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Review

Buccal and sublingual vaccine delivery

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ABSTRACT

Because of their large surface area and immunological competence, mucosal tissues are attractive administration and target sites for vaccination. An important characteristic of mucosal vaccination is its ability to elicit local immune responses, which act against infection at the site of pathogen entry. However, mucosal surfaces are endowed with potent and sophisticated tolerance mechanisms to prevent the immune system from overreacting to the many environmental antigens. Hence, mucosal vaccination may suppress the immune system instead of induce a protective immune response. Therefore, mucosal adjuvants and/or special antigen delivery systems as well as appropriate dosage forms are required in order to develop potent mucosal vaccines.

Whereas oral, nasal and pulmonary vaccine delivery strategies have been described extensively, the sublingual and buccal routes have received considerably less attention. In this review, the characteristics of and approaches for sublingual and buccal vaccine delivery are described and compared with other mucosal vaccine delivery sites. We discuss recent progress and highlight promising developments in the search for vaccine formulations, including adjuvants and suitable dosage forms, which are likely critical for designing a successful sublingual or buccal vaccine. Finally, we outline the challenges, hurdles to overcome and formulation issues relevant for sublingual or buccal vaccine delivery.

1. Introduction

Among public health interventions, vaccination is by far the most effective strategy in maintaining population health and combating infectious diseases, especially in developing countries and disaster areas. Vaccination saves millions of lives every year, while bringing numerous social and economic benefits [1]. Since the vast majority of pathogens infect their host through the mucosa, an ideal vaccine should induce protective immunity at mucosal sites in order to act as a first line of defense against infections. However, most of the vaccines currently in use are administered *via* injection, *e.g.*, *via* the subcutaneous or intramuscular route. This generally induces poor mucosal immunity, whereas vaccines administered *via* mucosal routes have proven to be effective for the induction of both systemic and local immunity [2]. Additionally, mucosal immunization makes vaccine delivery easier and safer than parenteral administration routes, is very suitable for mass immunization during pandemic situations, and improves acceptability especially among children [3,4].

Despite the advantages mentioned above, there are currently only few mucosal vaccines for human use on the market [5]. The reason for this is that mucosal vaccination poses several challenges, such as immune regulation and tolerance, as well as overcoming fast removal of the vaccine by body fluids and enzymes, as has been reviewed for the oral [3,6], the nasal [7] and the pulmonary route [8]. Compared to the above-mentioned traditional mucosal routes, sublingual and buccal vaccine administration has received less attention. For many years, these routes have been used for the delivery of low-molecular-weight drugs to the bloodstream. Currently, the only vaccines that are widely being used for delivery *via* the oral mucosae are therapeutic sublingual allergy vaccines. These vaccines are used for sublingual immunotherapy (SLIT) to treat allergic hypersensitivity. Sublingual delivery of allergens can activate regulatory T cells that can suppress undesired immune reactions [9]. This has resulted in several approved sublingual products for allergy immunotherapy, such as SLITone®, Sublivac®, Grazax®, Oralair®, and AllerSlit®forte. Allergy vaccines are reviewed by Valenta et al. [10] and are beyond the scope of this review since they are aimed at immune regulation (tolerance) instead of activation of the adaptive immune system.

In this review, we will describe the potential and limitations of the sublingual and buccal mucosae as vaccine delivery sites and the mucosal immune responses that are induced upon sublingual or buccal vaccination. Further, the current status of sublingual and buccal vaccine delivery will be discussed and suitable vaccine antigens and potent adjuvants (immune potentiators and/or delivery systems) will be highlighted. Appropriate dosage forms that are required for a successful

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sublingual or buccal vaccine will also be outlined. Finally, the forthcoming perspectives are given, including the existing research and development gaps in this field and the potential of improved or controlled release vaccine formulations for sublingual and buccal vaccine delivery.

2. Sublingual and buccal mucosal sites for vaccine delivery

Mucosal vaccine delivery in the mouth can be subdivided into sublingual and buccal delivery. Sublingual delivery occurs *via* the mucosa of the ventral surface of the tongue and the floor of the mouth under the tongue, whereas buccal delivery occurs *via* the buccal mucosa, which is located in the cheeks, the gums and the upper and lower inner lips (Fig. 1). The specific structure and cell composition of the sublingual and buccal regions in the mouth define whether they are more or less suitable for vaccine delivery (as described below).

Within the oral cavity, some mucosal regions are lined by a keratinized stratified epithelium (gingival, hard palate, outer lips), whereas other regions are lined by a non-keratinized stratified epithelium (Fig. 1). The epithelium is supported by a basement membrane, which separates the two major layers of the oral mucosa: the epithelium and the underlying connective tissue or lamina propria. The arrangement of the hard palate and gingival, including the pluristratified keratinized mucosal epithelium and the lamina propria that is anchored onto the periostium of the underlying bone, makes these regions chemically and mechanically resistant to withstand the shearing forces associated with chewing food. The floor of the mouth, the inner surface of the lips and cheeks, and the ventral side of the tongue are covered by a non-keratinized epithelium, rendering these relatively more elastic and pervious than keratinized mucosae, and thus potentially more suitable for drug or antigen delivery.

The epithelium serves as a mechanical barrier protecting the underlying tissues and consists of a basal layer, an intermediate layer, and a superficial layer. From the basal to superficial layer the cells become larger, more flattened, more proteinaceous in the form of protein monofilaments, and less viable due to the absence of organelles (Fig. 2). The compacted, flattened cells of the lower superficial and intermediate layers form the major physical barrier to transport, whereas the intercellular lipids play an important role in the permeability of the mucosa. Besides the epithelial cells, the oral mucosal epithelium also contains three other cell types. The basal layer includes Merkel cells, which are endocrine cells associated with nerve fibers that contribute to the overall barrier function of the epithelium. They have also been suggested to play a role in the regenerative processes of oral mucosa [11]. Further, the suprabasal layer contains two types of cells: melanocytes, which produce the pigment melanin and are thus responsible for the color of the mucosa; and Langerhans cells (LCs), which are the most superficial antigen-presenting cells and an important target for the induction of an immune response. Other antigen-presenting cells below the mucosa are the myeloid dendritic cells (mDCs) located along the lamina propria and the plasmacytoid DCs (pDCs) found in the submucosal tissue (Fig. 2).

Salivary glands, which are located just below the mucosa in the mouth, produce mucin, a major component of the mucus layer on the mucosal surface, and help to promote the production and secretion of saliva. Saliva is needed to moisten and lubricate the mucosae and assists the masticatory process by binding the food bolus prior to and during swallowing. Additionally, salivary secretions protect the oral epithelium from potential harmful substances and regulate the composition of the oral microbial flora by its enzyme activity and by maintaining the oral pH between 5.5 and 7.0 [12].

2.1. Implications for mucosal vaccine delivery and comparison with other mucosal sites

Sublingual and buccal mucosae are attractive vaccine delivery sites that may have advantages over other routes, because of their anatomy and physiology. Table 1 shows an overview of characteristics of different sites that have been investigated for vaccine delivery. The dermal delivery site has been included because it contains comparable features with the buccal and sublingual sites, such as the presence of LCs as a main target for the vaccine antigen. However, dermal vaccination has the disadvantage that the impermeable thick keratinized stratum corneum acts as a physiological barrier for the diffusion of antigens to reach LCs after topical administration. As a result, dermal vaccination in general needs chemical disruption and/or microneedle penetration of this wall in order to let antigens reach the LCs to induce an immune response [13]. In contrast, sublingual and buccal mucosae suitable for vaccine delivery lack keratinized epithelium.

Compared to gastro-intestinal mucosal routes, degradation by gastric fluids and gastrointestinal enzymes is avoided during sublingual or buccal delivery, although some enzymatic activity is present in the mouth.

Most of the mucosal routes have special 'gateways', the so-called microfold (M) cells that are present in the epithelium covering the follicles of mucosal tissues. These M cells take care of the transport of antigens to mucosa-associated lymphoid tissues (MALT). The Peyer's patches (PP), nasopharynx-associated lymphoid tissue (NALT) and bronchus-associated lymphoid tissue (BALT) are important inductive

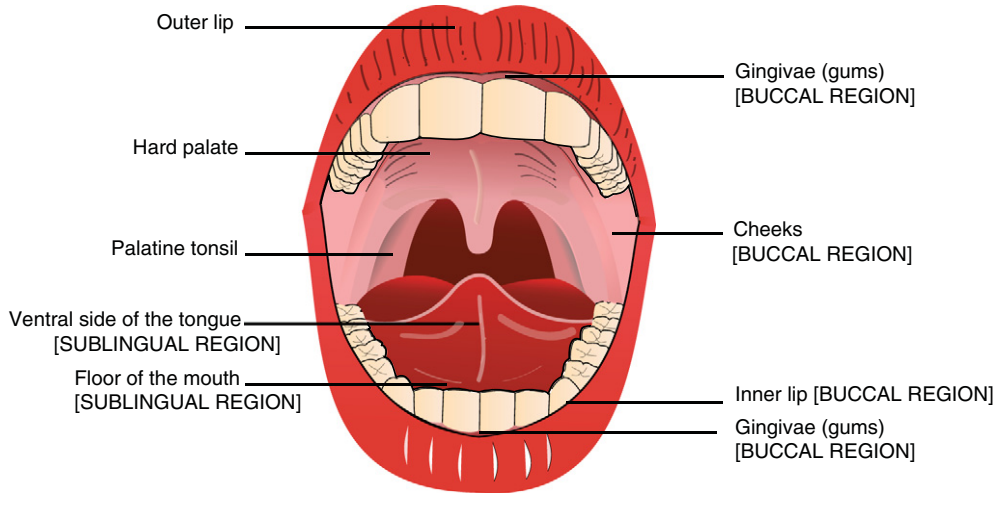

Fig. 1. The anatomy of the oral cavity. The sublingual and buccal regions for vaccine delivery are indicated.

