomatic infection in the intestine that is exploited for vaccination. All of the approved oral vaccines for these diseases employ live-attenuated or inactivated pathogens. As with the wild-type forms of these pathogens, the attenuated and inactivated forms can endure degradation or remain sufficiently intact through transit in the gastric and intestinal environment and elicit a stronger immunogenic response in the GI mucosa than other vaccine types (e.g., subunit and nucleic acid vaccines). Importantly, adenovirus immunization demonstrates the principle that GI immunization can be effective against systemic diseases without GI pathogenicity, and this has been extensively investigated as a vector for oral delivery of plasmid DNA vaccines (covered later). Some clinical trials have investigated the GI tract for immunization against diseases that are not related to the GI tract, such as influenza (60, 61), and that use other vaccine types and/or adjuvants, e.g., DNA vaccines (61) and RNA adjuvants (60), but these have not yet resulted in approved commercial products. Thus, there is significant opportunity for advances in vaccine delivery technology that can efficiently protect vulnerable vaccine components in the gastric environment, deliver them effectively to the epithelia and APCs, and co-stimulate (i.e., adjuvant) immune responses to typically less antigenic vaccines (e.g., subunit, DNA, and mRNA vaccines).

STRATEGIES AND DELIVERY SYSTEMS TOWARD ORAL VACCINATION

A broad range of approaches have been investigated to overcome the challenges of oral vaccine delivery (Figure 2). These approaches typically vary depending on the characteristics of the target inductor site, i.e., the oral cavity, stomach, or intestines (see Table 2). An overview of salient GI delivery challenges and strategies for overcoming them includes the following five points. First, fragile proteins, polysaccharides, and nucleic acids must be protected from degradation during gastric and intestinal transit, but less so in the oral cavity. This may be achieved by loading vaccine into particles (3, 4, 48), enteric capsules (62), or physical devices (20). Second, enhanced uptake across mucosal barriers is required and can be achieved by mechanical disruption of the epithelial barrier (35), the use of targeting ligands (63, 64), or vaccine-carrying particles with sizes that are preferentially absorbed mucosally (small intestine only) (50). Third, non-cell-based soluble antigens lack the key immunogenic characteristics of whole pathogens that the body’s immune cells are

effective at recognizing (46), which is particularly problematic in the tolerogenic environment of the GI tract. To overcome this, approaches have been developed that incorporate mucosal adjuvants (65) and/or delivery vehicles that mimic pathogenic properties and increase immunogenicity such as pathogen-sized particles as vaccine carriers (50), functionalization with molecular patterns typically presented by pathogens (46, 66), and the development of virus-like particles (VLPs)—individually expressed viral structural proteins that self-assemble into particles that resemble viruses (67). Fourth, the GI tract is a dynamic environment, giving potential vaccines limited resident times through each portion of the GI tract (Figure 1a). Contact time with the mucosa can be enhanced by the use of mucoadhesive strategies (68) or avoided altogether by physical devices that rapidly deliver a payload into the mucosa (20, 35, 69, 70). Fifth, the thermostability of vaccine formulations also needs to be considered, as it is critical in developing countries where cold-chain infrastructure is limited (71); this is particularly true for oral vaccines, given that their potential for self-administration is a key opportunity to improve coverage in remote and developing areas where infectious diseases are often most prevalent. The incorporation of stabilizing excipients and/or use of dry solid formulations has been shown to improve thermostability (72) and may need to be employed with oral vaccine formulations. This section summarizes major design considerations when developing oral vaccine approaches and the types of delivery systems that have been employed.

Microparticles and Nanoparticles

Microparticles and/or nanoparticles (MNPs) are the most widely investigated delivery system for oral subunit (protein and polysaccharide) vaccines (3, 4, 73–75). MNPs offer considerable versatility with the ability to both tailor and combine multiple functionalities into a single particle through variation in particle design, materials properties, and surface functionalization. MNPs also have potential for nucleic acid vaccine delivery (which requires additional transfer of nucleic acids across cell membranes) and have been used to deliver plasmid DNA encoding for antigens (76–80). Oral mRNA vaccination has not to our knowledge been reported; however, MNPs have been used to transfer mRNA vaccines into cells via other delivery routes, suggesting a potential for oral delivery (15, 81, 82). MNPs may be administered via solution (4, 83) or enteric capsules (62), where they are uptaken by GALT in the small intestine. MNPs have also been delivered via topical administration to the SL mucosa; however, due to the extremely short residence time of solutions in the oral cavity, MNPs typically require mucoadhesive (84) or localization (85) strategies to improve uptake or rapid delivery through physical injection (69, 70), particularly in the comparatively less absorptive buccal mucosa.

