*Minireview*

# Micro- and nanoparticulates for DNA vaccine delivery

**Eric Farris[1], Deborah M Brown[2,3], Amanda E Ramer-Tait[4] and Angela K Pannier[5,6,7,8]**

[1]Department of Biological Systems Engineering, University of Nebraska-Lincoln, Lincoln, NE 68588, USA; [2]School of Biological Sciences, University of Nebraska-Lincoln, Lincoln, NE 68588, USA; [3]Nebraska Center for Virology, University of Nebraska-Lincoln, Lincoln, NE 68588, USA; [4]Department of Food Science and Technology, University of Nebraska-Lincoln, Lincoln, NE 68588, USA; [5]Department of Biological Systems Engineering, University of Nebraska-Lincoln, Lincoln, NE 68588, USA; [6]Nebraska Center for Materials and Nanoscience, University of Nebraska-Lincoln, Lincoln, NE 68588, USA; [7]Center for Nanohybrid Functional Materials, University of Nebraska-Lincoln, Lincoln, NE 68588, USA; [8]Mary and Dick Holland Regenerative Medicine Program, University of Nebraska Medical Center, Omaha, NE 68198, USA

Corresponding author: Angela K Pannier. Email: apannier2@unl.edu

## Abstract

DNA vaccination has emerged as a promising alternative to traditional protein-based vaccines for the induction of protective immune responses. DNA vaccines offer several advantages over traditional vaccines, including increased stability, rapid and inexpensive production, and flexibility to produce vaccines for a wide variety of infectious diseases. However, the immunogenicity of DNA vaccines delivered as naked plasmid DNA is often weak due to degradation of the DNA by nucleases and inefficient delivery to immune cells. Therefore, biomaterial-based delivery systems based on micro- and nanoparticles that encapsulate plasmid DNA represent the most promising strategy for DNA vaccine delivery. Microparticulate delivery systems allow for passive targeting to antigen presenting cells through size exclusion and can allow for sustained presentation of DNA to cells through degradation and release of encapsulated vaccines. In contrast, nanoparticle encapsulation leads to increased internalization, overall greater transfection efficiency, and the ability to increase uptake across mucosal surfaces. Moreover, selection of the appropriate biomaterial can lead to increased immune stimulation and activation through triggering innate immune response receptors and target DNA to professional antigen presenting cells. Finally, the selection of materials with the appropriate properties to achieve efficient delivery through administration routes conducive to high patient compliance and capable of generating systemic and local (i.e. mucosal) immunity can lead to more effective humoral and cellular protective immune responses. In this review, we discuss the development of novel biomaterial-based delivery systems to enhance the delivery of DNA vaccines through various routes of administration and their implications for generating immune responses.

**Keywords:** DNA vaccine, biomaterials, transfection, vaccination, oral delivery

***Experimental Biology and Medicine* 2016; 241: 919–929. DOI: 10.1177/1535370216643771**

## Introduction

Vaccination is considered by many to be one of the most successful public health interventions of the modern era.[1] The advent and subsequent development of vaccines has led to a dramatic increase in worldwide life expectancy and has resulted in the complete eradication of several diseases such as small pox and polio.[2] Current vaccine methodologies are largely protein-based and require direct administration of either dead or attenuated bacteria/viruses, recombinant proteins, or virus-like particles.[3] These protein-based vaccines often fail to generate a complete immune response and typically only generate an antibody-mediated immune response, leading to incomplete immune protection, specifically from pathogens that replicate intracellularly (e.g. viruses).[3] Furthermore, traditional vaccines suffer from limited stability, dependency on ''cold chain'' storage and transport, and costly and time-consuming production,[4,5] all of which limit the potential of traditional vaccines for rapid, widespread deployment, especially to underdeveloped regions where infectious diseases remain prevalent.

Unlike traditional protein-based vaccination, DNA vaccination involves the delivery of plasmid DNA (pDNA) encoding a pathogen-specific target antigen driven by a eukaryotic promoter resulting in the intracellular production and subsequent immune sampling of the target antigen.[6] DNA-based vaccines are considered an attractive alternative to traditional vaccine strategies, as they can

more closely mimic live infections and induce both antibody and cell-mediated immune responses.[3] Furthermore, DNA vaccines eliminate the need for cold chain storage and transportation,[7] can be quickly altered by manipulating the transgene sequence to adapt to new and fast-emerging diseases,[8] and are considered safer than traditional vaccines as the pathogen is not involved in vaccine synthesis. pDNA used in DNA vaccines can also be quickly and easily replicated and amplified in bacteria, allowing for accelerated production time frames, and the capability of large-scale production.[9] In addition, DNA vaccination is applicable to a range of viral,[10] bacterial,[11] and parasitic[12] diseases. DNA vaccines are also uniquely suited for anticancer and antitumor therapies as their encoded antigen is produced intracellularly and introduced directly onto major histocompatibility complex (MHC) class I for antigen presentation to CD8+ T cells, which are essential effector cells for cytolytic activity.[13] The identification of the first human tumor-specific antigen recognized by CD8+ T cells introduced the possibility of cancer immunotherapy treatments,[14] and has since led to the identification of numerous tumor antigens that offer promising targets for anticancer DNA vaccination strategies.[15]