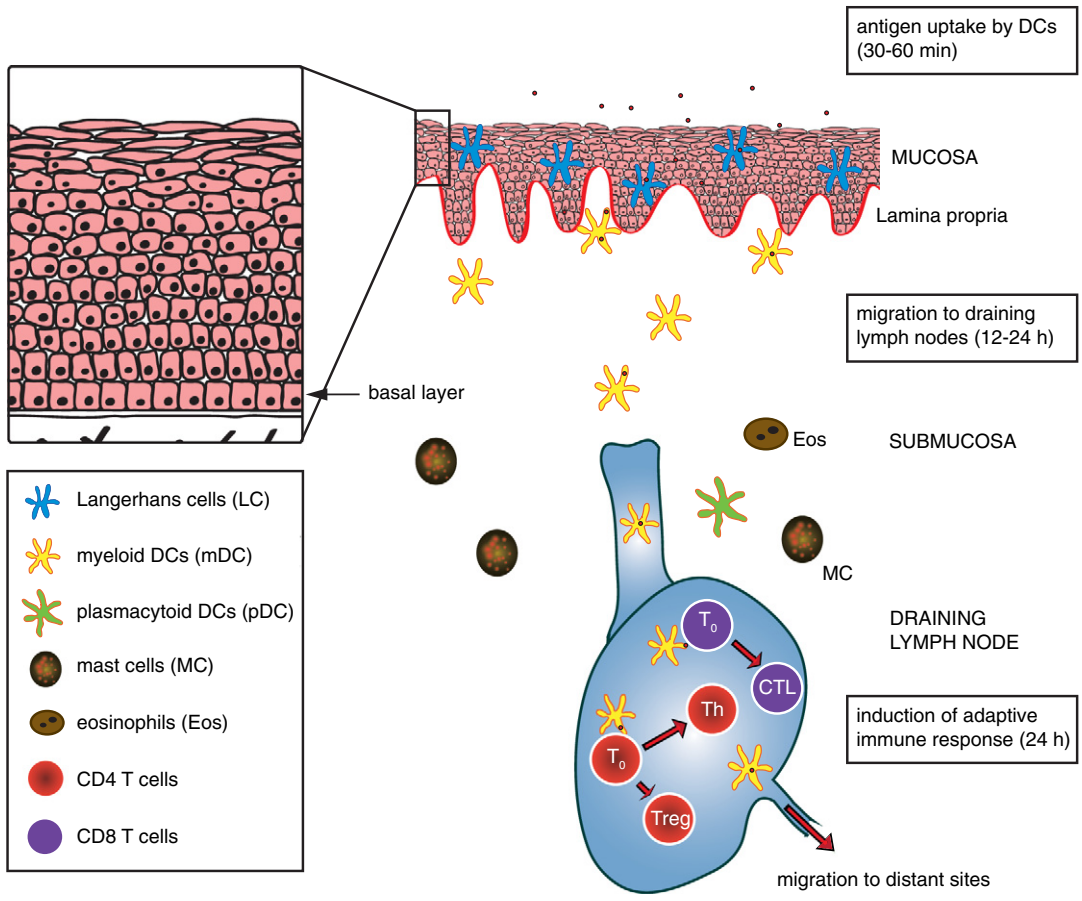

Fig. 2. Antigen delivery and antigen presentation following sublingual or buccal vaccination. Upon vaccine delivery, the antigen is likely to be captured by Langerhans cells (LC) within the mucosa itself and myeloid dendritic cells (mDCs) along the lamina propria. Antigen-bearing DCs will migrate to draining lymph nodes where they interact with naïve CD4 and CD8 T cells to support the differentiation into effector T cells (*i.e.*, helper (Th) and cytotoxic T cells (CTL)) and thereby induction of the adaptive immune response.

tissues that generate mucosal immunity upon vaccination *via* the gastro-intestinal (oral), nasal and pulmonary routes, respectively. In addition, the buccal and sublingual mucosal epithelia are covered with squamous stratified epithelium, whereas the epithelial cells of the small intestine, nasal cavity, trachea and bronchi are covered with columnar epithelium.

Table 1
Characteristics of different sites for vaccine delivery in humans. Mucosal immune responses (MIR) after immunization by different routes are indicated [82].

	Sublingual	Buccal	Oral/gastro-intestinal	Intranasal	Pulmonary	Dermal
Estimated surface area (cm^2)	26.5 ± 4.2	50.2 ± 2.9	350,000[a]	160–180	700,000 [c]	20,000
Epithelial structure (cell layers)	Stratified squamous, non-keratinized	Stratified squamous, non-keratinized	Simple columnar, non-ciliated [a]	Pseudo-stratified columnar, ciliated	Pseudo-stratified [d]/ Simple columnar, ciliated [e]	Stratified squamous, keratinized
Thickness, cell layer	8–12 cells (0.1–0.2 mm)	40–50 cells (0.5–0.8 mm)	Single cell	Single cell	Single cell	Multiple cells (2–3 mm)
Vaccine target	LCs (oral lymphoid foci)	LCs (oral lymphoid foci)	M cells (PP)	M cells (NALT)	M cells (BALT)	LCs
MIR distinct sites:						
Respiratory tract						
Upper	+++	?	−	+++	?	+++
Lower	+++	?	−	+/+++ [b]	++	+++
Gastrointestinal tract						
Stomach	+	?	+	−	?	?
Small intestine	+++	?	+++	−	?	+
Colon	?	?	++	−	?	+
Reproductive tract	+++	+	−	++	++	?
Systemic response	+++	++	+	+++	+	+++

Abbreviations used: MIR: mucosal immune response; LCs: Langerhans cells; MALT: mucosa-associated lymphoid tissue; PP: Peyer's patches; NALT: nasopharynx-associated lymphoid tissue; BALT: bronchus-associated lymphoid tissue.
[a] Based on delivery in the small intestine.
[b] Strong response by aerosol administration.
[c] Total surface area lungs.
[d] Epithelium lining the trachea.
[e] Epithelium lining the bronchi.

Compared to other routes, the sublingual and buccal routes have the potential to induce mucosal immune responses in a broad range of tissues (Table 1) as described in more detail in Section 2.2 ('Mucosal immune responses').

Nagai et al. (2014) recently reported the transport of sublingual antigens across sublingual ductal epithelial cells to ductal APCs in mice [14]. Since different studies failed to detect specific sampling cells (or M-like structures) in the sublingual or buccal mucosa [14,15], it seems most likely that antigens cross the ductal epithelium *via* paracellular and transcellular pathways [14]. So, probably the efficiency of vaccine delivery *via* these routes is directly related to the permeability of the mucosal membrane. This permeability is influenced by the thickness and the degree of keratinization of these membranes. The thickness of the human buccal mucosa has been estimated to be in the range of 500–800 μm, whereas the mucosal thickness of the sublingual region is about 100–200 μm [16]. In terms of permeability, the sublingual region is more permeable than the buccal region, which in turn is more permeable than the palatal region (Fig. 1). Sublingual administration can provide a rapid uptake of macromolecules and thus appears to be an attractive route for dosage forms with a short delivery period.

In the development of sublingual or buccal vaccines, the presence of saliva should be taken into account. Although saliva may be useful for the release of the antigen from certain dosage forms, it can be disadvantageous since the salivary composition, pH, and flow rate are variable. Excessive secretion and salivary flow may dilute the antigen or lead to swallowing of the dosage form before the antigen is absorbed through the mucosa, the so-called 'saliva washout' [12]. Moreover, the presence of digestive enzymes could lead to degradation of the antigen.

Since pH is a critical parameter for antigen absorption, the pH values at the different oral mucosal sites should be kept in mind for successful vaccine delivery. For example in adults, the pH of the floor of the mouth is about 6.5, whereas that of the buccal region is about 6.3 [17]. However, factors, such as diet and saliva flow rate, may affect the pH of the oral mucosa.

In Section 4 ('Dosage forms'), we describe different ways to circumvent the challenging characteristics of sublingual or buccal vaccine delivery.

