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REVIEW ARTICLE

Veterinary vaccine nanotechnology: pulmonary and nasal delivery in livestock animals

Daniella Calderon-Nieva[1] · **Kalhari Bandara Goonewardene**[2] · **Susantha Gomis**[2] · **Marianna Foldvari**[1]

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Abstract Veterinary vaccine development has several similarities with human vaccine development to improve the overall health and well-being of species. However, veterinary goals lean more toward feasible large-scale administration methods and low cost to high benefit immunization. Since the respiratory mucosa is easily accessible and most infectious agents begin their infection cycle at the mucosa, immunization through the respiratory route has been a highly attractive vaccine delivery strategy against infectious diseases. Additionally, vaccines administered via the respiratory mucosa could lower costs by removing the need of trained medical personnel, and lowering doses yet achieving similar or increased immune stimulation. The respiratory route often brings challenges in antigen delivery efficiency with enough potency to induce immunity. Nanoparticle (NP) technology has been shown to enhance immune activation by producing higher antibody titers and protection. Although specific mechanisms between NPs and biological membranes are still under investigation, physical parameters such as particle size and shape, as well as biological tissue distribution including mucociliary clearance influence the protection and delivery of antigens to the site of action and uptake by target cells. For respiratory delivery, various biomaterials such as mucoadhesive polymers, lipids, and polysaccharides have shown enhanced antibody production or protection in comparison to antigen alone. This review presents promising NPs administered via the nasal or pulmonary routes for veterinary applications specifically focusing on livestock animals including poultry.

Keywords Pulmonary vaccine delivery · Nasal vaccine delivery · Aerosol · Spray vaccine · Livestock · Chickens

Introduction

Vaccination is a powerful tool for the prevention and control of infectious diseases [1]. In humans, vaccination has made the eradication of small pox possible, with polio soon to follow [1]. Despite these tremendous advances in human health intervention, several infectious diseases are still high burdens for the global economy and public health [2]. Zoonoses accounts for 60% of all infectious human pathogens that have a possibility to cause pandemics [3]. The farm/livestock industry is a major source of zoonotic potential where animals are in constant close proximity providing greater opportunity for viral mutation, or bacterial gene transfer which can be transferred directly to humans after consumption. Perhaps one of the most feared zoonotic infectious diseases is avian influenza, which could be prevented quickly, and specifically, if a universal synthetic vaccine was available [4].

As such, not only does veterinary vaccination in livestock aim to prevent and control animal diseases, it also aims to prevent disease in food animals to avoid zoonosis or infection in human consumers and improve the efficiency of production of food animals [5]. For example, by replacing drug therapies in food animals with vaccination, environmental build up and residue in food animal products can be reduced [5]. However, due to the large-scale nature of food-producing facilities, cost-

✉ Marianna Foldvari
foldvari@uwaterloo.ca

[1] School of Pharmacy, Waterloo Institute of Nanotechnology and Center for Bioengineering and Biotechnology, University of Waterloo, 200 University Avenue West, Waterloo, ON N2L 3G1, Canada

[2] Department of Veterinary Pathology, Western College of Veterinary Medicine, University of Saskatchewan, Saskatoon, SK S7N 5B4, Canada

effectiveness is also a major consideration. Non-economical vaccines will not likely be widely adopted if cheaper alternative treatments are available [5]. While human vaccination also aims for cost-effective vaccines, individual health and well-being is a stronger consideration for compliance.

Current licensed vaccines in both livestock and humans are derived from live, modified, attenuated, or killed vaccines [6, 7]. Unfortunately, attenuation is an expensive long process. Live vaccines also have the potential to revert to virulence and are not recommended for the immune-compromised [8]. Another drawback of vaccines is that they likely require an adjuvant and must be administered by needle, which requires trained personnel and proper disposal [9]. Some vaccinations even require multiple doses of vaccine to induce a sufficient immune response against the agent [4].

On the other hand, needle-free nasal vaccination and pulmonary vaccination is attractive because of easy access, the high vascularity and permeability, and limited metabolism in the nasal cavity [10]. This could be of great importance in the livestock industry where administration of a large number of vaccinations could be limited by the availability of the number of trained personnel. Additionally, needle-free vaccination is significant in terms of safety due to both decreased risk of contamination from infected needles and potential irritation from injection [11, 12]. In fact, the pulmonary route of vaccination has been around since the 1950s during the development of an aerosol Newcastle disease vaccine in chickens, which is now widely used [13, 14]. In ruminants, aside from averting first pass metabolism and the rumen, the respiratory mucosal surfaces of an organism not only have the potential to initiate immunity at the local site of administration but also systemically due to the close proximity of the blood-lung barrier [15]. There is already evidence that immunization via the respiratory tract not only produces high local immune responses [11, 16] but also provides high systemic mucosal immunity in mice and non-human primates [11, 17]. This is especially important as many infectious diseases such as Influenza, *Escherichia coli* (*E. coli*), and *Mycobacterium tuberculosis* (*MTb*) are able to initiate their infectious process at mucosal surfaces [15].

In practice, both pulmonary and nasal deliveries have highlighted biological challenges that can prevent the proper delivery of vaccine to the lung. In mammals, particles delivered via the nasal or pulmonary route can be lost to the oropharynx because of the turbulent air flow and continuous branching and narrowing of the airways [18, 19]. However, synchronic inhalation seems to improve loss by bypassing the esophagus [18]. Additionally, the mucociliary blanket in the upper airways and the nasal cavity is designed to constantly clear particles [10, 18, 20]. While there are some recognized anatomical differences between large livestock animals and a complete anatomical dissimilarity with the avian lung (poultry), the mucociliary blanket is present in all of these species. The loss of particles delivered to the target site via inhalation in the air sacs of the avian system is also a concern despite their unidirectional air flow [21].