As with all vaccination strategies, the goal of DNA vaccination is to induce robust, protective memory immune responses, which typically require the activation and interplay of all three arms of the immune system, including the humoral and cellular arms of adaptive immunity, as well as the innate immune system.[3] Adaptive immune responses are induced by specialized immune cells including professional antigen presenting cells (APCs) and mediated by B- and T-lymphocyte responses. APCs, such as dendritic cells (DCs) and macrophages, are responsible for the capture, uptake, and processing of antigen for display on MHC Class I and II molecules to activate and drive differentiation of CD8+ and CD4+ T-effector cells, respectively. CD8+ T cells have cytotoxic function and serve as an effector of cellular immunity, while CD4+ T cells activate B cells and facilitate their differentiation into high affinity, antibody-secreting plasma cells, and memory B cells. APCs also express pattern recognition receptors (PRRs) which, when recognizing conserved microbe-associated molecular patterns, initiate signaling events that activate APCs. Therefore, APCs act to coordinate both the innate and adaptive arms of the immune response and are pivotal in generating complete immune protection. For effective immune protection, efficient delivery of DNA vaccines must occur in the appropriate target tissue and to the proper cell type. Developing strategies for controlled delivery of DNA vaccines that preferentially home to these sites and cells is essential for immune activation (Figure 1).

To date, three DNA vaccines have been licensed for veterinary use, and a fourth has been approved for use in pigs used in the food supply for human consumption. All four licensed DNA vaccines are administered as an intramuscular injection of naked pDNA,[7] the simplest method of DNA vaccine delivery. While naked DNA vaccination elicited measurable levels of antigen-specific immunity when administered intramuscularly, in most cases, the immune responses were weak.[3] Therefore, the use of biomaterials to develop delivery platforms that provide protection to pDNA and allow for controlled, cell-targeted, and site-specific release are the most promising strategies for improving the efficacy of DNA vaccination strategies. The formulation of DNA vaccines into micro- and nanoscale particles also has implications for the immunogenicity of the delivered vaccines. Particles ranging in size from 1 to 10 μm are the preferred platforms for targeted delivery to APCs due to their preferential uptake by APCs over other cells, and the bulk size of microparticles creates depots of DNA that allow for sustained exposure to cells.[16] In contrast, nanoparticles have the ability to directly reach the lymph nodes,[3] have multiple routes of uptake, and often achieve an overall higher transfection efficiency when compared to microparticles.[17]

In addition to the biomaterial delivery platform, another consideration in DNA vaccination efficacy is the route of administration used. Delivery route can impact a vaccine's ability to elicit the desired response by targeting various professional APCs associated with different tissues, while also requiring different materials that adequately meet the requirements of each route of delivery. Typical routes of administration that have been investigated for DNA vaccines include parenteral routes (e.g. intramuscular, intradermal, and subcutaneous injection) and mucosal routes (e.g. oral, intranasal, and vaginal). Delivery via the parenteral route can promote activation of tissue-specific APCs (e.g. Langerhans cells in the dermis in intradermal injection) to induce humoral and cellular immune responses. Recently, the mucosal route of vaccine administration has received much attention due to the ability to generate local immunity at body sites that serve as common routes of entry for many pathogens.[18] In particular, the oral route allows for DNA vaccine delivery to the professional APCs residing in the lamina propria underlying the intestinal epithelium.[19] Delivery via the oral route can subsequently result in both mucosal and systemic immunity due to the highly vascularized nature of the intestinal epithelium.[19] Similarly, intranasal and vaginal routes deliver DNA vaccines to the underlying professional APCs lining the respiratory and urogenital epithelium, which then travel to draining lymph nodes to induce adaptive B- and T-cell immunity.[20,21] This review highlights recent attempts at improving the efficacy of DNA vaccination though the development of novel biomaterial-based delivery platforms for use in various routes of administration, focusing on studies ranging from 2009 to the present, as well as focusing on parenteral administration and mucosal administration accomplished via oral delivery.

## Parenteral administration

Parental administration of DNA vaccines, including intramuscular, subcutaneous, and transdermal routes, often involves the injection or otherwise direct administration of the delivery platform. Vaccines delivered via this route typically induce systemic immune responses including humoral and cellular responses. The following sections will discuss the use of various biomaterial delivery systems, including synthetic and natural polymeric systems, cationic