2.2. Mucosal immune responses

Unlike small synthetic drugs, vaccine antigens do not reach the bloodstream after entering the sublingual or buccal mucosa, but are rather captured by antigen-presenting DCs, mainly LCs, in the mucosa. The antigen uptake, process and the presentation of their epitopes to T cells by antigen-presenting cells are required to induce effective adaptive immunity. For example, the ovalbumin antigen crosses the epithelial barrier within 15 to 30 min and the uptake by sublingual DCs occurs within 30 to 60 min after sublingual administration in mice [18]. Rare pro-inflammatory cells, *i.e.*, histamine-containing mast cells (MC) or eosinophils (Eos) (Fig. 2), are found in oral tissues, and these cells are mainly spread in the muscular layer beneath the mucosa [18,19]. Therefore, it is likely that most of the antigen is mainly captured by LCs and other oral DCs in the upper layers prior to reaching pro-inflammatory cells. The relatively high frequency of LCs and low numbers of mast cells in the buccal region [19] make the buccal mucosa an attractive site for vaccine delivery despite its thicker epithelium and lower permeability as compared to the sublingual mucosa.

Antigen-bearing DCs migrate to the lymph nodes draining the sublingual and buccal area where they are able to prime both naïve CD4 and CD8 T cells (Fig. 2, T_0 cells). For example, Eriksson et al. reported that a substantial number of DCs leave the buccal epithelium after topical buccal immunization and migrate to draining lymph nodes where they present processed antigen to CD4 T lymphocytes [20]. Another study revealed the buccal epithelium as inductive site of efficient priming of CD8 T lymphocytes [21]. Upon activation, T and B cells that leave the site of the initial antigen presentation, enter the circulation and then disperse to selected mucosal sites, where they differentiate into memory or effector cells. The activation of CD4 T lymphocytes leads to the induction of antigen-specific helper T (Th cells) and/or regulatory T cell (Treg) mediated immune responses (Fig. 2), whereas CD8 T cells facilitate the induction of cytotoxic T lymphocyte (CTL) responses (Fig. 2). The tissue destination of these cells appears to be largely determined by site-specific integrins, supposed 'homing receptors', on their surface that will bind to mucosal tissue-specific receptors (addressins) on vascular endothelial cells [22]. Recent work of Hervouet et al. demonstrates that antigen-bearing DCs that have captured the antigen in the sublingual mucosa are encountered in distant lymph nodes and spleen following sublingual immunization of mice [23], which suggests that sublingual DCs are capable to enter the blood circulation to seed distant lymphoid organs.

The migration of immune cells from the inductive MALT to distant effector tissues is the cellular basis for the so-called 'common mucosal immune system'. The MALT contains T-cell zones, B-cell enriched areas containing a high frequency of sIgA-positive B cells and a subepithelial area with antigen-presenting cells to induce specific immune responses. As mentioned earlier, the oral mucosa lacks a certain immunological structure as observed in Peyer's patches of the intestine where the antigen is sampled by specialized M cells. However, different studies describe the concept of oral lymphoid foci, the equivalent of the germinal centers observed in other MALT, suggesting that the oral mucosa serves as a site for immune induction. The role of oral DCs in deciding whether to induce adaptive immunity or tolerance, and whether there exist germinal centers in oral lymphoid foci are discussed extensively by others [24,25]. The distribution of immune cells, particularly the abundant presence of oral LCs, makes the oral mucosa an attractive site for vaccine delivery. Both sublingual and buccal immunization are able to promote mucosal immunity, as well as systemic immunity, against pathogens entering the human body at more distant sites than the mouth (mucosa), such as the respiratory tract or reproductive tract (Table 1).

3. Current status of sublingual and buccal vaccine delivery

In the section below, we will describe the use of live (attenuated) pathogens, recombinant (heterologous expression) and inactivated vaccines. Furthermore, we will outline adjuvants, *i.e.*, immune potentiators and/or delivery systems, that have been evaluated for vaccination *via* the sublingual or buccal route. A summary of the published studies on sublingual and buccal vaccination is given in Table 2.

3.1. Live attenuated vaccines

3.1.1. Live attenuated viral vaccines

Sublingual immunization of mice with live attenuated influenza virus (A/PR/8 strain, H1N1) has been found to be safe and effective for inducing protective immune responses in mucosal and systemic compartments. Song et al. concluded that the observed protection was mediated by the induction of influenza virus-specific IgG in the serum and secretory IgA (sIgA) in the respiratory mucosa, which limit virus entry and replication in the respiratory tract. A single sublingual dose of A/PR/8 virus prevented lung pathology induced by influenza virus challenge and provided a broad-range cross-protection against different influenza virus subtypes. Thereby, the risk of potential passage of vaccine virus to the olfactory bulb was avoided by using the sublingual route since no viral RNA was detected in brains of sublingually vaccinated mice, in contrast to mice that received the same vaccine intranasally [26]. Similarly, sublingual administration of live-attenuated virus lacking the non-structural protein 1 (DeltaNS1) was as protective against influenza virus challenges in mice as intranasal immunization. Sublingual immunization with these DeltaNS1 viruses induced high levels of virus-specific antibodies and stimulated immune cells in mucosa-associated and systemic lymphoid organs [27]. Moreover, the vaccine

was well tolerated and did not induce bodyweight loss in sublingually vaccinated mice.

3.1.2. Recombinant virus based (RNA-) vaccines

Replication-defective adenovirus vectors (rAdV) have been widely explored for the delivery of antigens. Infection by adenoviruses occurs through the airway epithelium and replication takes place in mucosal tissues of the respiratory tract. These characteristics make these vectors suitable for mucosal vaccine delivery. Sublingual immunization with rAdV encoding the conserved influenza nucleoprotein antigen [28] or the soluble globular head of hemagglutinin [29] protected mice against influenza virus infection. Furthermore, sublingual administration of rAd5 vectors encoding HIV proteins induced both significant antigen-specific humoral (serum and mucosal IgG and IgA) [30] and cellular (systemic and mucosal CTL responses) [31] immune responses. Moreover, sublingual vaccination with rAdV encoding a truncated S protein (rAdV-S), which is a major antigenic protein present on severe acute respiratory syndrome-associated coronavirus (SARS-CoV), induced systemic neutralizing antibodies and airway IgA antibody responses in mice. These immune responses were similar to those induced by intranasal administration. It is worth noting that intranasal delivery of rAdV redirected the virus vector to the olfactory bulb, whereas no adenoviral DNA was detected after sublingual delivery [32]. Choi et al. reported that both mice and guinea pigs were protected against a lethal Ebola challenge after a single sublingual immunization with an AdV-based vaccine expressing the Zaire glycoprotein in a manner similar to that of traditional intramuscular vaccination [33].

3.1.3. Recombinant bacterial vaccines

With the availability of genetic tools for heterologous gene expression, the concept of live vaccine vehicles has sparked renewed interest, especially for the mucosal routes [34]. Several studies have shown that engineered *Bacillus (B) subtilis* is able to generate systemic and mucosal antibodies against heterologous antigens. *B. subtilis* delivered sublingually and expressing tetanus toxin C-fragment, evoked protective immunity in both mice [35] and piglets [36]. Batista et al. reported the use of 'gut-colonizing' *B. subtilis* spores as a new mucosal vaccine delivery platform consisting of two antigen expression strategies. One is active during spore formation, which leads to the display of recombinant adhesins at the spore surface that facilitates adhesion to mucosal surfaces. In addition, the recombinant spores have been shown to germinate after oral delivery resulting in intracellular expression of the antigen. Mice immunized with three doses of *B. subtilis* spores *via* the sublingual route, developed higher specific serum IgG titers when compared with the mice orally immunized with a ninefold higher dose of spores of the same strains. Although not proven in this study, the authors speculated that sublingual delivery of these spores will also result in intracellular antigen expression once they are captured by intraepithelial antigen-presenting cells and germinate [37]. The better immune response to sublingual vaccination might be ascribed to the fact that sublingual delivery has a smaller distribution volume and a less aggressive environment than gastro-intestinal delivery.

3.2. Inactivated vaccines

In a study by Cho et al., different routes, *i.e.*, intranasal, intravaginal, transdermal, sublingual and intramuscular, were compared in a mouse study using human papillomavirus 16L1 (HPV16L1) protein vaccine. Among these routes, the intranasal and sublingual routes provided the highest HPV16L1-specific levels of vaginal sIgA and systemic IgG responses that were comparable to those elicited *via* the intramuscular route [45]. Sublingual vaccination against respiratory syncytial virus (RSV) with a purified G protein fragment (Gcf) without the addition of adjuvants induced strong serum IgG and mucosal IgA responses (similar to intranasal vaccination) in mice. Interestingly, these antibody responses could be elicited by Gcf without the need for an adjuvant. The study demonstrated that the chemotactic activity exhibited by Gcf was necessary to induce protective immunity. Therefore, the authors proposed that Gcf has a self-adjuvanting property [46].