Even if particles are able to bypass mucociliary clearance barriers, the lower respiratory passageways are also not lacking in clearance mechanisms. Alveolar components and lysozymes can break down products near the blood-epithelial barrier in mammals [18]. Although the presence of immune cells in the lung is favorable to vaccine applications, the formation of tolerance or rapid clearance of a particle via innate immunity could hinder immune activation [18].

In order to improve vaccine potency and achieve needle-free delivery, nanotechnology has been incorporated into vaccine research [1]. More specifically, delivery of nanoparticle vaccines via the nasal or pulmonary (inhalable) route has become highly attractive. While most studies have found that mucosal delivery of the antigens alone using the pulmonary route is not efficient enough, nanoparticle (NP) systems have been found to greatly improve delivery through the mucosa of the pulmonary system in humans and show potential in veterinary medicine as well [15, 22–24]. NPs are defined as structures with at least one dimension in the range of 1–100 nm that have been widely applied to drug delivery [1]. In vaccine delivery, "nano" platforms have mainly focused on developing delivery vehicles for vaccine antigens, but some materials such as the biopolymer chitosan have shown vaccine adjuvant properties [25, 26]. These systems are advantageous, since they have the potential for limited adverse side effects, better stability, and may also stimulate the immune response enough so that adjuvants or repeated administration is not necessary [4]. Additionally, more sophisticated designs to incorporate selective targeting by ligand attachment or co-delivery of several antigenic components have been emerging.

The application of nanotechnology in veterinary vaccination is still in early stages. Some of the knowledge in this regard is available from small animal models used for human vaccine development. In fact, nanotechnology has been adapted to enhance the performance of the delivery of therapeutics in several areas like lung cancer and cystic fibrosis. Combined with nanotechnology, needle-free mucosal immunization can ease vaccination in the food production and livestock industry while ensuring sufficient protection against diseases which could cause serious economic losses on the farm. Several NP delivery vehicles have already been tested in livestock veterinary vaccine development in order to achieve needle-free vaccination for mass immunization [7, 15, 22, 24, 27–29].

The advantages of vaccination via the pulmonary route and the feasibility of implementing such vaccination methods out in the field will be explored in this review. Additionally, the research and application of nanotechnology for inhalation or nasal vaccine developments in livestock, and especially poultry, will be discussed as an important aspect of protection for animals in the food chain and link to human safety.

Availability of devices for vaccine delivery via inhalation or nasal delivery and mass administration

Mucosal drug administration via the pulmonary route has been well established in humans for a long period of time for respiratory diseases such as asthma and chronic obstructive pulmonary disease (COPD) [30]. In fact, nebulizers and dry powder inhalers are standard aerosol devices designed to administer drugs via inhalation in humans [31]. Specific aerosol devices for drug delivery to the lung in veterinary species have not been described in livestock, but metered dose inhalers for companion animals do exist including the AeroKat™ for cats, AeroDawg™ for dogs, and the AeroHippus™ for equine species (Trudell Medical International©). The delivery of aerosol therapeutics may be more difficult in animals, as one cannot teach them to take controlled breaths when using inhalers or nebulizers [32]. On the other hand, nasal administration may be a better option for larger animals.

Inhaler or nasal devices specific to vaccine administration have not been developed. However, nasal or inhalable vaccines are attractive in humans for needle fearing individuals and children. Furthermore, inhalable or nasal vaccines are attractive strategies for mass immunization in livestock and humans. Depending on farm size, animal handling for vaccine administration could add to the already labor intensive nature of the food production industry [33].

Vaccine administration via intramuscular or subcutaneous injection is still the standard today even though an intranasal (i.n.) vaccine against bovine respiratory disease (PMH®IN) released by Merck in 2014 exists for cattle, and spray vaccination also exists in the poultry industry [7]. Especially, administration via a parenteral route ensures high bioavailability and drug absorption that can be accurately predicted, in contrast to nasal or inhalation administration where absorption at the systemic level or amount lost at the oropharynx is not easily measured. As a result, veterinary syringes are designed to administer repeat-injections to aid farmers in administering multiple dosages of a vaccine without having to draw the vaccine formulation into the syringe each time prior to vaccination of the animal (Allflex©).

Among needle-free delivery devices for livestock, there are controlled release devices available for oral administration which are made of nylon or permeable materials [34]. The oral devices filled with drug can either have high density or expand upon entering the rumen to avoid regurgitation and ensure long-term release of drug [34, 35]. Intravaginal devices similar to human intrauterine devices are also available mainly for hormonal, fertility, and anti-helminthic drugs, but not vaccine administration [34]. In the poultry sector, non-invasive approaches to vaccine administration seem to focus on oral or ophthalmic routes [35]. Drugs incorporated into skin tags and ear tags are also available [35]. Coarse spray vaccines in the poultry sector are designed for administration to the eye and upper respiratory tract, and these can be easily administered through automation at the hatchery [36].

The complications involved in the design of inhalable controlled release devices or products results from the variation in physiology of animal species. For example, in food-producing animals or livestock, there are two categories of species: the ruminants and the avian. Aside from the obvious differences that exist between the avian and mammalian respiratory system, interspecies differences also exist [33] (Fig. 1). The results are differences in rates of biotransformation, differences in breathing pattern, and tissue distributions [33]. The consequence of the species differences is that each vaccine delivery system proposed must be specifically designed for a particular species [35].