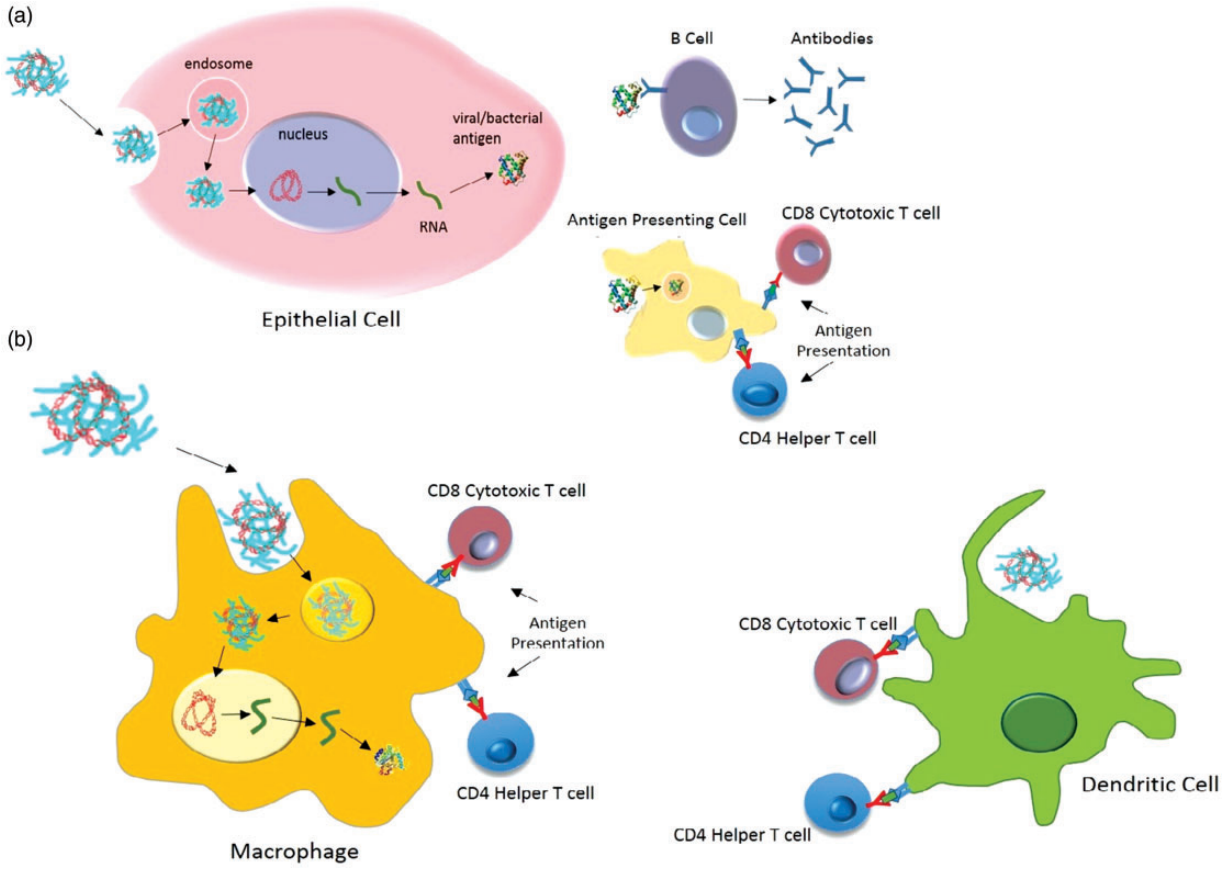


**Figure 1** Micro- or nanoparticulates encapsulate plasmid DNA used for vaccine delivery. In one model, particulates can be taken up by muscle cells or epithelial cells and pathogen-derived antigens are then transcribed and translated from plasmid DNA and secreted into extracellular spaces where they can be taken up by B-cell receptor mediated endocytosis or by professional APCs such as macrophages or dendritic cells. (a) Alternatively, APCs can be directly transfected by uptake of particulate encapsulated DNA. (b) Professional APCs such as macrophages are important for uptake of larger microparticles by phagocytosis, while dendritic cells are more effective at uptake of nanoparticulates by macropinocytosis. Dendritic cells in the draining lymph nodes are especially important for presenting antigen to naïve T cells for activation and differentiation (b) (A color version of this figure is available in the online journal)

lipid, and inorganic particles, as well microneedle-based platforms, for parenteral administration.

## Synthetic polymers

The use of polymeric delivery systems for the delivery of DNAs has been extensively investigated for a variety of applications including gene therapy, tissue engineering, and DNA vaccination. Polymeric delivery systems can complex or physically encapsulate DNA into nano- and microparticles to provide greater protection from nucleases, allow for tunable degradation and controlled release, and facilitate modification to achieve cell-specific targeted delivery. Perhaps, the most widely studied polymer for DNA vaccine development is poly(lactide-co-glycolide) (PLGA). PLGA nano- and microparticles have been used to encapsulate and deliver DNA vaccines against a variety of diseases including cancer,[22] swine influenza,[23] parasitic infections,[24] and hepatitis B.[25] Encapsulation of DNA vaccines into PLGA in these various studies increased systemic antigen-specific antibody responses. In addition, PLGA microparticles encapsulating pDNA encoding an antigenic protein of the human papillomavirus (HPV) have been investigated in phase II clinical trials and were shown to increase T-cell responses to HPV epitopes.[26] Although delivery of DNA using PLGA particles has been shown to induce immune responses, the encapsulation process can unfortunately lead to DNA degradation and ultimately lower transgene expression.