Murugappan et al. investigated whether sublingual administration of whole β-propiolactone (BPL)-inactivated influenza virus can prime the immune system for a later intramuscular boost with a heterologous vaccine. Although sublingual priming did not induce any detectable immune responses, it strongly enhanced hemagglutination inhibition (HI) titers against both the homologous as well as the heterologous vaccine after the intramuscular booster. In addition, sublingual priming induced IgA responses in the lung and nose, while intramuscular priming showed higher IgA responses in the lung, but not in the nose [40]. In a study by Song et al., a sublingual booster was given, instead of an intramuscular booster, and immune responses were analyzed more extensively. Immunization with formalin-inactivated influenza virus *via* the sublingual mucosa induced protective immune responses, elevated mucosal sIgA antibody levels, and enhanced virus-specific CTL responses [26]. The different procedures used for virus inactivation affect the membrane fusion properties of the virus to a certain extent, resulting in a less optimal activation of CTLs. Inactivation with formalin severely compromises fusion activity of the virus, while BPL-inactivation reveals preservation of the fusion activity [60]. However, a sublingual booster vaccination with formalin-inactivated influenza induced significant elevated virus-specific CTL responses in mice. Unfortunately, the analysis of CTL activation is lacking in the study using BPL-inactivated influenza.

As with mucosal immunization in general, sublingual (and buccal) vaccination with non-replicative antigens does not induce sIgA and serum IgG responses without the addition of adjuvants. Strong immune potentiators or delivery systems are needed to break mucosal tolerance and facilitate uptake through the oral mucosae. For example, a mucosal adjuvant (cholera toxin, CT) was needed to induce immune responses upon sublingual immunization with a human immunodeficiency virus (HIV) subunit vaccine [48]. Çuburu et al. evaluated the sublingual route for vaccine delivery using ovalbumin (OVA) as a model antigen. The mice received three doses of 200 μg OVA and although systemic IgG responses were measurable, mucosal OVA-specific antibody responses were absent after sublingual immunization of OVA alone. Co-administration of a mucosal adjuvant (CT) generated high OVA-specific IgA responses in saliva and nasal wash. These responses were of the same magnitude as those induced by intranasal administration [58].

Recently, sublingual administration of a subunit influenza vaccine was evaluated in mice. After three doses, detectable, but rather low, antigen-specific systemic IgG and HI titers were found after sublingual immunization, whereas mucosal IgA antibodies were below the detection limit. The addition of the mucosal adjuvant LTK63 was needed to those obtained after conventional intramuscular immunization [41]. A *Salmonella* vaccine consisting of sonicated *Salmonella* proteins induced protection only after sublingual immunization in the presence of adjuvants (CpG DNA or CT). This was observed in both adult [51] and neonatal mice [50].

Although mucosal adjuvants improve immune responses upon sublingual immunization using non-replicating/inactivated antigens, in some studies systemic and/or mucosal immunity was obtained with sublingual delivery of non-adjuvanted inactivated (subunit) vaccines [45,46,58]. In general, high doses were used and even the size of the antigen seemed to be an issue for successful immunization since small proteins/antigens showed the induction of both systemic and mucosal immune responses upon sublingual administration.

3.3. Adjuvants used in buccal and sublingual vaccines

3.3.1. Immune potentiators

3.3.1.1. Bacterial enterotoxins. The most powerful and hence the best-studied mucosal adjuvants are the bacterial enterotoxins cholera toxin (CT) and the *Escherichia coli* heat-labile toxin (LT), which have structural and biological similarities.

Table 2
Preclinical development of sublingual and buccal vaccines.

Antigen	Adjuvant	*In vivo* model	Dose (*no. of doses*)	Dosage form (*volume*)	Protection	Immune response/characteristics	Ref
Live attenuated vaccines							
Influenza A/PR/8		Mouse	1×10^2 *pfu* 1×10^4 *pfu*	L <7 *ul*	+/−	- Both systemic and mucosal Abs - Dose dependent protection against both homologous and heterosubtypic influenza virus challenge	[26]
deltaNS1 influenza A[1]		Mouse	2×10^7 *pfu*	L 4 × 5 *ul*	+	- Protection against challenge with homologous and heterosubtypic influenza virus - High levels specific Abs in both mucosal and systemic compartments - Stimulated immune cells in mucosa-associated and systemic lymphoid organs	[27]
Heterologous antigen expression — live vaccine carriers							
NP (nucleoprotein) of influenza A/PR/8	rAd5	Mouse	1×10^7 *pfu* 1×10^8 *pfu*	L <5 *μl*	−	- Single sublingual immunization failed to confer protection by different influenza strains	[28]
HA soluble globular head	rAd5	Mouse	1×10^8 *pfu*	L 10 *μl*	+	- Complete protection after challenge with lethal dose homologous virus - Induction of significant levels of HA-specific mucosal IgA and IgG	[29]
RSV - sFsyn[2]	HDAd[2]	Mouse	1×10^8 *pfu*	L 5 *μl*	+	- RSV F protein-specific systemic and mucosal neutralizing Abs - RSV-specific IFN-γ producing $CD8^+$ T cell responses in the spleen and lung - Effective protection against RSV infection; reduced lung viral titers upon challenge compared with control group	[38]
SARS-S protein (Spike)	rAd	Mouse	2×10^7 *pfu* 1×10^8 *pfu*	L 20 *μl*	n.d.	- SARS-CoV neutralizing antibodies in serum - Airway IgA - Induced CD8+ T cells responses in lungs - Unlike intranasal vaccination, no redirection of AdV to olfactory bulb	[32]
HIV-Env (Envelope glycoprotein)	rAd5	Mouse	1×10^8 *pfu*	L 10 *μl*	n.d.	- Serum IgA response - Vaginal IgA and IgG	[31]
HIV-Gag	rAd5 rEA	Mouse	1×10^{10} *pfu*	L 8 *μl*	n.d.	- Induction of CTL responses in spleen and SMLN - Higher innate immune responses and improved T cell responses after co-administration with rEA (TLR agonist)	[30]
SIV-Env/rev SIV-Gag	rAd5	Macaque	1×10^9 *pfu* Each (2×)	L 0.25 *ml*	n.d.	- Macrophage targeting in BAL fluid and rectal tissue - SIV-specific cellular responses, serum binding Abs and mucosal sIgA	[39]
Ebola ZGP (Zaire glycoprotein)	rAd5	Mouse Guinea pig	1×10^8 *pfu* 1×10^9 *pfu*	L 10 *μl* 40 *μl*	+	- Induced IFN-y T cells in spleen, BAL, MLN and SMLN - Elicited population of effector memory CD8+ cells and strong CTL responses in spleen and SMLN	[33]
Tetanus toxin fragment C (TTFC)	*Bacillus subtilis mLT*	Mouse	1×10^9 *pfu* (3–4×)	L 10 *μl*	+	- Tetanus-specific systemic IgG and mucosal sIgA - Full protection against lethal toxin challenge in mice immunized with TTFC vegetative cells (without mLT)	[35]
Tetanus toxin fragment C (TTFC)	*Bacillus subtilis mLT*	Pig	1×10^9 *pfu* (4×)	L 1 *ml*	n.d.	- Tetanus-specific systemic neutralizing Abs - Induction of salivary and vaginal IgA responses	[36]
Streptococcus mutans P1	*Bacillus subtilis*	Mouse	1×10^8 *pfu*	L 10 *μl*	n.d.	- Higher specific IgG titers when compared to group orally immunized with higher dose	[37]
Inactivated vaccines							
Influenza WIV (β-propiolactone-inactivated)		Mouse	20 μg (1×) 5 μg (i.m. booster)	L 10 *μl*	n.d.	- Enhanced HI titers after sublingual priming followed by an intramuscular booster when compared to the intramuscular priming - Enhanced lung and nose IgA titers with sublingual priming	[40]
Influenza WIV (formalin-inactivated)		Mouse	20 μg (2×)	L <7 *μl*	+	- Specific systemic and secretory Ab responses - 80% survival	[26]
Influenza WIV (formalin-inactivated)	mCTA-LT[3] (5 μg)	Mouse	20 μg (2×)	L <7 *μl*	+	- 100% survival, complete clearance of virus in the lungs (BAL fluid) - More IFNy-producing CD4+ and CD8+ T cells in spleens and MLNs than without mCTA-LT - More virus-specific CTL-responses than with PBS or killed A/PR/8 alone	[26]
Influenza HA subunit	LTK63[4] (5 μg)	Mouse	10 μg (3×)	L	n.d.	- Systemic responses (IgG and HI) comparable to intramuscular immunization - Influenza-specific Th17 cells and neutralizing mucosal IgA in the nose (comparable to intranasal immunization)	[41]
Influenza A virosome	c-di-GMP[5] (7.5 μg)	Mouse	2 μg (2×)	L 7 *μl*	n.d.	- Induction of systemic and local Abs capable of hemagglutination inhibition - Significant adjuvant effect on both systemic and local Ab responses - High frequencies of influenza-specific homo- and hetero-subtypic $CD4^+$ Th1 cells - Balanced Th1/2 profile and Th17 response after immunization with adjuvanted virosomes	[42]
Influenza 3M2eC protein	CT (2 μg)	Mouse	10 μg		+		[43]

(continued on next page)

Table 2 (*continued*)