Additionally, the administration approach is not only dependent on the type of animal but also on their housing facilities. For instance, in poultry, aerosol administration may be practical due to the close proximity and smaller housing facilities. Additionally, their smaller size and unidirectional airflow through their lung may favor deposition of aerosol vaccines in their respiratory tract. For example, an inactivated influenza vaccine has been shown to induce protection against lethal influenza challenge in chickens [44]. However, an influenza vaccine for example may not be desirable environmentally as an aerosol due to its zoonotic potential. In ruminants, due to their large body structure and nature of housing, it may be more difficult to use inhalable sprays that achieve proper dosing. However, if devices were designed specifically for inhalation or direct intranasal application for felines, dogs, and horses, these might be incorporated into their upkeep. With all aspects considered, mucosal immunization could replace the hazardous potential of needle administration.

Current nanopharmaceuticals in the market

Research from small animal models and clinical trials have shown that NP carriers can enhance therapeutic and vaccine action in many routes of administration (subcutaneous, intravenous, inhalable, and intramuscular) [9, 20, 45, 46]. NP carriers are thought to protect the active substance from the physiological environment and aid the interaction between the active substance and its target. In fact, there are a variety of nanopharmaceuticals already available on the market [47]. The available nanopharmaceuticals are mainly used to encapsulate cancer drugs. However, there is one nanovaccine available in Switzerland for influenza. Other NP drugs carry antifungal and hormone replacement active ingredients. The approved NP pharmaceuticals are formulated from lipid, surfactant, polymer, metal materials, and even viral components with the ability to carry not only active molecules but proteins as well [47]. This encompasses the variety of NPs that can be