Protection of the vaccine from the GI environment can be achieved in a number of ways, including by loading vaccines within the MNP carrier or adsorbing them to its surface (Figure 2). While surface loading provides less protection, it may render antigens more available for immune recognition. In either case, further protection can be achieved through an additional enteric surface coating on particles (86). Importantly, many MNPs also intrinsically improve uptake across epithelial barriers and stimulate the immune system (87). It is well established that certain particle sizes, ranging from several hundred nanometers in diameter (88, 89) to approximately 10 μm (50, 51, 90), are recognized and transcytosed by

M cells and enterocytes, though the exact optimal size range has been debated and may be affected by other physicochemical properties of the particle, complicating comparisons between studies. Smaller particles (<5 μm) are transported by macrophages to MLNs and ultimately the spleen, clearing them from the mucosa (47, 50, 91). Larger particles that remain in mucosa for longer periods have been shown to result in higher sIgA (50), suggesting a sustained-release mucosal depot may be an effective strategy for vaccination. Furthermore, MNPs are also taken up by APCs and DCs in a size-, shape-, and charge-dependent manner (49, 92), which could be further exploited to enhance the immunogenicity of MNP vaccines. A major advantage of MNPs is that they can be imparted with multiple functionalities through the incorporation of targeting ligands for receptors on epithelial cells and with adjuvants to stimulate immune responses. Several peptides have been shown to increase both binding to M cells and mucosal uptake when attached to MNPs (64, 93). Arginylglycylaspartic acid (or RGD peptide) has also been shown to increase uptake to M cells (63), as have mucoadhesive lectins (94). In one study, multiple Toll-like receptor (TLR)-targeting ligands (macrophage-activating lipoprotein, polyinosine-polycytidylic acid, and CpG oligodeoxynucleotide) encapsulated in MNPs were shown to target the large intestine (66), which has an abundance of GALT.

Biodegradable polymers are the most frequently used material for MNPs due to their biocompatibility and ability to provide sustained vaccine release (83, 95). Sustained release has resulted in increased antibody and T cell responses compared to pulsatile delivery (50, 96). The most widely used biodegradable polymers are polylactic acid (PLA) and poly(lactic-coglycolic) acid (PLGA) (47, 48, 50, 83, 86, 97–100), owing to their biocompatibility, biodegradability, relative ease of use, capability to tailor release rate such as for sustained release, and their wide approval by the US Food and Drug Administration for drug-release applications. Additionally, coblock polymers of PLA/PLGA with other polymers, for example poly-D,L-lactide–copoly(ethylene glycol), provide additional tuning of release kinetics (97). While PLA/PLGA MNPs protect vaccines from external degradation, their biodegradation/hydrolysis with water forms acidic breakdown components, which may affect vaccine stability and needs to be thoroughly evaluated. PLGA (76, 77) and chitosan (78) microparticles have also been used to deliver antigen-encoding DNA plasmids for oral vaccination, while biodegradable particles for DNA vaccination have been widely investigated through other routes, suggesting further potential for oral DNA vaccination (101).

Polyanhydrides (PAs) (102), polystyrene (98), poly-ε-caprolactone (103), polyethyleneimine (PEI) (104), and naturally occurring polymers such as alginate (105, 106) and chitosan (73, 78) have also been used for oral vaccine delivery (95, 107). PA has been shown to be beneficial compared with PLA/PLGA, as it does not form localized acidic breakdown components (95). PEI has been used to form polyplexes suitable for DNA vaccination (104). Similar polyplex approaches may potentially be applied in the future for mRNA vaccine delivery. Naturally occurring polymers such as alginate and chitosan typically have the benefit of relatively benign gelation conditions, allowing for easy encapsulation of proteins and nucleic acids (68). Chitosan specifically possesses mucoadhesive properties and reversibly opens epithelial tight junctions, improving mucosal permeability (54, 73). However, its high solubility in gastric pH conditions necessitates additional stabilization

through cross-linking (108) or protective coatings (109). Inorganic MNPs have also been used for oral vaccination, including using gold (110) and silica (88). These MNPs are highly tailorable and have a stable rigid structure that is not affected by gastric conditions (110), while their surfaces can easily be functionalized with antigens, targeting moieties, and polymers for stability enhancement.

Lipid-Based Approaches

Liposomes are enclosed vesicles of concentric self-assembling lipid bilayers composed of phospholipids and cholesterols (Figure 2). The oral delivery of liposomes has a long history in the drug delivery field, which began in the 1970s with encapsulated insulin, but has only more recently been explored for oral vaccines. The advantages of using liposomes for mucosal vaccine delivery are that they can protect against the degradation of antigens in the harsh GI environment and facilitate uptake of antigens by APCs. Liposomes are also compatible with an array of cargo: e.g., subunit proteins, DNA, RNA, carbohydrates, and peptides, which are loaded within the aqueous core. Oral delivery to preclinical animal models of liposomes containing cargo for influenza A, *Salmonella enteritidis*, and *Mycobacterium* was shown to elicit antigen-specific humoral and cellular immune responses (111–113). In addition, immunogenicity can be enhanced by incorporating lectins into liposomes, which leads to them targeting intestinal M cells in PPs and the induction of even higher antibody titers than with liposomes alone (114). Despite these initial promising results, liposomes need to be further optimized for survival within the GI tract, as intestinal bile salts have been shown to disrupt liposome stability.