In addition to utilizing PLGA for DNA encapsulation, DNA-coated PLGA microparticles have been reported to facilitate increased DNA loading, reduce DNA degradation throughout the formulation process, and enhance the delivery of DNA vaccines to APCs.[27] Reddy et al.[28] coated cationic PLGA microparticles with pDNA encoding the 1D gene of the foot and mouth disease virus (FMDV) and delivered intramuscularly to guinea pigs. The DNA-coated microparticles resulted in higher FMDV-specific antibody and neutralizing antibody titers, as well as increased lymphocyte proliferation compared to naked plasmid, for one year post vaccination in the guinea pig model of FMDV, highlighting the ability of microparticle-based delivery systems to induce long-lasting immune responses. While microparticles are an attractive delivery platform for DNA vaccines due to their ability to passively target APCs based on size exclusion, the micron size often leads to decreased transfection efficiencies. This is particularly true for

PLGA microparticles, where the large size can lead to acidification of the microenvironment upon degradation of the particles, DNA degradation, and lowered immunogenicity of vaccines. To overcome the limitation associated with PLGA microparticles, several teams have investigated PLGA nanoparticles as an alternative for DNA vaccine delivery.

Lee et al.[29] developed quantum dot-loaded PLGA nanoparticles with a glycol chitosan shell for dual live cell tracking and DNA vaccine delivery.[29] The cationic glycol-chitosan shell allowed for electrostatic interaction with pDNA vaccines to increase loading and promote pH-dependent intracellular release. These particles were able to directly transfect Langerhans cells, tissue-specific professional APCs residing in the dermis, with the enhanced green fluorescent reporter gene after transdermal administration. Transgene expression in the draining lymph nodes was increased following Langerhans cell migration, highlighting the ability of the PLGA nanoparticles to activate APCs, and induce their migration to the draining lymph nodes, which is necessary for APC interaction with and activation of naïve B and T cells.[29] In addition to PLGA nanoparticles, the use of other cationic polymeric materials to form polymer/DNA nanoparticles has also been investigated for DNA vaccine delivery.

Another synthetic polymer that has been widely used in DNA delivery, including DNA vaccines, is poly(ethylene imine) (PEI). Although the use of PEI as a non-viral gene delivery vector is well documented, PEI/DNA complexes do suffer from toxicity issues as well as aggregation in the presence of serum proteins and rapid clearance from circulation, which combine to limit the efficiency of DNA vaccine delivery. Therefore, Shuaibu et al.[30] developed PEI/pDNA complexes coated with γ-polyglutamic acid (γ-PGA) for intravenous delivery of malaria DNA vaccine. The addition of γ-PGA greatly reduced the surface charge of the particles, leading to decreased aggregation and greater stability in physiological conditions. Furthermore, the addition of γ-PGA led to a Th2-dominant immune response, which is crucial for protection against parasitic infections.[31] The addition of γ-PGA was hypothesized to act as an adjuvant by activating receptors of the innate immune response, as γ-PGA is produced by certain strains of bacilli.[32] Thus, synthetic polymers for DNA vaccine delivery are capable of inducing immunogenicity, APC targeting, and potential PRR triggering, all of which are important for enhancing adaptive immune responses. Still, challenges remain in efficiently targeting APCs to trigger the appropriate immune responses needed for protection against various infectious diseases. For example, the Th2 response induced by γ-PGA was protective against a parasitic infection, but may not be effective against intracellular viral infections that rely on CD8+ cytotoxic T cells for complete clearance.

## Natural polymers

Natural materials have long been investigated for many biological applications, including tissue engineering and drug and gene delivery, due to their inherent biocompatibility and biodegradability. In particular, chitosan, the partially deacetylated form of chitin from crustacean and insect shells, has been extensively investigated for DNA vaccine delivery. Due to its positive charge, chitosan can form electrostatic interactions with the phosphate groups of DNA, condensing and complexing it to form nanoscale complexes.[33] Chitosan can also be ionically crosslinked into nanogels containing DNA through the use of a crosslinking anion such as sodium tripolyphosphate.[34] Due to the presence of primary amines on the polymer backbone, chitosan is well suited for a variety of chemical modifications to increase DNA delivery such as enhanced intracellular dissociation of the DNA from the polymer[35] and cell-specific targeting.[36–38] Moreover, chitosan has been shown to activate the NLRP3 inflammasome, a cytosolic PRR of the innate immune system responsible for enhancing proinflammatory cytokine production.[39] Chitosan has been widely used as a delivery platform for DNA vaccines for a variety of pathogens including Leptospirosis,[40] Coxsackievirus B3,[41] and influenza.[42,43] Chitosan nanoparticles have also been formulated with pDNA encoding HPV-16 E7, a tumor-specific antigen for immunotherapy against HPV-associated tumors. After intramuscular injection of the HPV-16 E7/chitosan nanoparticles, the vaccine platform induced CD8+ T-cell activation and proliferation, stimulated interferon (IFN)-γ and interleukin (IL)-4 production, and reduced tumor size in a mouse model.[44] Similarly, chitosan nanoparticles encapsulating DNA encoding the swine flu hemagglutinin antigen resulted in robust serum IgG titers for up to eight weeks and increased T-cell proliferation following intramuscular immunization.[45]