Antigen	Adjuvant	*In vivo* model	Dose (*no. of doses*)	Dosage form (*volume*)	Protection	Immune response/characteristics	Ref
				L *15 μl*		- Both systemic and mucosal Abs - Protection against both homologous and heterosubtypic influenza virus challenge	
HPV16L1 VLP		Mouse	5 μg	L *10 μl*	+	- Protection against genital challenge with HPV pseudovirions - Neutralizing Abs in serum and genital IgG and IgA Abs - Neutralizing Abs in cervicovaginal secretions	[44]
HPV16L1 VLP	CT (2 μg)	Mouse	5 μg	L *10 μl*	+	- Protection against genital challenge with HPV pseudovirions - Higher systemic IgG Ab titers than after sublingual VLP administration without CT - Lower vaginal IgG, but higher vaginal IgA Ab responses than intramuscular immunized mice with alum-adjuvanted VLPs - Neutralizing Abs in cervicovaginal secretions	[44]
HPV16L1 VLP		Mouse	30 μg 50 μg	L *10 μl*	n.d.	- Higher vaginal and salivary sIgA responses when compared to untreated animals - Increased number IFN-γ producing CD8 + T cells in spleen	[45]
HPV16L1 VLP	CTB (10 μg)	Mouse	30 μg	L *10 μl*	n.d.	- Elevated mucosal sIgA induction after co-treatment with CTB - Enhanced production of IL-4 and IFN-γ from stimulated CD4 + T cells - Higher number IFN-γ producing CD8 + T cells in spleen and SMLN when compared to HPV16L1 alone	[45]
HPV16L1 VLP	Several adjuvants (see characteristics)	Mouse	30 μg	L *10 μl*	n.d.	- No enhanced effects on systemic IgG nor on vaginal and salivary IgA Ab responses (Adjuvants that were used are: Poly I:C (0.1 mg), MPLA (10 μg), Imiquimod (50 μg), L18-MDP (1 μg), Murabutide (10 μg), peptidoglycans (50 μg), Vitamin D3 (0.5 μg), γ-polyglutamic acid (1 mg))	[45]
RSV G protein (Gcf)	CT (2 μg)	Mouse	20 μg	L *15 μl*	+	- Strong serum IgG and mucosal IgA responses - Protection against RSV challenge without significant lung eosinophilia - No adjuvant effect of CT	[46]
Measles virus NP		Mouse	30 μg (1×)	L *15 μl*	+/−	- Single buccal immunization (injection or topical application) induced antigen-specific CD8 CTLs - Rapid recruitment of DCs into the mucosa - Protection against lethal challenge following buccal vaccination by injection (100%) or topical administration (40%)	[47]
HIV-1 Pol	CTB CT (1 μg)	Mouse	25 μg (3×)	L *5 μl*	n.d.	- CTB-Pol conjugate induced IFN-γ producing CD8 T cells - Induction of mucosal CTLs in the genital tract after immunization with CTB-Pol mixed with CT, but not with CTB-Pol alone or Pol mixed with CT	[48]
HIV-1 gp41	CT (1 μg)	Mouse	10 μg (3×)	L *5 μl*	n.d.	- Strong specific IgG and IgA responses in serum and genital secretions after gp41 + CT immunization - gp41-specific IgA and IgG ASCs in genital tract	[48]
HIV-1 CN54gp140 (gp140)	FSL-1 Poly I:C MPLA Pam3CSK4 R848 CpG B (20 μg each)	Mouse	10 μg (3×)	L *10 μl*	n.d.	- Increased serum IgG and IgA titers when co-administered with Poly I:C - Diminished systemic specific Ab responses with MPLA - Detected IgA titers in vaginal washes of all animals where antigen was administered with FSL-1, poly I:C, Pam3CSK4 or CpG B	[49]
HIV-1 CN54gp140 (gp140)	Chitosan (100 μg)	Mouse	10 μg (3×)	L *10 μl*	n.d.	- No significant adjuvant effect of chitosan	[49]
Salmonella proteins (SPP)		Mouse (newborn)	6/40 μg (3 + 1)	L *1.2 μl*	−	- Slightly enhanced systemic IgG titer and mucosal IgA responses compared to the PBS control group	[50]
Salmonella proteins (SPP)	CT (0.2/1 μg) CpG (1/10 μg)	Mouse (newborn)	6/40 μg (3 + 1)	L *1.2 μl*	+	- Enhanced antigen-specific systemic IgG and mucosal sIgA responses in CT or CpG groups compared to mice immunized with SSP alone - Protection against intestinal necrosis and higher survival rates for adjuvant groups	[50]
Salmonella proteins (SPP)	CT (1 μg) CpG (10 μg)	Mouse	40 μg (7×)	L *10 μl*	+	- Significant higher antigen-specific sIgA responses for both groups immunized with SPP alone or together with CT or CpG as adjuvant - Higher IFN-γ production in spleen upon SPP-CpG vaccination - Higher IL-4, IL-5 and IL-6 production in spleen upon SPP-CT vaccination - Protection against intestinal necrosis and higher survival rates for adjuvant groups	[51]
Pneumococcal whole cell (chloroform inactivated)	dmLT	Mouse	10 μg (3×)	L *5 μl*	+	- Dose-dependent protection in sublingual immunized mice (reduced bacterial load in nasal wash compared to control mice) - Induced systemic IL-17A levels upon sublingual vaccination	[52]

Helicobacter pylori lysate	CT (10 μg)	Mouse	500 μg (2×)	L *10 μl*	+	- Enhanced proliferative responses to H. pylori antigens in CMLNs - Immune protection against *H. pylori* infection - Strong specific serum IgG and IgA titers in stomach and intestine - Strong proliferation and IFN-γ and IL-17 production by T cells from spleen and MLNs - Increased IFN-γ and IL-17 gene expression in stomach	[53]
Helicobacter pylori lysate	CT (10 μg)	Mouse	500 μg	L *10 μl*	n.d.	- Increased expression of chemokines and chemokine receptors known to attract eosinophils, T cells and neutrophils - Higher counts of CD4 + T cells, eosinophils, neutrophils and CD103 + DCs in the gastric lamina propria of immunized mice	[54]
Helicobacter pylori lysate	CT (10 μg)	Mouse	400 μg	L *10 μl*		- Decrease in bacterial load after challenge when compared to non-immunized control group	[55]
Helicobacter pylori lysate	dmLT (10–20 μg)	Mouse	400 μg	L *10 μl*	+/−	- Decrease in bacterial load after challenge when compared to non-immunized control group - Enhanced *ex vivo* proliferative and cytokine responses from cells from spleen and MLNs after restimulation with *H. pylori* antigens	[55]
Chlamydial major outer membrane protein (MOMP)	CTA1-DD (20 μg)	Mouse	100 μg	L *7 μl*	+/−	- Reduction of severity and incidence of genital tract pathology after challenge with *Chlamydia muridarum* after sublingual vaccination with or without adjuvant - 80% of the MOMP-CTA-DD-immunized animals protected in genital tract	[56]
Chlamydial major outer membrane protein (MOMP)	CT-CpG (5 μg CT) (10 μg CpG)	Mouse	100 μg	L *7 μl*	+/−	- Reduction of severity and incidence of genital tract pathology after challenge with *Chlamydia muridarum* after sublingual vaccination with or without adjuvant - 60% of the MOMP-CT-CpG-immunized animals protected in genital tract - Reduction of duration of vaginal shedding after challenge, but not of the total bacterial burden - MOMP-specific IFN-γ, TNF-α and IL-17 cytokine production by lymphocytes isolated from MLNs	[56]
Tetanus toxoid (TT)	LT (1 μg)	Mouse	10 − 20 μg (4×)	L *5 μl*	n.d.	- Induced systemic TT-specific IgG and mucosal IgA levels after TT-LT immunization when compared to TT alone - Long lasting TT-specific ASCs in bone marrow and CD4 + and CD8 + T cells in dLNs and spleen	[57]
Tetanus toxoid (TT)	LTK63 (10 μg)	Mouse	10–20 μg (4×)	L *5 μl*	n.d.	- Induced systemic TT-specific IgG and mucosal IgA levels after TT-LTK63 immunization when compared to TT alone - Long lasting TT-specific ASCs in bone marrow and CD4 + and CD8 + T cells in dLNs and spleen	[57]
Tetanus toxoid (TT)	FSL-1 Poly I:C MPLA Pam3CSK4 R848 CpG B (*20 μg each*)	Mouse	10 μg (3×)	L *10 μl*	n.d.	- Significant increase in specific systemic IgG when co-administered with FSL-1, poly I:C, CpG B and an increase in IgA for FSL-1 - Detectable IgG titers in vaginal washes of all animals where antigen was administered with FSL-1, poly I:C or CpG B - Decreased specific systemic and vaginal IgA responses for TT-MPLA-immunized mice	[49]
Tetanus toxoid (TT)	Chitosan (100 μg)	Mouse	10 μg (3×)	L *10 μl*	n.d.	- Increase in specific systemic IgG and IgA above TT alone when co-administered with chitosan - Increased IgG1/IgG2a ratio relative to TT alone	[49]
Ovalbumin (OVA)	CT (2 μg)	Mouse	200 μg (3×)	L *<10 μl*	n.d.	- Systemic and mucosal Ab responses - Balanced Th1/Th2 cytokine responses - Induction of $CD8^+$ T cells in lung tissues and systemic lymphoid organs	[58]
Ovalbumin (OVA)	Ad2F (25 μg) CT (2 μg)	Mouse	25 μg (3×)	L *7 μl*	n.d.	- Ad2F-delivered OVA was efficiently taken up by DCs and migrated mostly to SMLNs - Highest OVA-specific serum IgG, IgA and mucosal IgA titers for OVA-Ad2F + CT-immunized mice - Mixed Th-cell response by enhanced IL-4, IL-10, IFN-γ and TNF-α-specific cytokine-forming cells	[59]
Ovalbumin (OVA)	CT (2 μg)	Mouse	200 μg (3×)	L *5 μl*	n.d.	- OVA-specific IgG and IgA Abs in blood and cervicovaginal secretions - IgA ASC in genital mucosa upon sublingual immunization similar to intranasal or vaginal immunization and superior to intragastric vaccination - OVA-specific effector CD8-positive CTLs in genital mucosa following sublingual immunization with OVA and CT	[44]
Hcβtre (from Botulinum neurotoxin A)	Ad2F (25 μg) CT (2 μg)	Mouse	25 μg (5×)	L *7 μl*	+	- 100% protection against BoNT/A intoxication for Hcβtre-Ad2F + CT-immunized mice - ~60% protection against BoNT/A intoxication for Hcβtre + CT-immunized mice	[59]