To increase the stability of oral immunizations in the GI tract in the presence of bile salts, distinct lipid-based carriers known as bilosomes, which directly incorporate bile salts into their formulation, are under investigation. The increased stability allowed enhanced protection of a variety of vaccine antigens, including tetanus toxoid (115). When delivered orally, bilosomes containing hepatitis B surface antigen and cholera toxin B subunits have also been shown to generate both systemic and local immunity (65), potentially due to M cell targeting. Importantly, bilosomes were able to induce sIgA throughout the GI tract and drive Th1 and Th2 responses in animal models (116). Further evaluation in human clinical trials will be critical to fully understand bilosomes as potential oral immunization agents.

An additional innovative form of lipid-based vehicles for vaccine delivery are immune-stimulating complexes (ISCOMs). These use unique materials (saponin, cholesterol, and phospholipids) that can self-assemble to entrap bacterial and viral envelope proteins and both protect antigenic cargo and act as immune stimulants. ISCOMs have been shown to drive potent immune responses to several antigens (75), and importantly induce both cellular and humoral immunity, likely due to the engagement of materials with both innate and adaptive immune systems. Unlike bilosomes, however, ISCOMs have not been shown to increase sIgA levels, which may limit their effectiveness in preventing GI infections (117). ISCOMs will also require further innovation and rigorous clinical testing to determine their potential as an oral immunization modality in humans.

Viral and Bacterial Vectors

The use of viral and bacterial vectors expressing recombinant antigens for other pathogens is particularly attractive for oral vaccination, because pathogens that naturally infect the GI tract are efficient vectors for mucosal delivery/colonization. Bacteria such as *Salmonella typhi*, *Escherichia coli*, *Listeria*, *Vibrio*, and *Shigella* and viruses such as those in the poxvirus and adenovirus families have been used for intestinal delivery (118–122). *S. typhi* is perhaps the most studied bacterial vector in animals, but translation to humans has proved challenging (118). Recombinant adenoviruses (rAds) are the most widely used viral or bacterial vector for oral vaccination, owing to their ability to infect and replicate in the intestinal mucosa and deliver a DNA cargo (122). rAds have also been delivered to the SL mucosa (123). IM delivery of rAds has been a mainstay in the vaccinology field, and in clinical studies, both antiviral humoral and cellular immunity have been induced (124). However, the presence of naturally occurring neutralizing antibodies against a diverse number of rAds reduces their utility for systemic immunization. In contrast, oral delivery of rAds is not affected by preexisting immunity and could represent a promising route of adenoviral immunization.

Oral vaccination with rAds has been conducted with both replication-competent and replication-defective rAds. For replication-competent vectors, adenovirus serotypes 4 and 7 have been utilized, which have a natural tropism for GI tissue. rAd4 and rAd7 vectors containing a hepatitis B surface antigen transgene were delivered to chimpanzees and human volunteers, respectively (125, 126), but neither vaccine was particularly immunogenic. This was also similar to a live rAd4 vector expressing the hemagglutinin gene of the H5N1 influenza virus, which when formulated as enteric-coated tablets and delivered to healthy human volunteers did not elicit strong immunity (61). Interestingly, when these orally vaccinated individuals were subsequently boosted by a systemically delivered H5N1 subunit vaccine, the titers and avidity of virus-specific antibodies were markedly higher than in individuals only vaccinated systemically (127), suggesting that oral delivery of replication-competent rAd4 vectors could be a useful priming agent for the induction of robust, protective immunity following systemic boost.

Orally delivered, replication-defective rAd serotype 5 vectors have been shown to be substantially more immunogenic. Clinical trials testing a rAd serotype 5 vector encoding an influenza hemagglutinin, in conjunction with a TLR3 adjuvant, as an enteric-coated tablet led to the induction of transgene-specific $CD8^+$ T cell responses and antibodies (128). Additionally, unlike systemic immunization, oral immunization was not affected by preexisting adenovirus vector immunity (60). Follow-up studies demonstrated that delivery of rAd5 vectors using radio-controlled capsules, which allowed for the release of particles within the jejunum or ileum, led to higher rates of vaccine responders in comparison to systemically immunized individuals (129). These compelling results provide the impetus for the future investigation of oral delivery of replication-deficient adenoviral vectors.