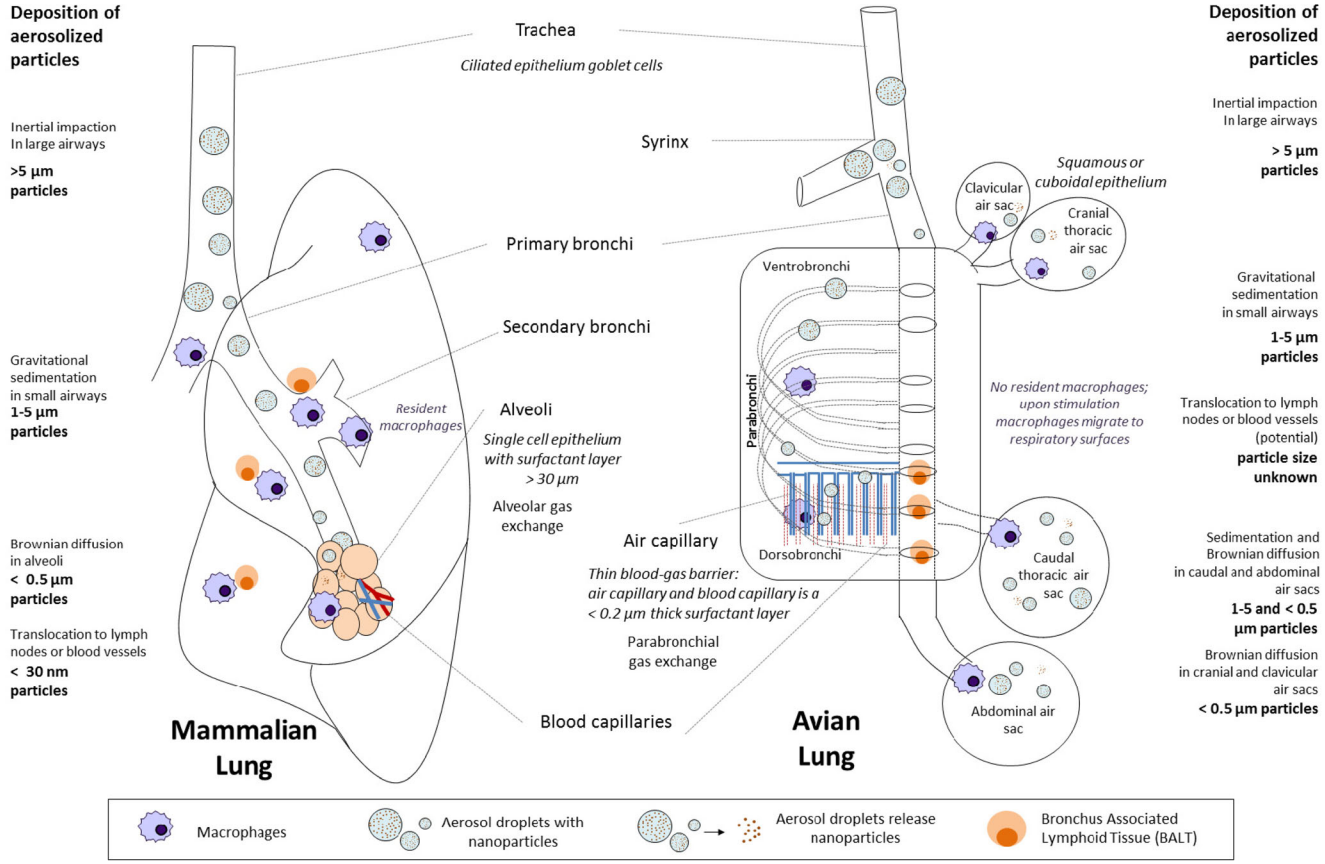

Fig. 1 Comparative schematic diagram of the anatomical and immunological features of mammalian and avian respiratory system with respect to vaccine administration. Also shown are the size-dependent deposition pattern of aerosol droplets and the relevance of novel vaccine design involving nanoparticle carriers for antigens and adjuvants within aerosol droplets. Inhalation of particles >5 µm results in inertial impaction in the large airways and are mainly cleared by mucociliary mechanisms in the trachea of both species which are swallowed. The main trend of deposition in both species is that smaller 1–5 µm particles have the ability to penetrate deeper into the lungs, either the alveoli or air capillary, i.e., the blood-air interface. In birds, aerosol particles of <1 µm have been shown to deposit in the cranial thoracic air sacs, and even smaller <0.1 µm particles can deposit in the caudal thoracic air sacs of birds, although the fate of these particles is not well known [37, 38, 39–42]. Unlike the mammalian lung, the avian lung lacks the constant surveillance of foreign particles by resident macrophages, but they are rapidly recruited upon stimulation. The presence of BALT at the junctions of the primary and caudal secondary bronchi in the avian lung may aid in immune stimulation and lymphocyte recruitment. In the mammalian lung, BALT forms after activation of the immune system, and the possibility of inducing tolerance over immune activation is a consequence of constant surveillance by lung macrophages present in the tissue and in the alveoli. Nanoparticles can be released from aerosol droplets in the alveoli and translocate to the lymph nodes and/or blood vessels. This was shown in mammalian (mouse) models [43]. Since in avian species, the blood-air interface epithelium is 60% thinner than the mammalian epithelium; it is anticipated that the delivery of nanoparticle vaccines to the systemic circulation is also possible

created and the versatility of applications and packages that they can hold.

Absent from this list are any approved particles designed for pulmonary or nasal administration. Although the use of human aerosol devices has improved to deliver greater amounts of dose to the lung, achieving systemic delivery is still suboptimal [31]. Yet, in terms of vaccine application, dosing is critical to proper immune stimulation. A suboptimal dose may induce tolerance or no immune stimulation at all. On the other hand, over dose could result in detrimental immune stimulation. Regardless, the design of NPs for drug, gene, protein, and vaccine delivery via the airways is currently an exciting research field.

Physical and biological parameters involved in aerosol delivery

The fate of particles entering the airways is dependent on three aerodynamic properties: impaction, sedimentation, and diffusion (Fig. 1). Whether or not a particle settles in the respiratory system by impaction, sedimentation, or diffusion depends on the particle size distribution generated by the delivery device and the type of breathing pattern during inhalation of the dose [31, 48]. Upon inhalation of a deep forceful breath, particles greater than 1 µm tend to impact as their higher density and momentum prevent it from changing direction if there is a change in airflow pattern. In the airways, these larger particles (3–6 µm) get trapped in the pharynx,

mouth, or the mucus of the trachea, which results in them being removed by swallowing [49, 50]. Upon slower air velocity or a slower breathing pattern, particles between 1 and 5 μm (NPs) in size tend to settle in the smaller airways and respiratory bronchioles by sedimentation (gravity), since their residence time within the lung increases [50]. Also, NPs have better chance of reaching the bronchioles and respiratory mucosa in the lower airways [48]. The smallest NPs less than 0.5 μm tend to deposit in the alveolar spaces resulting from Brownian motion [48, 50, 51]. Though these smaller particles tend to get exhaled but if less than 34 nm in size, they enter the blood stream and are cleared via renal filtration [52]. Since systemic immune activation is critical to initiating cell-mediated immune responses, targeting to the alveolar region at the interface of the blood-air boundary is highly desirable for a NP vaccine.

Since aerosols can be dry powders, liquid suspensions, or liquid solutions, the type of formulation is also important in the development of aerosol vaccines. The final vaccine formulation must be compatible with the device chosen to administer the vaccine. For example, if a multi-dosing inhaler device is used, the interaction of the formulation with the holding chamber must be considered to ensure consistent dosing after every administration [31]. If a nebulizer is chosen, the NP formulation designed must be a liquid to allow the output to generate small droplets. Furthermore, different types of nebulizers are only compatible with certain types of formulations. For instance, ultrasonic nebulizers which generate aerosol droplets using high energy soundwaves are ineffective in nebulizing more viscous solutions such as suspensions or liposomes [31]. But vibrating mesh or plate nebulizers which physically break up the liquid into smaller droplets work very efficiently for suspensions or liposomes [31].

Unlike the aerosol delivery to the lung, the nasal cavity is a lot smaller, and the aerodynamics does not play as large a role in deposition of particles. In nasal delivery, the goal of systemic vaccination is to reach the respiratory region. The respiratory region of the nasal cavity containing nasal turbinates have a high surface area and create turbulent air flow to allow better contact between the inhaled air and the mucosal surface. Nasal turbinates are in close proximity to blood vessels. Additionally, the mucosal-associated lymphoid tissue in the nose (nasal associated lymphoid tissue (NALT)) that is separated from the epithelial barrier containing the mucociliary blanket is the main target of mucosal vaccination in the nose.

The first physiological barrier in the nose is a mucociliary layer that clears entering particles which then go to the back of the throat and esophagus to get cleared by the digestive system [19]. Furthermore, enzymatic activity within the nasal cavity mucus is a concern to drug delivery [20]. Perhaps the most important factor that affects particle delivery in the nasal mucosa is actually membrane permeability. Large polar molecules do not pass through the epithelial cell membrane easily and must be accompanied by absorption enhancers such as bile salts and phospholipids to change the permeability of the epithelial cell layer [19].