Abbreviations: Abs: *antibodies*; *ASC*: *antibody secreting cells*; *CTL*: *cytotoxic T cell*; *HA*: *hemagglutinin*; *HIV*: *human immunodeficiency virus*; *(m)CT*: *(mutant) cholera toxin*; *CTA/B, A/B subunit of CT*; *(m)LT*: *(mutant) heat labile toxin*; *LTB, B subunit of LT*; *dmLT*, *double mutant LT*; *MPLA*: *monophosphoryl lipid A*; *rAd*: *recombinant adenoviral vector*; rEA: recombinant *Eimeria tenella*; *RSV*: *respiratory syncytial virus*; *SARS*: *severe acute respiratory syndrome-associated coronavirus*; *SIV*: *simian immunodeficiency virus*; *WIV*: *whole inactivated virus*.

[1] Influenza virus lacking the Nonstructural Protein 1.

[2] Vaccine based on helper-dependent adenoviral vector expressing the soluble fusion glycoprotein of RSV.

[3] Subunit mutant of cholera toxin (CT) E112K with the pentameric B subunit of LT.

[4] Mucosal adjuvant, K63 mutant of LT.

[5] Mucosal adjuvant, (3′,5′)-cyclic dimeric guanylic acid.

The adjuvant effect of sublingually administered CT has been documented with a number of antigens, including influenza [43] and HIV [48] subunit vaccines, *Salmonella* proteins [50,51] and *Helicobacter pylori* lysates [54,55] with the latter two as undefined vaccines. LT, when co-administered sublingually with tetanus toxoid (TT) induced higher specific IgG and mucosal IgA antibody titers when compared to TT alone [57]. However, CT and LT cause severe diarrhea in humans and are involved in the clinical occurrences of cholera and enterotoxigenic *E. coli* enteritis, and therefore not suitable as an oral or sublingual adjuvant for human use [61–63]. Since enterotoxicity is mainly caused by the enzymatically active A-subunit, mutated enterotoxins with reduced toxicity but retained adjuvant properties, have been developed. LT(R192G), also named mLT, showed reduced toxicity in mice, but maintained its adjuvanticity to a level nearly equivalent to that of LT [64,65]. Building on this mutant, a double mutant of LT, R192G/L211A or dmLT, showed adjuvanticity for a co-administered antigen equivalent to mLT upon oral administration (gastro-intestinal delivery) [66]. Recently, dmLT has been evaluated as an adjuvant for sublingual and buccal vaccination with a whole-cell pneumococcal vaccine that induces protection in mice [52]. In the search for an alternative adjuvant for a *H. pylori* lysate vaccine, dmLT was compared to CT. Earlier studies have shown that a strong mucosal adjuvant like CT was needed to induce protective immune responses against *H. pylori* infection. Sublingual immunization with *H. pylori* lysate and dmLT significantly decreased the bacterial burden after *H. pylori* infection compared to unimmunized mice and to the same extent as when using CT as adjuvant [55]. Moreover, cellular immune responses that are known to correlate with protection were also fully comparable when using dmLT and CT as adjuvants.

In a study by Cho et al., HPV16L1 protein provided both vaginal and salivary sIgA, and serum IgG responses after sublingual administration in mice (150 μg) [45]. Several adjuvants were tested, including the B subunit of cholera toxin (CTB), three toll-like receptor agonists (*i.e.*, Poly(I:C), MPL, imiquimod), three nucleotide-binding oligomerization-domain agonists (L18-MDP, murabutide, PGN), vitamin D3 and poly-gamma-glutamic acid. Among the adjuvants tested, only CTB provided improved mucosal sIgA and systemic IgG induction. Sublingually applied CTB also enhanced the production of IL-4 and IFN-y by stimulated $CD4^+$ T cells from the spleen, as well as the number of IFN-y producing $CD8^+$ T cells that were isolated from the spleen or submandibular lymph node (SMLN). The other adjuvants had no effect on the immune response when compared to the unadjuvanted control [45].

3.3.1.2. Toll-like receptor (TLR) ligands. Despite the negative results mentioned above, TLR agonists can significantly improve immune responses after sublingual vaccination. A mouse study evaluating TLR agonists (*i.e.*, FSL-1, Poly(I:C), monophosphoryl lipid A (MPLA), Pam3CSK4, R848, cytosine–phosphate–guanosine (CpG)) in different mucosal routes using HIV gp140 and a tetanus toxoid revealed clear differences in immunogenicity [49]. MPLA, a TLR-4 agonist, suppressed systemic responses when administered sublingually, while the responses were enhanced after intranasal or subcutaneous immunization. CpG, a TLR-9 ligand, evoked enhanced immune responses upon sublingual and intranasal immunization whereas it did not affect the responses after subcutaneous immunization [49]. Another study revealed that sublingual immunization with an Ad5 vector expressing a TLR agonist derived from *Eimeriatenella* significantly activated NK cells, natural killer T (NKT) cells, B cells, and $CD4^+$ T cells in the spleen. In addition, the number of cells expressing MHC-II increased [30].

Bacterial DNA or synthetic oligodeoxynucleotides (ODNs) containing CpG motifs act as mucosal adjuvants. These TLR 9 ligands induced strong Th1 responses in mice after sublingual delivery of a *Salmonella* vaccine [67].

3.3.2. Delivery systems

Virus-like particles (VLPs) and virosomes have been evaluated for sublingual delivery. Cho et al. showed that sublingual delivery of HPV16L1 VLPs in mice induced systemic IgG and mucosal sIgA responses that were similar to the intranasal route, but significantly higher compared to other delivery (intravaginal, transdermal, intramuscular) routes [45]. In contrast to this study in mice, a clinical trial by Huo et al. with Gardasil® (Sanofi Pasteur), which contains the same L1-based VLPs from HPV but is co-administered with aluminum hydroxyphosphate as an adjuvant, showed that sublingual immunization is much less effective than intramuscular immunization [68]. However, this alum adjuvanted VLP formulation is not suitable for sublingual HPV vaccination, which is likely due to the fact that alum adjuvants consist of relatively large (micrometer range) particles that are probably poorly taken up by mucosal epithelial cells. Other adjuvants as well as improved dosage forms (see Section 4) may help to increase the immune response to the VLPs.

A sublingual vaccine containing influenza H5N1 virosomes (2 μg HA) in combination with the mucosal adjuvant (3′,5′)-cyclic diguanylic acid (c-di-GMP) effectively induced local and systemic H5N1-specific humoral and cellular immune responses in mice. The systemic IgG and nasal sIgA antibody levels were lower than those induced by intranasal administration, but the IgG levels were comparable to those obtained after intramuscular administration, whereas nasal sIgA levels were higher than the levels upon intramuscular administration [42].

4. Dosage forms

Optimized dosage forms may improve the performance of sublingual and buccal vaccines substantially. Several dosage forms exist for sublingual and buccal delivery of marketed drugs. These range from droplets, sprays, and orally disintegrating tablets to oral films. However, only a few dosage forms have been used to explore sublingual or buccal delivery of vaccines. Almost all sublingual vaccination studies reported here have been performed by the simple application of droplets of a vaccine under the tongue. There are no studies on the role of potentially crucial variables, like contact time, vaccine viscosity and antigen release kinetics on immunogenicity.

In pre-clinical studies, vaccine droplets are applied under the tongue on the floor of the mouth and the animal is kept under sedation for less than 1 h to allow the vaccine to be taken up (see Table 2). For sublingual vaccination of mice 5 μl can be applied sublingually without transfer of the vaccine to the stomach (including a sedation time of 30 min) [48,57].