Mucosal Adjuvants

Adjuvants are immunomodulatory agents that improve the immunogenicity of vaccine vectors. Numerous adjuvants have been developed and validated for systemic immunization,

but they have proven ineffective at eliciting effective mucosal immunity, in part due to their inability to withstand the harsh conditions at mucosal sites and initiate sufficient cross-talk between innate and adaptive immune cells. Studies identifying adjuvants capable of inducing immune responses at mucosal tissue sites have utilized three mechanisms: toxin-mediated immune stimulation; TLR agonism; and natural immunomodulation using cytokines, as described below. Depending on the adjuvant and vaccine delivery vehicle, these adjuvants may be coadministered in a solution or capsule with the oral vaccine formulation or directly conjugated or incorporated within a delivery particle (e.g., an MNP or liposome).

Two of the most promising toxin-mediated adjuvants studied thus far have been the ADP-ribosylating enterotoxins cholera toxin (CT), produced by *Vibrio cholerae*, and heat-labile toxin (LT), made by enterotoxigenic *Escherichia coli* (ETEC). Both CT and LT are potent enterotoxins, but they have been modified to eliminate their endogenous toxicity while still retaining their immunostimulatory properties. In the case of LT, a double-mutant form (dmLT), containing mutations of arginine to glycine at position 192 and leucine to alanine at position 211 in the active A subunit, promoted robust mucosal immune responses when codelivered within an antigen (130) in preclinical models (131) and was subsequently deemed safe, well-tolerated, and immunogenic in human clinical trials. A phase I dose-escalating safety study of 5–100 μg of orally delivered dmLT resulted in no serious adverse events (132). Coadministration of dmLT with either whole-killed or live-attenuated ETEC in two additional trials resulted in enhanced immune responses to less immunogenic antigens and protective efficacy from oral challenge 6 months after immunization (133, 134). This is the result of dmLT having no detectable effect on intestinal epithelial cells but marked effects on DCs responsible for immune stimulation (135), making dmLT a highly promising adjuvant for future oral vaccines.

TLRs belong to the family of PRRs that are expressed on DCs, macrophages, monocytes, and epithelial cells within intestinal tissue sites. Thus, TLR agonists serve as an additional class of mucosal adjuvants, with two of these, unmethylated cytosine-guanine-containing oligonucleotides (CpG DNA) and monophosphoryl lipid A (MPL), among the most promising. MPL in particular, which is a TLR4 agonist and detoxified version of lipopolysaccharide from *Salmonella minnesota* R595, has been demonstrated to increase IgG and IgA levels when coadministered with a number of antigens (136). Additional TLR agonists with demonstrated mucosal immunogenicity include polyinosine-polycytidylic acid [poly(I:C)], flagellin, and imiquimod, which engage TLR3, TLR5, and TLR7/8 receptors.

Cytokines are additional soluble cell-derived mediators that are capable of exerting a profound effect on APCs, allowing them to serve as potent adjuvants at mucosal sites. Orally delivered interleukin-12 (IL-12) complexed with liposomes was able to induce elevated IgG levels to coadministered tetanus toxoid (137). In addition, IL-15 was demonstrated to be an effective mucosal adjuvant when delivered with herpes simplex virus 2, with augmented humoral and cellular responses in both the mucosal and systemic compartment (138). Collectively, these studies reveal the diversity of mucosal adjuvants, which will require further investigation to identify which are most suitable for a given vaccine application.

Physical Devices

Physical devices could also be employed to achieve vaccine delivery through GI mucosa. These may take a variety of forms, including using stored electrical or chemical energy to drive processes that permeabilize epithelia (e.g., reverse iontophoresis, electroporation, thermal ablation) (12), high-energy liquids or particles that penetrate tissue (69), or physical protrusions that mechanically disrupt epithelia [e.g., microneedle arrays (MNs)] (35, 139). In the last two decades, these approaches have been pioneered primarily to permeabilize the skin barrier for both diagnostics (140–142) and drug and vaccine delivery (143, 144). Physical devices may have the added capability to transport vaccine in an internal compartment to protect it from degradation until delivery at the intended mucosa. The accessibility of the target mucosa, however, may impose challenges requiring miniaturization and triggering/targeting release.

The oral cavity has recently been of interest for the use of physical devices for vaccination (35, 69, 70). Its ease of access enables greater freedom in device design and the translation of many approaches developed for the skin. MNs have been widely investigated to mechanically penetrate buccal mucosal/epithelial layers and deliver vaccine to APCs (35). MNs have been studied for both subunit and DNA (145) vaccines against HIV (145), influenza (146), hepatitis B virus, herpes simplex virus, and human papillomavirus (35). Additionally, MNs are a versatile delivery platform that may deliver any vaccine type in combination with particulate delivery vectors (such as MNPs or liposomes), stabilizing excipients, or adjuvants. Liquid and solid formulations may be delivered through hollow needles and dissolvable projections/coatings, respectively. Solid formulations in particular have also been widely reported to offer increased vaccine thermostability (72). Furthermore, the process of mechanically disrupting epithelial barriers has been reported to stimulate antibody responses in what has been described as physical adjuvantation (147, 148).