Although chitosan-based nanoparticles show great promise as DNA vaccine delivery systems, these systems often suffer from low transfection efficiency and subsequent low immunogenicity. This low transfection efficiency can be attributed to poor dissociation of pDNA from chitosan in the intracellular environment, limited stability in the presence of serum proteins due to the highly positive charge density, and, in the case of DNA vaccine delivery, poor cell specificity for targeting professional APCs. To overcome these barriers and increase the efficiency of chitosan-based delivery systems, a variety of modification techniques have been employed. Csaba et al.[34] investigated the effects of poly(ethylene glycol) (PEG) conjugation on particle stability and transfection efficiency. This team found that PEGylation increased the *in vivo* stability of chitosan/DNA nanoparticles due to a reduction in surface charge and reduced interaction with serum proteins.[34] The most extensively investigated functionalization strategy for targeting chitosan to APCs is particle mannosylation to increase binding to the macrophage mannose receptor (MMR). C-type lectin receptors (such as the MMR) are one family of PRR that contain carbohydrate-recognition domains that bind sugar moieties, including mannose. Mannose functionalization of chitosan increases nanoparticle association with APCs and increases internalization via mannose receptor-mediated endocytosis.[46–48] Layek et al.[49] developed an L-phenylalanine-modified chitosan for increased adsorptive endocytosis and intracellular dissociation of DNA, further functionalized with mannose for APC-specific targeting (Man-CS-Phe/DNA). Following intradermal

delivery of Man-CS-Phe/DNA complexes encoding hepatitis B surface antigen (HBsAg) to mice, high anti-HBsAg titers were observed for up to six weeks, as well as increased lymphocyte proliferation and increased IL-4 and IFN-γ production.

Both natural and synthetic polymeric delivery platforms serve to increase immune activation in response to DNA vaccines through increased APC targeting and uptake. Despite increases in the immunogenicity of DNA vaccines due, in part, to targeting strategies and carrier modifications, there still remains challenges in achieving the appropriate immune response, specifically balanced Th1 and Th2 helper T-cell differentiation to drive both T cell and antibody-based immunity. In addition to synthetic and natural polymeric DNA vaccine delivery platforms, non-polymeric delivery systems, including cationic lipid and inorganic particulates also hold potential for increasing the efficiency of DNA-based immune responses.

### Cationic lipids and inorganic nanoparticles

Cationic lipids have been widely used as non-viral gene delivery platforms since first introduced by Felgner et al.[50] The amphiphilic nature of cationic lipids allows for self-assembly into lipoplexes and liposomes. The use of cationic lipids for the delivery of DNA vaccines in particular has received much interest due to their abilities to control liposome size, functionalize the carrier, and activate innate immune receptors such as PRR.[51] Moreover, novel synthesis techniques for lipid-based systems allow for APC targeting of lipoplex and liposome DNA vaccine delivery platforms. Srinivas et al.[52] developed cationic amphiphiles containing mannose-mimicking shikimic acid head groups to enhance the delivery of DNA vaccines to professional APCs via targeting of the MMR. The lipoplexes were able to mediate transgene expression in an MMR-dependent manner in a macrophage cell line and were also shown to transfect bone marrow-derived DCs. Furthermore, this delivery system was employed to deliver melanoma tumor-associated antigen and resulted in long-lasting protective immunity in mice.[52] Similarly, Perche et al.[53] developed a mannose-functionalized lipopolyplex delivery system to deliver antimelanoma antigen mRNA. The platform consisted of PEGylated, histidylated polylysine/mRNA complexes further encapsulated in mannosylated liposomes. The functionalized lipopolyplexes led to increased specific internalization into DCs and suppressed tumor growth in vaccinated mice due to the pH-sensitive destabilization of endosomal membranes and cytosolic release of the mRNA vaccine.[53] Such RNA vaccines represent a promising approach to vaccination, as the mRNA payload can be translated into the antigenic protein of interest within the cytosol. Importantly, this approach eliminates the need for nuclear import, which has been established as a major intracellular barrier to successful gene delivery. The development of biomaterials able to protect RNA, which is highly susceptible to enzymatic degradation, and target its delivery to APCs will be instrumental to successful immune protection.