Potential for enhanced pulmonary and nasal immune stimulation with various nanomaterials

In vaccine delivery, direct interaction between an adjuvant and an antigen presenting cell is critical to immune activation. Therefore, the interaction of NPs at the cellular level is very important to understanding mechanisms of NP adjuvanticity. Chitosan NP sizes around 400–1000 nm have been reported to elicit higher serum immunoglobulin A (IgA) levels than 3000 nm NPs [53, 54]. However, polylactic-co-glycolic acid (PLGA) NPs around 1000 nm have also been found to induce stronger serum immunoglobulin G (IgG) than 200 or 500 nm particles after i.n. immunization. At the cellular level NP size, surface charge, and surface morphology are known to influence the uptake and trafficking by pulmonary antigen presenting cells [55]. For example, it was found that 50 nm polystyrene particles are taken up by alveolar and non-alveolar macrophages, B cells and dendritic cells in the lung, but only by dendritic cells in the lung-draining lymph nodes (inguinal, mesenteric, and mediastinal) [56]. The surface charge of a particle can also influence type of cells recruited to the site of action. In fact, hydrogel rod-shaped cationic particles have been found to associate with dendritic cell subtypes while alveolar macrophages were found to preferentially take up negatively charged particles [55, 56].

Contradictory theories between the correlation of size and immune activation are likely due to the different particles that have been directly characterized for NP-adjuvant-cellular interactions in vitro. Additionally, orientation of the antigen within or on the surface of the particle could influence the mechanism of antigen presentation [54, 57]. Theoretically, nanoparticle drug therapies are thought to reduce dosing frequency due to the increased accumulation of drug per particle at specific sites [51]. Similarly, in vaccine delivery NPs can be made to carry several antigens at once. This is advantageous, since it more closely mimics real pathogens which stimulate the immune system through recognition of various antigens.

Further emerging advantages of NP vaccines involve cell specific targeting by antibody or small molecule conjugation to the surface of the particle [51, 58–62]. Particle functionalization and targeting toward certain environments, tissues, cells, and even intracellular components could greatly enhance the stimulation of the immune response and reduce clinical signs of disease. The NP systems that have been applied to vaccinology and also tested in food-producing veterinary species are discussed below.

Vaccine platforms against livestock and poultry diseases

While research on nasal or pulmonary vaccine delivery options for humans is quite extensive, for food animals and especially large animal livestock, delivery methods are much more limited (Table 1). Inhalable vaccine delivery is preferred in the chicken industry, whereas nasal vaccine delivery is more applied to ruminants in livestock. The following sections are focused on veterinary species with developments in the ruminant and poultry industry separately mentioned.

The poultry industry

The poultry industry mostly consists of turkeys, broiler chickens, and layer hens. Most studies of inhalable or nasal delivery focus on broiler chickens, although there are a few studies in turkeys and layer hens. As mentioned previously, broilers and layer hens are subject to intensive vaccination against many infectious diseases [7]. As a matter of fact, spray vaccination in poultry is standard against Newcastle disease virus (NDV) and infectious bronchitis virus. However, spray vaccination in this regard refers to 100–200 μm liquid particles which do not specifically target inhalation but also seem to induce immunity through ocular, oral, and nasal mucosas. There is a grey area in the definition of spray vaccination in the literature to whether a spray drier is used versus a liquid spray generator or a nebulizer. However, the commonality of the three devices is that they all generate aerosols in which inhalation plays a role in the generation of immunity via the pulmonary or nasal mucosa. In this regard, this paper will state whether a dry or liquid spray formulation was administered, and if mentioned, whether nebulization was used to generate the vaccine formulation.

The two major pathogens targeted for NP immunization are NDV and influenza, although, vaccination against *E. coli* and Salmonella have also been investigated using NP carriers [22, 63, 73–75]. Studies of microparticle inhalable vaccines or nasal vaccines do exist in poultry, although there are few studies comparing the two delivery routes directly or the performance of the microparticle versus nanoparticle formulations. The preliminary studies will be described below.

Nasal vaccination using NPs in chickens has been tested against NDV and influenza using chitosan [63], liposome [64], and liposome-polymer particles [29]. Polymeric chitosan particles have been an attractive NP vaccine platform because of biocompatibility, mucoadhesive, and permeating properties [18]. Additionally, chitosan itself is thought to have adjuvant-like properties which could enhance immune stimulation [76]. In a study comparing chitosan and calcium phosphate particles, it was shown that both particles carrying inactivated NDV produced high antibody titers in blood and mucosa [63]. However, the chitosan particles performed better than calcium phosphate particles against NDV lethal challenge [63]. It is of note that the protection study involved three immunizations prior to challenge, and no physical characterization of the particles was stated.

Liposomal carriers are among the most characterized in the nanotechnology field. Conventional liposomes are lipid structures formed by one or more bilayers of amphiphilic lipids, and they are thought to cross through epithelial barriers [20]. Liposomes are not immune-stimulatory themselves; however, they have been found to induce higher IgA and IgG titers after immunization [20]. The charge of the liposome based on lipid composition has also been found to be important after i.n. immunization [20]. Both positively and negatively charged liposomes have been reported to be immune-stimulating [20]. The effect of liposome surface charge has been tested in chickens in efforts to improve the antigenicity of formalin-inactivated NDV after i.n. immunization [64]. Three differentially charged liposomes composed of phosphatidylcholine (PC), phosphatidylserine (PS), and stearylamine (SA) were tested for their ability to elicit mucosal and systemic humoral responses. Interestingly, the neutral liposome made with PC induced the highest secretory IgA and systemic humoral responses and protection against challenge. The co-administration of LPS with the vaccine NP formulation further enhanced vaccine efficacy. The effectiveness of the PC liposome formulation was attributed to the fact that the transition temperature of the liposome is closer to the chicken body temperature than the others. Additionally, the head group was thought to play an important role in the recognition of APCs, but the mechanism is not known [64].