High-pressured liquid jets have also been used to deliver vaccine to the oral cavity. A capsule-sized device, the MucoJet, delivered a liquid-jet vaccine when placed against buccal tissue (69). The minimally invasive and self-administrable device did, however, require some user assembly prior to administration. A similar approach used a larger handheld liquid-jet device to deliver a vaccine for HIV antigen in combination with a modified vaccinia Ankara vector into buccal or SL tissue (70). Furthermore, the SL mucosa allows for slow absorption of macromolecules, which can be exploited by approaches that localize release over extended periods. Both mucoadhesive patches (84) and nonadhesive orally disintegrating films (85, 149) have been shown to limit diffusion by releasing vaccine at the mucosal surface and increase antigen uptake.

RECENT ADVANCES IN BIOLOGIC DELIVERY

Ingestible Devices for Gastric and Intestinal Biologic Delivery

The use of physical devices for vaccine delivery to the gastric and intestinal mucosa requires more advanced approaches compared to the oral cavity. Such approaches require the miniaturization of components (e.g., within ingestible capsules) and/or mechanisms to trigger actuation and vaccine delivery at the site of interest. Our group has recently reported

proof-of-concept delivery of biologic therapeutics to both the stomach (20) and small intestine (22) using physical devices. The self-orienting millimeter-scale applicator (SOMA; see Figure 2) is an ingestible capsule that inserts a solid millipost composed of a biologic (with or without excipient) through the mucosa (20). The soluble millipost then dissolves, releasing drug into the gastric wall, and we have demonstrated this for the systemic absorption of insulin. We also demonstrated a device for intestinal delivery of biologic therapies: the luminal unfolding microneedle injector (LUMI) (22). The LUMI consists of a three-armed device, folded into an ingestible capsule and held together by an elastomeric core. Upon reaching the intestines, the arms and elastomeric core are ejected from the capsule and unfold to contact the lumen. Arrays of soluble microneedles at the ends of the arms deliver the biologic drug into the intestinal mucosa. Perforation of the relatively thin intestinal mucosa is avoided by the use of ultra-short (<1 mm) microneedles. Both these devices incorporate actuation (i.e., triggering mechanisms) based on contact with gastric or intestinal fluid that dissolves a soluble plug to release a compressed spring. Rani Therapeutics has also proposed a capsule device with an extendable, dissolvable solid needle controlled by an expanding balloon for delivery to the intestinal mucosa called the RaniPill™ (21, 150), which was recently tested in a phase I clinical study (151; https://clinicaltrials.gov/ct2/show/NCT03798912). Collectively, these physical delivery devices have been widely regarded as disruptive technologies for biologic delivery to the stomach and intestines (18, 19).

Because these devices are intended to be self-administrable, they may have significant potential for mass oral vaccination campaigns needed during pandemic outbreaks of novel viruses [e.g., the severe acute respiratory syndrome coronavirus 2 causing COVID-19 (coronavirus disease 2019), swine flu, avian/bird flu, Zika virus] and/or for vaccines such as seasonal influenza, human papillomavirus, and common travel vaccines that are administered to adults. As platform devices, they may be loaded with potentially any type of vaccine (i.e., pure subunit/protein, particulate, lipid based, viral/bacterial vector, VLP) or adjuvant, and their compatibility with solid formulations offers potential for thermostability prior to delivery (72, 152), with or without stabilizing excipients, giving them a high degree of versatility. A key limitation of ingestible devices for vaccination, however, may be infant immunizations, where such devices may represent choking hazards.

A Role for Permeation Enhancers?

PEs increase the uptake of peptides through the intestinal epithelium for systemic delivery and have been of major interest for pharmaceutical companies for several decades, with over 250 having been investigated (7, 153). Success has been moderate, with single-digit bioavailability of biologics commonly reported. Given this, and the existence of alternative mucosal absorption strategies (e.g., via intestinal PPs), PEs have not been rigorously investigated for oral vaccination. However, recent advancements may offer renewed promise. Ionic liquids have been shown to generate high oral bioavailability for biologics (up to 50%) via intestinal absorption (23) and may have potential for delivery of vaccines. High oral bioavailability of insulin has also recently been reported using anionic nanoparticles (154). Another study identified that the PE *N*-[8-(2-hydroxybenzoyl)amino] caprylate (or SNAC) (155) enabled the gastric absorption of a glucagon-like peptide-1 receptor agonist. This

process mimicked thin film strategies for SL delivery in that release was localized beneath a slowly eroding tablet in the stomach, which could potentially be investigated to deliver vaccines. Several surfactants have also been investigated and show some promise for peptide delivery (7, 153, 156). The efficiency of PEs, however, is typically inversely proportional to the molecular weight of the peptide/protein (156), and this limitation needs to be considered for vaccine delivery, where shorter peptides are typically only weakly antigenic (157).