Sublingual administration of allergens to humans by droplets has been used for years in Europe in sublingual immune therapy (SLIT) against allergies. Typically, in SLIT, droplets of a highly viscous glycerol formulation containing the allergen extract are applied under the tongue. The high viscosity contributes to antigen retention under the tongue and as such facilitates the efficacy of the immune therapy.

In a recent clinical trial, HPV vaccine was applied sublingually to humans. Compared to SLIT with glycerol droplets, a relatively complicated administration protocol was used in this study. In brief, subjects rinsed their mouths with water, the sublingual area was dried and then 0.5 ml of vaccine was applied on the floor of the mouth. Adsorbent pads were applied in the mouth to absorb the saliva during and after the vaccine application. Despite the elaborate administration protocol, in only three of the twelve subjects were virus-neutralizing antibodies induced in serum after three standard doses of HPV vaccine *via* the sublingual route. These neutralizing antibody titers were still 1000-fold lower than in the intramuscular group. Researchers concluded that alternative delivery systems and adjuvants would be required to enhance and evaluate immune responses following sublingual immunization in humans [68].

The disappointing results of this clinical trial may be related to several factors. First, a significant amount of solution (0.5 ml *versus* 'a couple of droplets' in SLIT therapy) was applied. Despite the extensive administration protocol, a certain high volume will easily be digested and will follow the gastro-intestinal route. Secondly, the vaccine used in the study (Gardasil®) is a VLP based vaccine containing aluminum

hydroxyphosphate sulfate as adjuvant. An alum-adsorbed vaccine has a large particle size (in the range of 1 μm) and thus is not ideal for transfer over membranes. Finally, alum is not known to be a good adjuvant for mucosal vaccination.

Droplets for improved (extended) mucosal retention are currently under development by PATH [69]. Their technology is based on a thermo-responsive gel of undisclosed composition. The vaccine is presented as a liquid solution at room temperature, which enables sublingual delivery with an oral dropper, and transforms into a gel upon contact with the oral mucosa. Typical thermo-responsive gel formulations that gelate at a temperature above 30 °C are based on polymers, such as poloxamer and mucoadhesive polysaccharides. The gel matrix enables adherence (retention >20 min.) to the sublingual mucosa thereby preventing rapid clearance caused by salivation or swallowing and protecting the vaccine antigen from degradation caused by salivary enzymes. Vaccinating mice with a gel formulation containing tetanus toxoid elicits high levels of IgG and IgA in serum as well as in secretions of the mouth, gastrointestinal and reproductive tract. Currently, PATH is evaluating (pre-clinically) whether the addition of a mucosal adjuvant, dmLT, can contribute to the efficacy of sublingual vaccination using thermo-responsive gel formulations [69].

4.1. Sprays

Sublingual sprays have been used for years for the sublingual administration of nitroglycerine to patients in order to counter an acute angina pectoris. The most used nitroglycerine sprays are based on a formulation containing the drug substance, ethanol, a small amount of mint oil and a propellant, like tetrafluoroethane, to aerosolize the formulation. We are not aware of any investigations into direct spraying of the vaccine onto the sublingual or buccal mucosa.

A few studies, however, have applied vaccine sprays into the whole mouth. Immunization of humans with influenza whole inactivated virus (H1N1) without additives, such as an oral spray using a simple nasal spraying device (no use of propellant), did not result in IgA antibody responses in nasal secretions and only resulted in marginally increased IgA antibodies in saliva. Although no stimulation of cytotoxic T cells was shown, an increase in systemic influenza-specific antibodies was found [70].

To what extent the immune response elicited by sublingual vaccination is the result of direct immune activation of the tonsils remains a matter of debate. For example, it has been shown that direct immunization *via* the tonsils can give rise to adequate immune responses. Oral spray immunization with replication-deficient viral vector vaccines encoding simian immunodeficiency virus (SIV) sprayed directly on the tonsils of rhesus macaques induced cellular and humoral immune responses. Additionally, after SIV challenge viral RNA levels were equally reduced after systemic vaccination and vaccination through the use of the oral mucosal spray [139].

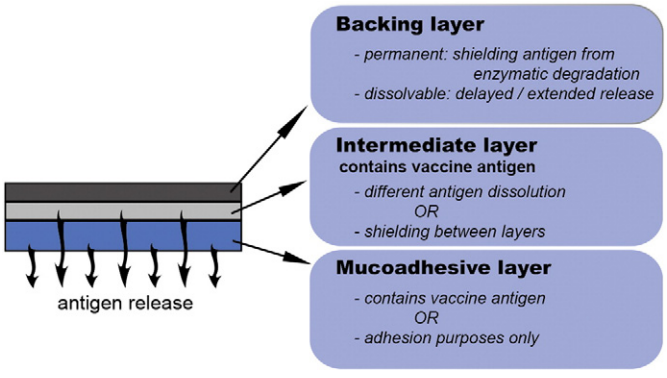

Fig. 3. Multi-layered films or tablets. The layers of multi-layered dosage forms have different functions.

4.2. Controlled release formulations

Several oral formulations are already licensed or are in development for drug delivery in the mouth. Based on their drug release kinetics and manufacturing method they can be categorized as: I) orally disintegrating preparations (tablets and fast-dissolving films) and II) extended (slow) release tablets and films. Although their value for sublingual and buccal delivery of antigens has not yet been extensively evaluated, they will be described briefly in the next section since they may play an important role in the development of potent and thermo-stable sublingual vaccines.

4.2.1. Orally disintegrating preparations

Orally disintegrating tablets for sublingual or buccal delivery are in general relatively small and porous. Tablets for buccal delivery facilitate fast disintegration and drug or vaccine release without inclusion of a disintegrant. In general, these tablets dissolve within a few seconds after being placed in the mouth without water, making them dried (stable) alternatives for droplets.

Recently, several excipients were studied for their wafer formation abilities (highly porous tablets made by direct compression) with the intention to formulate wafers containing HPV vaccine for sublingual vaccination [71]. A typical fast-disintegrating formulation for the generation of wafers with a diameter of 4 mm that showed a dissolution time of less than 25 s (in 3 ml at 37 °C) consisted of approximately 65% (w/w) myo-inositol (the placebo HPV powder) and 35% microcrystalline cellulose (MCC) [71].

4.2.2. Extended release films and tablets

To effectively vaccinate *via* the sublingual and buccal mucosae, the contact time of the antigen with the mucosa is expected to be critical, although this remains to be demonstrated. Sustained release dosage forms adhere to the mucosa and direct transport of the antigen to the mucosa may improve the efficacy of sublingual and buccal vaccination. In this regard, extended release films or tablets that consist of multiple layers with different functions (Fig. 3) may be applied. The sublingual mucosa is exposed to a high saliva flow, which might be advantageous for a better swelling and dissolution of oral tablets. On the other hand, it might also cause loss of the antigen and adjuvant due to salivary washout. Therefore, the much lower saliva flow of buccal mucosa makes this route probably a better target for a sustained release system.

Extended release tablets are typically based on gelling hydrophilic polymers, so-called hydrophilic matrix tablets. The polymers form a gel layer around the tablet when they make contact with water. The release profile is controlled by the overall swelling and erosion [72]. Most polymers that are used as mucoadhesives are hydrophilic polymers that gelate upon contact with saliva, allowing adherence to the buccal mucosa by interfacial forces, such as hydrogen bonding, electrostatic interaction as well as van der Waal's bonds.

Borde et al. [73] prepared extended release tablets for the model antigen ovalbumin, based on two-layer tablets consisting of a mucoadhesive layer, which is composed of carbopol, and a controlled release layer. Since the tablets were not able to adhere to the floor of the mouth in mice, the tablets were applied upside down to the ventral side of the tongue, resulting in the release of the antigen toward the sublingual region. Immunization studies were performed with tablets containing 250 μg ovalbumin directly followed by sublingual administration of a 1 μg/ml CT solution (7.5 μl) as adjuvant. Immunization with a fast-releasing tablet was favorable over extended release formulations and comparable to the liquid reference (ovalbumin with CT) for the immune response. However, the production of saliva was induced by administering pilocarpine subcutaneously before administering the tablet to provide better swelling and adhesion of the tablets [73]. The authors concluded that extended release formulations for sublingual vaccination have to be investigated more in detail. Extended release formulations for

mice have to be optimized to direct the antigen toward the ventral sublingual mucosa. This will improve the antigen diffusion and prevent the salivary washout and the impact of enzymes by shielding.

Extended release films based on multiple layers are generally made by the solvent casting method [74] for which the antigen is added to a coating mass (*e.g.*, cellulose, polysaccharides) and subsequently cast onto a film application apparatus. After drying and solvent evaporation, the films can be cut into single doses.

The viscosity and density of the coating mass are critical for successful film casting (layer-by-layer coating). Moreover, the vaccine antigen needs to be formulated in the coating mass with proper excipients to guarantee resistance to the applied drying stresses [75]. Formulations that have been proven to protect vaccines during the fabrication of coated solid dosage forms have been described in literature but are not yet used to produce vaccine-containing films. For example, the group of Prausnitz applied formulations based on trehalose, surfactant (poloxamer) and a polymer (PVP) to coat microneedles with vaccines such as the whole inactivated influenza virus [76] and measles [77] for dermal vaccination. An additional advantage of dried formulations is the improved thermal stability during storage.