In addition to polymers and lipids, hybrid particles consisting of inorganics combined with polymers have also been investigated for DNA vaccination delivery strategies. Ye et al. developed iron oxide nanoparticles coated with γ-glutamic acid and PEI for the delivery of pDNA encoding both IL-21 and *Mycobacterium tuberculosis* (*MTb*) antigen for inducing protective immunity in mice. Intramuscular immunization with these nanoparticles resulted in co-expression of immunostimulatory IL-21 and target antigen, leading to increased activation of T lymphocytes, and subsequent protection following *MTb* challenge. Wang et al.[54] developed core-shell silicon oxide-layered double hydroxides ($SiO_2$ LDH), encapsulating pDNA encoding HBsAg. The $SiO_2$ LDH/DNA nanocomposites were effectively internalized by macrophages, leading to high reporter gene expression *in vitro*. Upon parenteral administration to mice, the $SiO_2$LDH/pHBsAg leads to enhanced HBV antibodies and antigen-specific T-cell responses. Moreover, vaccination with the $SiO_2$LDH/pHBVsAg nanoparticles activated macrophages and promoted Th1 differentiation via activation of the NF-κB pathway. LDH-DNA particulates have also been investigated for enhancing the efficacy of melanoma tumor vaccines. Subcutaneous injection of the LDH-DNA particles led to increased antigen-specific Ig titers and significantly reduced tumor growth.[55] In the case of tumor vaccine therapies, activated cytolytic CD8+ T cells are the main effector cells responsible for eliminating tumor cells; therefore, it is crucial to generate a type 1 immune response for cytotoxic T-cell differentiation. Biomaterial-based DNA vaccine delivery systems allow for the targeted delivery of immunostimulatory cytokines (such as IL-12), either through encapsulation within the biomaterial matrix or via coexpression of an antigen/cytokine dual plasmid. The targeted delivery of such stimulatory molecules along with a specific antigen would be capable of inducing strong immune responses, while also limiting the negative effects often seen with systemic circulation of immunostimulatory cytokines.

Recently, the use of cationic solid lipid nanoparticles (cSLN) has been investigated for gene delivery applications due to their ability to increase DNA stability and loading as well as overcome some of toxicity concerns that are associated with lipoplex delivery systems. Doroud et al.[56] developed cSLN containing a cocktail of DNA vaccines against *Leishmania major*, a protozoan parasite responsible for cutaneous leishmaniasis. These cSLN represent a unique delivery system for enhancing the immune response due to the presence of 1,2-dioleoyl-3-trimethylammonium-propane, a cationic surface-active lipid that has been shown to activate DCs and drive their maturation through binding of PRR,[57] highlighting the adjuvant activity of some lipid delivery platforms. Upon subcutaneous footpad injection in mice, the cSLN delivery system was able to reduce the parasitic burden in lymph nodes and induce high levels of IFN-γ and IL-5, indicators of Th1 and Th2 immune responses, respectively. The above studies highlight the use of micro- and nanoparticles consisting of polymeric and non-polymeric materials that often require direct injection to activate an immune response, therefore requiring administration from medically trained personnel.

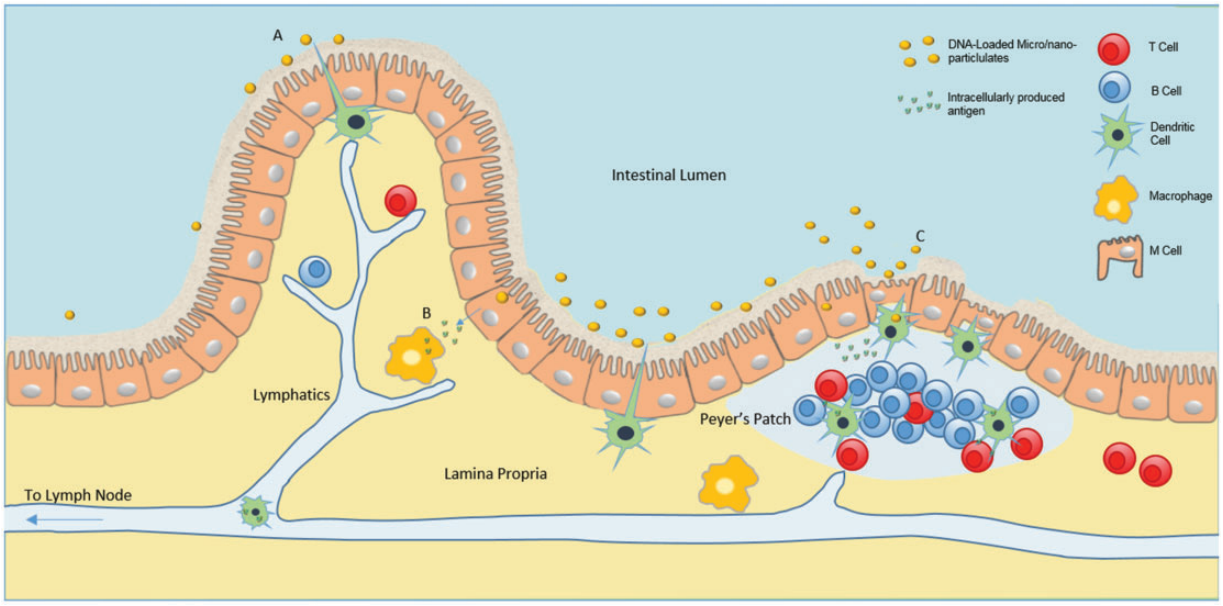


**Figure 2** Cross-sectional representation of the intestinal epithelium and underlying immune cells of the Lamina Propria. DNA encapsulated micro- and nanoparticulates delivered via the oral route can take advantage of multiple routes to transfect cells or APCs for antigen expression and immune activation. (a) Nanoparticles can be directly sampled from the intestinal lumen to transfect DCs. (b) DNA vaccine-loaded particulates can be taken up by intestinal epithelial cells which then produce and secrete the desired antigen for sampling by professional APCs. (c) DNA vaccine-loaded particulates can undergo transcytosis across the intestinal epithelium by specialized M cells where they may transfect cells of the mucosa-associated lymphoid tissue (specifically Peyer's Patches) (A color version of this figure is available in the online journal)