CONCLUSIONS AND FUTURE OUTLOOK

To date, the only approved orally delivered vaccines are live-attenuated and inactivated vaccines. Oral delivery of subunit and nucleic acid vaccines has many potential clinical advantages; however, the GI tract poses many challenges to the successful development of such technologies. Particulate, lipid-based, bacterial, and viral vectors designed for intestinal mucosal uptake have traditionally dominated oral vaccine design. There has been success in animal studies using these strategies primarily for intestinal delivery; however, there is a remaining need to enhance immunogenicity, improve low antigen uptake, and continue the development of mucosal adjuvants. Recent breakthrough advances in physical devices and PEs for oral biologic and vaccine delivery are enabling the investigation of alternative routes for vaccination to both the intestines and previously understudied mucosal targets in oral and gastric mucosae. These offer the promise of versatile platforms for highly efficient oral vaccine delivery, which may lead to new generations of oral subunit, DNA, and mRNA vaccine development, which are of particular use in response to the growing threat of pandemic outbreaks.

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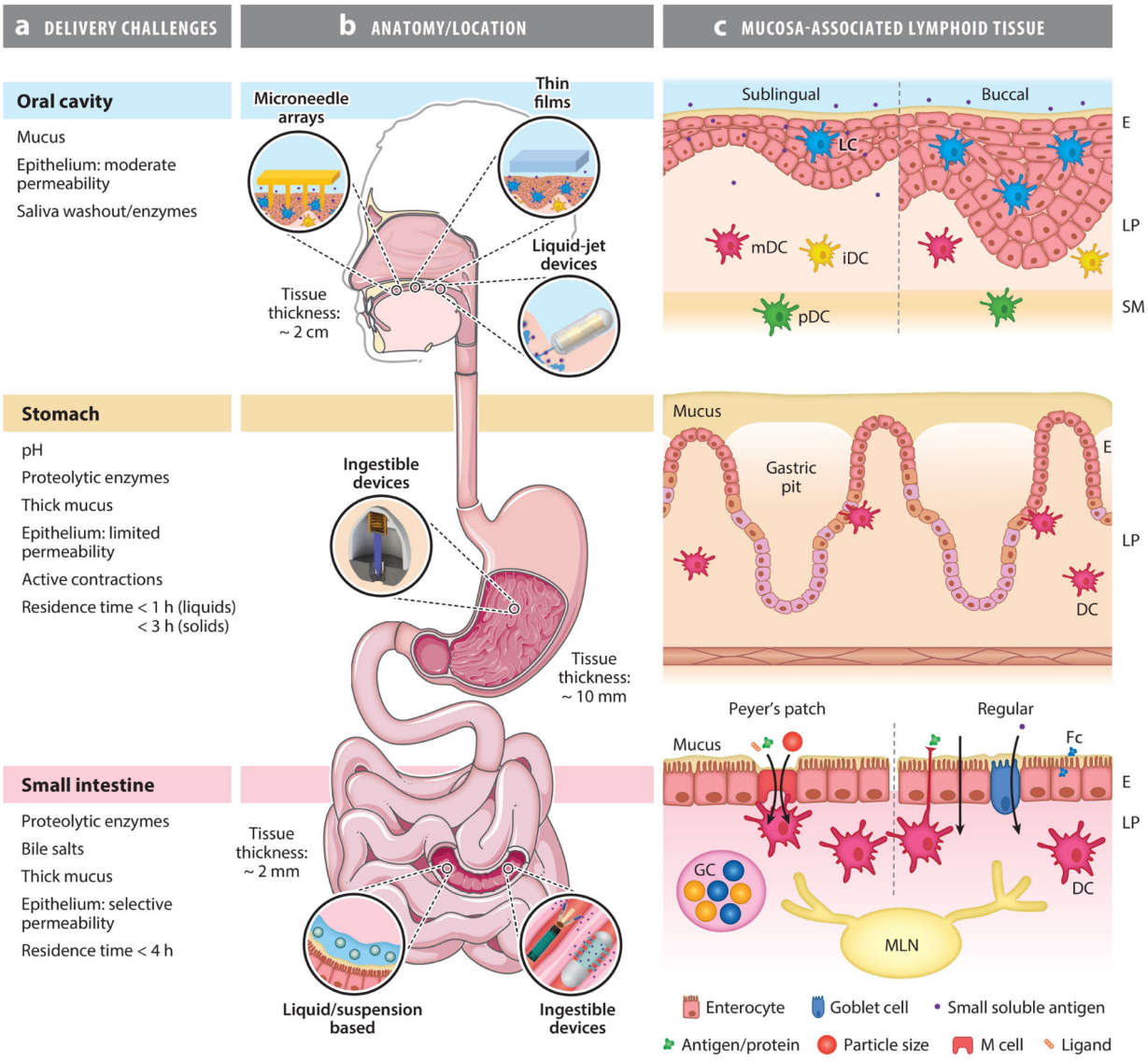

Figure 1.