5. Perspectives

The oral mucosae, in particular the sublingual and buccal regions are attractive sites for the delivery of antigens, since their accessibility, non-invasive and (immunological) advantages over other (mucosal) routes (Section 2.1). Therefore, sublingual vaccine delivery has gained significant attention during the past few years, as shown by the numerous pre-clinical studies published in the last decade (Table 2).

The preclinical proof of concept of the sublingual route for vaccine delivery has been proven for several antigens. In general, high doses of antigen (10–500 μg) are administered in mice using a multiple dosing regimen (Table 2). However, well-designed studies including proper dose response and tracking (PK/PD) studies are still lacking. Nevertheless, the use of strong mucosal adjuvants seems to be necessary to induce protective immune responses upon sublingual vaccination using inactivated vaccines. Reported studies on screening and comparison of adjuvants for sublingual vaccination, unfortunately, are still limited. Of the adjuvants investigated thus far (CT, CTB, LT, LTB, mLT, dmLT, CpG, c-di-GMP, L18-MDP, FSL-1, Poly(I:C), MPLA, Pam3CSK4, R848, murabutide, peptidoglycans, vitamin D3, chitosan and poly-gamma-glutamic acid), enterotoxins (and their mutants) and TLR-9 agonists resulted in the highest antibody responses whereas the other adjuvants did not improve immune responses upon sublingual delivery. The most used adjuvants for sublingual vaccine delivery are the enterotoxins CT and LT, which are considered too toxic for human use. However, genetically defined mutants of these toxins, which have reduced or minimal toxicity, seemed to be promising adjuvants to augment both systemic and mucosal immunity in response to sublingual administration.

Recombinant live carriers expressing the vaccine antigen seemed to elicit protective immunity by inducing both antigen-specific systemic as well as mucosal antibodies at distinct mucosal sites [28,32]. The exact advantage of such live vaccines over inactivated vaccines is still not clear, but it could be related to factors such as receptor-mediated uptake, intracellular replication of antigens and co-delivery of immune potentiators and antigens to APCs.

An approach that resembles the characteristics of the live pathogens, *e.g.*, by co-delivery of immune potentiator and antigen, is needed to develop an effective inactivated (subunit) vaccine for the sublingual route. Unfortunately, to date, limited studies have focused on such approaches that use tailored formulations, such as nanoparticles or conjugated antigen–adjuvant formulations. In the design of these formulations, mucoadhesive or receptor-binding properties may be built in to increase interaction with the oral mucosa and thereby facilitate antigen uptake.

In addition to delivery systems, dosage forms may improve the efficacy of sublingual and buccal vaccines. Today, research on dosage form optimization for sublingual or buccal vaccination appears marginal. The few reported studies use mouse models to evaluate sublingual vaccination *in vivo*. Nevertheless, the small space under the tongue of animals used for preclinical studies makes testing of sublingual solid dosage forms challenging. For example, Muragappan et al. faced the problem that sublingual tablets were not able to dissolve under the tongue of mice [40]. Moreover, two-layered extended release tablets failed to adhere to the floor of the mouth in mice, resulting in a suboptimal administration that applied the tablet upside down on the ventral side of the tongue [73]. Potential problems related to the sublingual application of slow release formulations are due to the lack of an expanse of smooth or (relatively) immobile mucosa, which make it difficult to keep the dosage form in contact with the sublingual mucosa. Other dosage forms, such as wafers, films and thermo-responsive gels, should be explored as these may likely increase the potential of sublingual vaccine delivery. Sustained dosage forms (*e.g.*, multi-layered oral films or tablets) are probably better suited for application on the buccal mucosa, which is considerably less permeable than the sublingual mucosa. The few current studies on buccal vaccine delivery use buccal injections instead of a topical application. However, the development of the extended release formulations would make topical buccal cheek vaccination easier. Compared to the sublingual route, buccal administration in small animals, such as mice, is more complicated since the buccal compartment has a minimal anatomical barrier with the sublingual compartment. Besides the animal size, the anatomical features of the oral cavity should be taken into account, because of the specialized histological characteristics and immunological competences of the oral mucosae in humans. However, most rodents, such as mice, rats and hamsters, have buccal mucosa that contains keratinized epithelium, in contrast to non-keratinized epithelium of sublingual and cheek mucosae in human. For buccal cheek administration, other animal models such as rabbits, dogs and pigs are more appropriate since they contain non-keratinized buccal mucosae. Moreover, the thickness of the buccal mucosa in these animal models (rabbit, 600 μm; dog and pig, 770 μm) is comparable to that of humans (500–800 μm) [78]. Current literature does not address to which extent mucoadhesive formulations increase the delivery of the antigen to oral mucosal APCs after sublingual or buccal administration, requiring proper tracking (pharmacokinetics, PK) studies in relation with immunological outcome (pharmacodynamics, PD) in adequate animal models (Table 3).

To select suitable formulations and dosage forms, an *in vitro* model for the mucosa that can predict the transport of an antigen might be useful. Porcine buccal mucosa is often chosen for *in vitro* studies on buccal delivery of medicines because of its close resemblance to human buccal mucosa with respect to structure, enzyme activity as well as permeability characteristics [79]. However, mechanistic studies evaluating the conditions for sublingual or buccal antigen delivery, such as optimal contact time of the dosage form and the differences between the oral mucosal routes, are lacking in literature. Moreover, the importance of specific molecular features of the antigen and/or antigen formulation, such as size (*e.g.*, 1–10 nm proteins *versus* 10–250 nm viruses *versus* 250 nm–2 μm bacteria), surface charge or specific receptor ligands, is still not investigated systematically.

Most pre-clinical studies (*in vivo* animal models) are used to evaluate the vaccine candidate's efficiency to induce protective mucosal (and systemic) immunity. Secretory IgA (sIgA) provides antigen-specific immune protection in mucosal tissues. As a result, most preclinical studies on sublingual vaccination include the detection of sIgA in mucosal secretions. Unfortunately, most studies are designed for an optimal readout of systemic immune responses, making assumptions on mucosal immunity not fully conclusive. Moreover, in general, the presence of sIgA is determined in saliva, which is not the best readout for mucosal immunity, especially for rodents [80]. A more predictive method to measure mucosal immunity in mucosal tissues of rodents is

Table 3
Current challenges in the development of sublingual and buccal vaccines.

Challenges	Research should focus on:
Get more insight into pharmacokinetics and pharmacodynamics (PK/PD)	- Dose response studies Dose-sparing possibilities Multiple dose regime needed? Potent and safe adjuvants - *In vivo* imaging (tracking) studies
Get more insight into immunological mechanisms	- Role of oral DCs upon buccal/sublingual immunization - Antigen uptake and transport - Interactions of antigen and immune cells - Use of proper animal models - Readouts for mucosal immunity
Development optimized vaccine formulations and proper dosage forms	- Tailored vaccine delivery Oral DCs as vaccine target - Stable vaccine formulations - Optimal contact time (mucoadhesive) - Optimal antigen release time - Use of proper animal models
Development of predictive assays for mucosal immunity	- Optimal sampling for mucosal readout - Validated assays for (pre-)clinical studies

the 'Perfext method', which is a direct method for quantitative assessment of *in vivo* antibody or cytokine production at the local level [81]. The method uses the collection of heparinized mucosal tissue of the animal followed by detergent treatment and the detection of antigen-specific antibodies by ELISA [53,55].

Based on several pre-clinical studies, it would be highly interesting to follow the expected upcoming clinical studies on sublingual vaccine delivery (using live attenuated vaccines). These studies should build on the experience of previous preclinical studies that were designed to evaluate mucosal immunity as a correlate of protection, which would require standardized and validated assays predictive of mucosal immune protection [82]. For example, a method described by Saletti et al. allows measurements of both systemic and mucosal antibody responses to vaccines by detecting antigen-specific plasmablasts with a specific mucosal pedigree (*e.g.*, $\alpha4\beta7$, CCR10) on small amounts of whole blood [83]. Although it was developed for clinical samples, a certain type of mucosal readout would also be useful for pre-clinical studies, amid limited blood volumes that can be sequentially withdrawn from small animals. The full benefits of sublingual vaccination can only be revealed by the determination of both local and systemic responses.

Current literature on sublingual and buccal vaccination with a broad range of antigens provides a strong base for further testing of these non-invasive vaccine delivery routes. This testing should include mechanistic studies on the superiority of live vaccines over inactivated antigens, including dose–response studies (Table 3). Based on the findings, tailored vaccine formulations for inactivated antigens may be designed in the near future. Development of these sophisticated formulations and optimized dosage forms that facilitate antigen uptake by the oral mucosa will be an important step forward toward successful sublingual and/or buccal vaccination. Finally, upcoming clinical studies that confirm the suggested safety and efficiency (by proper readout of both mucosal and systemic immunity) may result in the first approved sublingual vaccination strategy.

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