Consequently, patient compliance is compromised, and widespread, rapid deployment of DNA-based vaccines, especially to resource poor environments, is crippled. To truly appreciate the translational potential of such systems, biomaterial-based delivery platforms must facilitate patient compliance and utilize delivery routes conducive to simple administration. One promising alternative to direct injection that still makes use of the parenteral route is the use of transdermal microneedle patches, which can also improve both patient compliance and the immunogenicity of DNA vaccines.[58–60]

### Microneedles

Delivery of vaccines transdermally has shown to increase vaccine immunogenicity, as well as serve a simple and safe delivery strategy.[61] Microneedles consist of solid micron-scale needles that can be composed of biodegradable materials (e.g. PLGA, chitosan or polylactic acid) for sustained release of vaccines as well as non-degradable materials (e.g. stainless steel)[62] that serve to penetrate the epidermis of the skin to deliver DNA vaccines to the resident APCs within the dermis, the Langerhans cells. Hu et al.[63] developed mannosylated cell-penetrating peptide-conjugated PEI/DNA complexes encoding tumor antigen delivered via microneedles. The transdermal delivery system was able to efficiently target skin DCs and induce strong Th1 differentiation and CD4+ and CD8+ cell infiltration into solid tumors. Moreover, microneedles present an ideal substrate for the use of polyelectrolyte multilayers to co-deliver DNA vaccines along with adjuvant materials. DeMuth et al.[64] developed poly(L-lactide) microneedles coated with alternating layers of pDNA/polyI:C adjuvant and biodegradable poly(β-amino-ester) (PBAE). The coated microneedles were able to sustain the release of pDNA and PBAE to form *in situ* polyplexes and mediate robust immune responses against a model HIV antigen. Microneedle arrays have also been coated with virus-like particles (VLP) encapsulating HPV antigen. VLPs are non-replicating molecules that consist of an empty particle that has a structure similar to that of pathogenic viruses. The VLP vaccine-coated stainless steel microneedles elicited strong neutralizing antibodies as well as CD4+ and CD8+ T-cell activation upon transdermal administration.[60] All of the above studies indicate the potential of parenterally administration of DNA vaccines to generate effective humoral and cellular-mediated immune responses; however, these routes of administration typically do not result in the generation of mucosal immunity, which will be discussed in the next section.

## Mucosal administration: Oral delivery

Mucosal immunity is considered an important first line of defense against many pathogens; consequently, vaccines that generate both systemic and mucosal immunity are of great interest. DNA vaccines delivered via mucosal routes (i.e. oral, intranasal, and vaginal) offer the advantages of high patient compliance and the ability to generate both mucosal and systemic immunity. However, delivery via mucosal routes introduces additional challenges to the successful delivery of DNA vaccines and subsequent activation the immune system. Specifically, the delivery vehicle must penetrate the mucous layer and be transported across the epithelial layer while still specifically targeting the underlying APCs (Figure 2). The

following sections discuss approaches in developing biomaterial-based DNA vaccine delivery systems that address some of these obstacles and also highlight challenges that must be overcome for successful mucosal administration.

### Inorganic particles, liposomes and virus-like particles

Of all the possible routes for vaccine administration, the oral route is often considered preferable due to patient compliance given the ease of administrating and dosing without medically trained personnel.[19,65] For oral delivery of DNA vaccines, the intestinal epithelium represents a unique target due to its large cellular surface area, highly vascularized nature, and ability to generate mucosal immunity. One constituent of the intestinal mucosa, the lamina propria, is rich in APCs, including macrophages and DC, which are able to sample antigens directly from the intestinal lumen as well as antigens that have been transported across the intestinal epithelium.[66] These APCs are instrumental in generating B-cell and T-cell responses and providing protection against pathogens that enter via mucosal sites.[67] Moreover, due to the highly vascularized nature of the intestinal epithelium, oral DNA vaccines also have the potential to generate systemic immunity in addition to mucosal immunity.[68] Oral delivery of DNA vaccines has been an area of interest for multiple research groups but has only seen limited success, mainly due to the degradation of DNA by endogenous nucleases and the harsh conditions encountered in the gastric environment.[66] Additionally, the mucosal epithelium presents additional challenges such as a highly viscous mucus layer and specialized enzymatic processes that only specific biomaterials may be able to overcome.