Since mucoadhesive polymers are thought to improve residence time in mucosal tissues, the addition of tremella or xanthan gum to liposome vaccine formulations containing inactivated influenza H5N3 were tested as i.n. vaccines [29]. The multilamillar mucoadhesive liposome vesicles induced higher immune response than the virus alone and liposome without the polymer. Additionally, the lower viscosity xanthan gum particle increased the efficiency of nasal vaccine delivery, which suggests that there may be a critical viscosity in which the formulation becomes too thick to effectively release the antigen to the nasal mucosal tissues despite the longer residence time in the nasal mucosa.

Aside from nasal NP vaccine delivery systems, a variety of studies have investigated nebulized or spray-dried vaccines in chickens. Both are inhalable formulations, but unlike nebulization that produces liquid inhalable particles, spray vaccines can involve transforming liquid to a dried inhalable powder. The final product is an inhalable dry spray. They are highly attractive for immunization via the lung, because they are stable and tend to be delivered efficiently [18]. In humans, spray vaccines against influenza and tuberculosis have been tested [77–80]. In fact, an inhalable dry powder measles

Table 1 Nasal and pulmonary nanoparticle and microparticle vaccines in development for livestock and poultry

Nanoparticle type	Composition	Antigen	Species	Delivery route	Efficacy	Ref
Polymeric	Poly(d, l -lactide-co-glycolide) (PLG) polyvinyl alcohol microparticle and 60% nanoparticle mix	*Toxoplasma gondii* Tachyzoite protein extract: SAG1 Cholera toxin (CT)	Ovine (sheep)	i.n.	Systemic and local immune response. Consistent and higher IgA in nasal secretions and serum than soluble antigen. Not clear whether CT improved immune response in comparison to PLG-SAG1 alone.	[43]
Polymeric	Poly lactic-co-glycolic acid (PLGA)	Bovine parainfluenza 3 virus (BPI3V) proteins	Dairy calves (bovine)	i.n.	Enhanced and sustained mucosal IgA response compared to i.n. modified live virus commercial vaccine.	[24]
Liposome-mucoadhesive polymer	Phosphatidylcholine (PC) (zwittterionic) and tremella or xanthan gum	Inactivated influenza H5N3	SPF Leghorn chicken	i.n.	Mucoadhesive liposome vesicles induced higher immune response than the virus alone and liposome without the polymer. Viscosity affects vaccine efficacy.	[29]
Polymeric	Chitosan	Inactivated NDV	Broiler chicken, layer hens	i.n.	Increased IgA humoral response in layers, not broilers.	[63]
Liposome	Phosphatidylcholine (PC) (zwittterionic); Phosphatidylserine (PS) (−ve) or Stearylamine (SA) (+ve)	Formalin-inactivated NDV	SPF Leghorn chicken	i.n.	PC induced the highest secretory IgA and systemic humoral responses. LPS co-administration increased vaccine efficacy.	[64]
Liposome	Hydrogenated soybean phospholipids	Inactivated APEC strain KAI-2, O-78	SPF chicken	Coarse spray Eye drop	Reduction in the number of challenged bacteria and clinical signs was observed in chickens after a challenge with APEC.	[65]
Montanide™ IMS adjuvant NP	Unknown	Live IBV	SPF chicken (also commercially used in all farm animals)	i.n. coarse spray	Better than non-adjuvanted vaccine and montanide oil-in-water emulsion. i.n. administration performed better than coarse spray.	[66]
Adenovirus	BAdV-3	Bovine-specific viral antigens: BHV-1 glycoprotein gD, BRSV IL-6	Bovine (cattle)	i.n.	Induces antigen-specific immune responses. Co-expression of different vaccine antigens seems to produces similar response with lower viral titer.	[67–69]
ISCOMs	Glycoside Quil A	BHV-1 viral membrane proteins	Bovine (calves)	i.m.	Better protection than commercial attenuated vaccine and higher antibody response produced.	[70]
Polymeric	Chitosan spray-dried microparticle with recombinant polymeric protein antigen (BLSOmp31)	Brucellosis	Ovine (sheep)	i.n.	Induced local and systemic immune response in sheep, biphasic release of antigen from microsphere.	[71]
Polymeric	Fungal chitosan	Foot and mouth disease whole virus	Guinea pig	i.n.	Higher IgG production in comparison to vaccination with virus alone. Systemic immune response comparable to traditional intra peritoneal alum-inactivated virus vaccine, IgA production resulting from NP vaccine was higher than alum-inactivated viral vaccine.	[72]

vaccine has undergone a phase 1 clinical trial and was proven to be safe and produced high levels of measles antibody [81]. In chickens, coarse spray vaccination has performed better in comparison to drinking water after challenge of *Salmonella enteritidis* strain and reduced colonization and shedding of bacteria [82]. Moreover, coarse spray administration of liposomes carrying inactivated avian pathogenic *E. coli* (APEC) showed protection against lethal *E. coli* challenge [65].