Challenges and opportunities for oral vaccine delivery to the oral cavity, stomach, and small intestine and anatomy of their respective mucosal inductor sites. Each region has unique physiochemical barriers to vaccine absorption and organization of immune inductive tissue. (*a*) Summary of the delivery challenges to these regions. (*b*) Relative location of target mucosae and examples of broad delivery approaches that have been used to deliver vaccines or biologics to each site. Panel *b* partially adapted from Servier Medical Art (https://smart.servier.com/smart_image/complete-digestive-apparatus/) and Reference 69. Reprinted with permission from Servier Medical Art and AAAS. (*c*) The structures of the mucosae and inductor sites at each location. The location of key known APCs, including DCs, and lymphoid tissue are indicated. The sublingual and buccal mucosae contain nonkeratinized stratified squamous epithelia, which differ in thickness (100–200 μm and 500–800 μm, respectively). This allows for increased diffusion of macromolecules in the sublingual mucosa compared to the thicker buccal mucosa. LCs are present in the epithelium, mDCs

and iDCs in the lamina propria, and pDCs in the submucosa. The gastric mucosa is lined with gastric pits, a comparatively thick mucus lining, and a simple columnar epithelium with tight cell-cell junctions that prevent macromolecule absorption. Characterization of the gastric mucosa as an inductor site is limited; however, DCs have been observed associated with the epithelia of gastric pits. The intestinal mucosa contains a protective mucus lining and simple columnar epithelium that is capable of recognizing and uptaking pathogens/particles/antigens via several routes, depending on the properties of the pathogen/particle/antigen. These routes occur via specialist M cells (in the follicle-associated epithelium of Peyer's patches only, which have a thinner mucus lining), interdigitated DCs, enterocytes and goblet cells, and through intercellular junctions. Note the intestinal epithelium forms high-surface-area villi, which are not shown schematically due to scale. Abbreviations: APC, antigen-presenting cell; DC, dendritic cell; E, epithelium; Fc, fragment crystallizable; GC, germinal center; iDC, interstitial dendritic cell; LC, Langerhans cell; LP, lamina propria; M cell, microfold cell; mDC, myeloid dendritic cell; MLN, mesenteric lymph node; pDC, plasmacytoid dendritic cell; SM, submucosa.

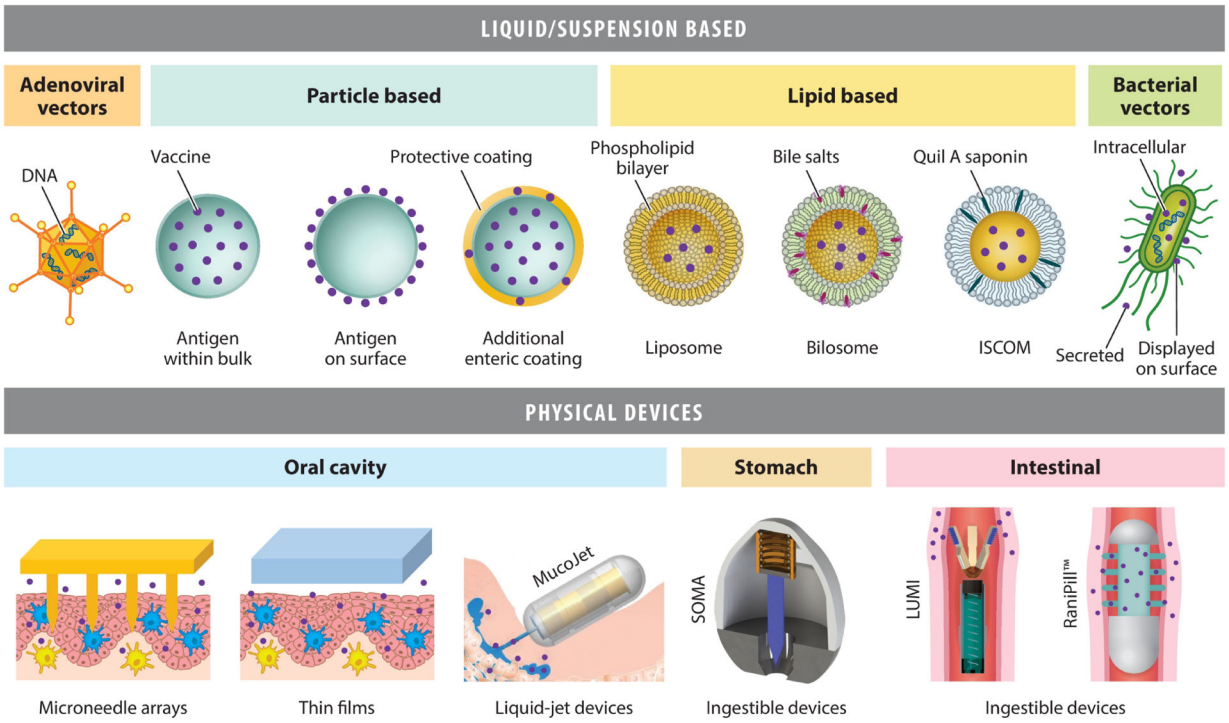

Figure 2.