NP vaccine formulations have been most commonly tested against *E. coli* infection, particularly with synthetic CpG-ODN adjuvants. Nanoparticle formulations containing CpG-ODNs have been found to protect against several diseases in mice [83, 84] and *E. coli* and Salmonella in chickens [22, 73–75, 85–87]. However, these particle platforms are not delivered via the pulmonary route, yet they are effective against lethal *E. coli* challenge via *in ovo*, intramuscular, and subcutaneous routes. Our group is investigating NPs for the pulmonary route of vaccination in broilers which present an easier vaccination method at the industrial scale [88, 89].

Specific NP vaccination studies in chickens are sparse; however, there are investigations of NP vaccines administered via the spray route [90, 91]. These studies have found that spray vaccines provide local and topical treatment in air sacs [92]. Some particle deposition studies can give clues about the characteristics of particle uptake to aid the design of optimal NP vaccine delivery systems. In order to establish local drug levels in the lung and air sacs, it has been found that particles less than 3 μm are able to bypass the mucociliary transport [37]. However, larger particles deposit in the upper airways, particularly the tracheal bifurcation [37, 38]. Particle deposition is also dependent on age, and it was shown that in comparison to 2- and 4-week-old broilers, 1-day-old chicks contained more >3 μm particles in the nose and eyes and in the lower respiratory tract, while 1–3 μm particles deposited less compared to older chickens [38].

Interestingly, one study compared i.n. and spray administration against protection of infectious bronchitis virus using the commercial adjuvant Montanide [66]. Montanide can be used with a variety of veterinary antigens, and it can come in NP, polymer, or oil-in-water formulations. In comparison to a non-adjuvanted commercial vaccine, it was found that both the NP and polymer technology of Montanide was better than the oil emulsion. However, i.n. immunization seemed to perform better than spray immunization, and the polymer adjuvant performed best in spray form. Like the factors involved in nebulization of NPs and drugs in humans, the delivery of aerosol vaccines in chickens could be dependent on the device output and the interaction between the NP and the device itself. This is perhaps why the controlled administration of the i.n. formulation performed the best. However, there are no investigations of the interactions between vaccine formulations and coarse spray or nebulization devices for chickens.

Pulmonary and nasal vaccines in ruminants

From the literature, it can be concluded that nasal delivery of vaccines is preferred over aerosol delivery in the ruminants due to the lack of NP applications tested via inhalation. NP and in some cases, microparticle delivery systems have been developed and tested in mainly the ovine (sheep) and bovine (cattle) species. Initially, the sequence of vaccine development begins with testing small animal models, and testing parenteral administration prior to mucosal application in the target species. However, some studies have formulated NP vaccines and tested directly in the large animal model. Among these is one of the most commonly used vaccine viral vectors, adenovirus. Adenoviral vectors have been widely used in research for human vaccination against tuberculosis, HIV, and other respiratory diseases [93–98]. Since adenovirus is a species-specific virus that naturally infects the respiratory tract, it has been extensively studied for pulmonary and nasal administration. Additionally, they have the ability to infect both dividing and non-dividing cells, they have the capacity to package large foreign genes, they elicit strong antigen-specific T cell responses, they are relatively easy to produce recombinant virus, and they lack virulence [67]. Even concerns with integration and safety profile of viral vectors have faded [11, 16].

The human adenovirus 5 vector has been used to immunize cattle intra-nasally against Bovine herpes virus 1 (BHV-1) and was able to produce a specific antibody response stronger than the commercially available live attenuated vaccine. It also clinically protected cattle after challenge with high infectious dose of BHV-1 [99]. Due to safety concerns regarding zoonosis with using human viral vectors in domestic animals, bovine adenovirus 3 (BAdV-3) a natural non-pathogenic virus has been modified specifically for a vaccine delivery vehicle for cattle [67, 68]. Although primarily tested in cotton rats, BAdV-3 has been used to incorporate bovine-specific viral antigens against BHV-1 or Bovine respiratory syncytial virus (BRSV) [67, 68]. After immunization, antibodies specific against both viral antigens were detected in the sera and nasal secretions of the rats [69]. Additionally, the co-expression of two viral antigens by BAdV-3 required less viral titer to induce the same quantity of antibody expression than BAdV-3 expressing either BHV-1 or BRSV antigens. It is suggested that the co-expression of two antigens may be more economically favorable than individual antigen expression [69]. The cotton rat is considered a suitable animal model for cattle. However, BAdV-3 has also been developed further as a BHV-1 vaccine expressing the cytokine interleukin 6 (IL-6) to reduce viral shedding in cattle [100], which was not achieved with the sole expression of BHV-1 glycoprotein gD despite clinical protection in cattle after challenge [101]. The IL-6 did not improve protection or immune response in this investigation, but it was suggested that IL-6 may not be

enough to influence the mucosal immune response in calves, and other potent adjuvants could be used to reduce viral shedding.

Immune stimulating complexes (ISCOMs) have also been developed to vaccinate against BHV-1 in calves [70]. Traditionally, ISCOMs are a 40-nm cage-like structure held together by hydrophobic interactions between saponin and lipids [25]. However, for the BHV-1 vaccine, the ISCOM (30–35 nm) was made of glycoside Quil A, a plant adjuvant, which formed a honeycomb structure with BHV-1 viral membrane proteins. The ISCOM adjuvant NP vaccine produced higher antibody response and resulted in better protection than the available commercial attenuated vaccine. It is important to note that the ISCOM was administered through intramuscular injection and resulted in protection against viral challenge. Note, ISCOMs are known to be particularly strong mucosal adjuvants similar to parenteral and subcutaneous influenza vaccination and have resulted in higher IgA in serum, lung, and nasal washings [102, 103]. It would be interesting to determine whether the BHV-1 ISCOM vaccine would perform better at lower dosing than intramuscular injection and compare it to the commercial attenuated vaccine.

Polymer particles are among the most popular vaccine formulations in ruminants. However, a variety of the polymer particle vaccines developed have not been NPs but are in the microparticle size range (>1 μm). Despite the main populations of particles in the 1–2-μm size range, BHV-1 vaccine-loaded chitosan microparticles have been shown to be effectively taken up by bovine kidney cells, from both spray dried and gel chitosan microparticle formulations [27].

Chitosan microparticles are frequently used as i.n. vaccine delivery vehicles for cattle and sheep [27, 71]. However, they have mainly been studied for their ability to induce local and systemic humoral antibody responses and not necessarily have been tested for inducing protection. In sheep, spray-dried chitosan microspheres containing a polymeric protein antigen (BLSOmp31) decorating the surface were able to induce local and systemic immune response after three i.n. immunizations over 40 days [71]. The microspheres produced a biphasic release of the antigen and were able to induce a nasal immune response despite the lower mucin adhesion with protein-loaded particles versus blank chitosan particles. Although this was just a preliminary study, it would have been interesting to see if blank chitosan microparticles would also induce a slight immune response in sheep.

There is evidence of effectiveness using chitosan NP vaccines which have been prepared for immunization against Foot and Mouth Disease in livestock [72]. Unlike traditional chitosan, this group used fungal chitosan derived from a fungal cell wall, since it can produce higher yields, has low molecular weight, and has high degree of deacetylation [72]. The low molecular weight and high degree of acetylation is found to influence chitosan particle formation toward more stable complexes [104]. Since guinea pigs are a suitable animal model for cloven hoofed animals (pigs, cattle, and sheep), the extent of the immune response was measured through antibody titer measurements from serum, intestinal tract, and broncho-alveolar tissues after delivery of whole virus to the nasal tissue in guinea pigs [72]. All the particles compared ranged in size from 220 to 280 nm with low polydispersity index, unlike the commercial chitosan NPs which had the largest size. In comparison to vaccine delivery with just virus, all formulations (including commercially derived chitosan) produced higher IgG titers in sera over time. Even the systemic immune response produced by NPs was comparable to the traditional intraperitoneal alum-inactivated virus, vaccine and nasal IgA produced from the NP vaccines was also higher in comparison to the injected vaccine. Effective mucosal IgA production was also seen in the intestinal mucosa, which was not produced from intraperitoneal injection with alum-FMD-v vaccine. It would also be interesting to compare the gel chitosan formulation [105] with the chitosan NP formulation to determine which would stimulate stronger immune responses.

Immunization with other mucoadhesive polymers like alginate have also been tested in the cattle species but only to determine whether alginate microparticles can produce local immune responses [106]. The particles carried pig serum albumin as an antigen but were not geared to any specific disease. Since the alginate microparticle study aimed to compare the oral versus i.n. route of administration, the particles formed were mainly under 5 μm to optimize delivery. However, the study was only able to conclude that immunization with alginate microparticles may be plausible with both nasal and oral administration to provide specific immune responses against other antigens.

Other polymer particles that have been used to determine if they can enhance the immune response of vaccines in bovine and ovine species are poly(d, l-lactide-co-glycolide) (PLG) and PLGA. PLG particles were carrying SAG1 surface antigen from a *Toxoplasma gondii* tachyzoite [43]. These particles were under 2 μm and polydisperse, but with more than 60% of the population being NPs. Antigen was present both inside the particle and adsorbed to the surface upon particle formation. After three i.n. immunizations over 2 weeks, there was evidence of consistent local IgA in comparison to the soluble antigen; however, the formulation failed to protect against oocyst challenge. Addition of cholera toxin to the PLG-SAG1 particle also did not seem to improve the immune response significantly. In this particular study, even IgG production in the nasal mucosa and serum was very low, which is in contrast to previous studies in mice [43].

Perhaps a more insightful report compares the immune response created by a commercial vaccine against the Bovine parainfluenza 3 virus respiratory pathogen in dairy calves to the same vaccine formulated in PLGA NPs

(225 nm, −22.7 mV) [24]. Unlike the commercial vaccine, the PLGA vaccine elicited greater IgA response in the mucus which persisted over the whole study period. The serum IgG response was also similar to the commercial vaccine but appeared to be more of a sustained release of antigen due to transient antibody production. It would be interesting to see in the future how the release profile of the antigen correlates with protection against respiratory disease in comparison to the commercial vaccine, as this platform also produced IgG to a comparable level of that of the commercial vaccine.

Conclusions and future directions

The pulmonary route of vaccination is promising for eliciting effective immune responses. Although many researchers are investigating pulmonary vaccines of human disease, it is important to remember that vaccinating livestock and food-producing animals is also important to prevent animal and zoonotic pathogens. The development of veterinary vaccines is highly dependent on cost-benefit ratio. However, this should not limit the major aim of veterinary vaccines of ensuring the health of animals and herd immunity. While the nasal and pulmonary route of vaccine administration has not quite made it to the market in humans, the use of NP delivery systems can help enhance vaccine effectiveness and help to ensure better delivery through devices that are specifically tailored for each species. In fact, materials that overcome delivery barriers determined from human findings have been translated into investigations of vehicles in livestock and poultry vaccines. Studies of nasal immunization with NP systems are common in both ruminants and chickens; however, data involving spray or nebulization of vaccines is lacking. It is expected that both research and translation of pulmonary vaccine delivery using NPs in livestock and poultry will be rapidly expanding (Table 1).

Compliance with ethical standards

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