Summary of the main delivery technologies in development for oral vaccination. A variety of liquid/suspension-based approaches have been investigated, including microparticles and nanoparticles, adenoviral and bacterial vectors, and lipid-based approaches. Adenoviral vectors contain a DNA-based cargo that is delivered to and expressed as antigen by host cells in vivo. Bacterial vectors are engineered with DNA to express vaccine antigens that may be either secreted, presented on the cell surface, or contained intracellularly and released in vivo. Particle- and lipid-based approaches may potentially carry vaccine either as preformed antigens or as nucleic acids for expression in vivo. More recently, there has been an expanding range of physical devices that localize vaccine release and/or mechanically disrupt mucosa for highly efficient delivery of a broad range of biologics to target sites in the gastrointestinal tract. These include microneedle arrays, thin films/patches (e.g., mucoadhesive and/or disintegrating), liquid-jet injectors (such as the MucoJet), SOMA, LUMI, and the RaniPill™. MucoJet panel reproduced from Reference 69. Reprinted with permission from AAAS. Abbreviations: ISCOM, immune-stimulating complex; LUMI, luminal unfolding microneedle injector; SOMA, self-orientating millimeter-scale actuator.

Table 1

Clinically approved oral vaccines

Disease (pathogen)	Vaccine	Antigen/vaccine type	Adjuvant	Formulation
Cholera (*Vibrio cholerae*)	Dukoral	Inactivated *V. cholerae* recombinant CTB	NA (CTB)	Liquid
	Vaxchora	Live-attenuated *V. cholerae* O1 Inaba 569B strain	NA	Liquid
Gastroenteritis (rotavirus)	RotaRix	Live-attenuated	NA	Liquid
	RotaTeq	Live-attenuated pentavalent reassortment human/bovine viruses G1, G2, G3, G4, and P1A[8]	NA	Liquid
Polio	Orimune	Live-attenuated poliovirus serotypes Sabin 1, 2, and 3	NA	Liquid
Typhoid fever (*Salmonella typhi*)	Vivotif	Live-attenuated *S. typhi*	NA	Enteric capsule
Acute respiratory syndrome (adenovirus)	Approved for military populations only	Adenovirus types 4 and 7	NA	Enteric capsule

Abbreviations: CTB, cholera toxin B; NA, not applicable.

Table 2

Delivery technologies for oral vaccination

Delivery system/ mode of delivery	GI mucosal target	Vaccine type	Adjuvant	Targeting ligand/ mucoadhesion/ localization	Common materials and/or form
Microparticles/ nanoparticles	Oral (85) (limited) sI (47, 48, 51, 63, 93, 158)	Subunit (47, 48, 159) DNA (76, 77) mRNA[a]	Self-adjuventing (47, 88, 159) Codelivery (160)	Targeting ligand (63, 66, 93) Mucoadhesion (68) Localization (85)	PLGA/PLA (47, 48, 76, 93), PA (102), PEI (104), chitosan (68), PCL (103), alginate (105)
Liposomes/ bilosomes/ISCOMs	sI (111)	Subunit (116) DNA (111) mRNA[a]	Codelivery (117)	Targeting ligand (114) Mucoadhesion (161)	Lipids (162), lipid + salts (65, 116), lipid + adjuvant (163)
Bacterial and viral vectors	Oral (123) sI (125, 164)	DNA (125) mRNA[a]	Self-adjuventing	Mucoadhesion (165)	Adenovirus (125) *Salmonella* (166) Poxvirus (167) VLPs (66)
Physical devices	Oral (146) Stomach[a] (20) sI[a] (22, 150)	Subunit (69) DNA[b] mRNA[a]	Mechanical (147, 148) Codelivery[c]	Device localization (20, 22, 69, 70, 145, 146, 150)	Microneedles (145, 146), liquid jet (69, 70), ingestible devices (20, 22, 150), thin films (85)

[a]Delivery technology is highly suitable for mRNA vaccine but to our knowledge has not been demonstrated for vaccination through the oral route.

[b]Delivery technology is highly suitable for DNA vaccine but to our knowledge has not been demonstrated for vaccination through the oral route.

[c]Platform technology is capable of delivering biologics orally, including vaccines and/or adjuvants; however, it has not yet been demonstrated for vaccination.

Codelivery refers to incorporation within a micro- and/or nanoparticle or lipid-based vehicles.

Abbreviations: ISCOM, immune-stimulating complex; mRNA, messenger RNA; PA, polyanhydride; PCL, poly-ε-caprolactone; PEI, polyethyleneimine; PLA, polylactic acid; PLGA, poly(lactic-coglycolic) acid; sI, small intestine; VLP, virus-like particle.