The use of liposomes for oral DNA vaccine delivery has been well documented. Wang et al.[69] developed liposomal systems for delivery of DNA encoding *MTb* antigen and oral administration which generated antigen-specific mucosal and systemic humoral immunity against tuberculosis. Moreover, this platform produced efficient antigen expression by Microfold (M) cells, which are implicated in transporting antigen across the intestinal epithelium for sampling by professional APCs. In addition to traditional liposomes, non-ionic surfactant-based vesicles (niosomes) have also been reported to possess strong adjuvant properties. Jain et al.[70] produced mannosylated niosomes encapsulating pDNA encoding HBsAg for oral mucosal vaccination. The mannan coating stabilized the niosomes throughout the gastrointestinal (GI) tract and targeted the mannose receptor present on APCs. Oral vaccination of mice with the modified niosomes induced strong cellular and humoral immune responses, while also inducing the production of neutralizing secretory IgA, a key antibody produced during mucosal immune responses. While lipid-based systems can deliver DNA vaccines via the oral route, these platforms have limited stability in the GI tract and often undergo degradation due to enzymes and the presence of bile salts that serve to solubilize the lipids.

### Synthetic and natural polymers

While the previous studies have described the use of lipid based systems for oral delivery, the most studied delivery platforms for oral vaccination consist of synthetic and natural polymer platforms due to their highly tunable nature and ability to be modified for enhanced biodegradability,[71,72] controlled release,[73] and cellular targeting.[74–76] As previously mentioned, the natural polymer chitosan has been extensively investigated for the delivery of DNA vaccines via intramuscular and peritoneal routes, but chitosan is also uniquely suited for mucosal delivery applications. Chitosan delivery systems have characteristics that make them an ideal choice for oral DNA delivery, including good biocompatibility and biodegradability, high affinity for DNA, and mucoadhesive properties,[77,78] which allow an increased residence time in the intestinal mucosa and increased sampling by APCs in the underlying lamina propria. In addition, targeting of APCs is possible with ligand modification of the chitosan polymer.[79,80] While chitosan nanoparticles have been used to orally deliver DNA vaccines, including successes against *Toxoplasma gondii*,[67] *Schistosoma mansoni*,[81] and Coxsackie B virus-induced myocarditis,[82] the amount of transgene/antigen production can be low due to chitosan instability in the acidic gastric environment and DNA degradation by digestive enzymes. While these synthetic and natural polymeric delivery vehicles hold great promise for oral DNA vaccine delivery, the instability of many polymeric systems in the GI tract, coupled with the variability in the GI environments, emphasize the need for the development of hybrid delivery platforms that protect DNA vaccines through complete GI transit.

### Hybrid particles

The use of multiple materials for developing oral delivery systems is considered an attractive strategy for overcoming the challenges of complete DNA protection through GI tract transit, as well as the controlled delivery and targeting of the cargo to APCs in the lamina propria of the intestinal epithelium. Dual material systems offer the advantage of selecting properties to match the requirements of each compartment of the GI tract, including protection from gastric conditions and subsequent release in the intestinal environment. Materials that can serve as a protective coating or form an encapsulating matrix around DNA complexes are of special interest. Bhavsar et al.[83] developed an oral gene delivery platform consisting of gelatin/DNA nanoparticles encapsulated in poly($\varepsilon$-caprolactone) (PCL) microspheres. Encapsulation of the gelatin particles in the PCL matrix provided protection of the DNA from the gastric environment and increased the delivery of intact gelatin/DNA nanoparticles in the intestine.[83] However, the slow and potentially toxic degradation of PCL and the use of harsh particle formulation methods can lead to undesired DNA release kinetics, toxicity, and DNA degradation during processing. To overcome some of these limitations, Bhowmik et al.[84] developed a composite microparticle delivery platform consisting of synthetic and natural polymers for the oral

delivery of pDNA encoding HBsAg. The microparticles, containing chitosan as well as consisting of equal ratios of albumin, hydroxyproplymethylcellulose acetate succinate, and eudragrit, were formed via a spray drying method. The combination of polymers served to enhance the oral stability of the microparticles, as well as impart mucoadhesive properties to enhance delivery to cells in the intestinal epithelium. The microparticles were further functionalized with the M-cell targeting ligand *Aleuria Aurantia* Lectin to enhance targeted delivery to APCs. Oral administration of the microparticles led to increased serum IgG and fecal IgA titers when compared to subcutaneous injection, indicating the induction of both systemic and mucosal immune responses. Similarly, Channarong et al.[85] investigated chitosan/DNA-loaded liposomes, further modified with a chitosan coating, for improving targeting to Peyer's patches. Chitosan/DNA complexes were entrapped in liposomes consisting of phosphatidylcholine and cholesterol using a thin film fabrication method. The chitosan coating increased the protective abilities of the chitosan/DNA-loaded liposomes and resulted in stable lipopolyplexes in both gastric and intestinal fluid. *In vivo* studies with orally delivered chitosan/DNA-loaded liposomes indicated transgene expression throughout the upper and lower intestine, an observation attributed to the bioadhesive nature of chitosan, which allows for increased residence time in the intestine.

While these synthetic and lipid-based platforms highlight the advantages of using several materials in designing oral delivery systems, issues with toxicity and complete protection remain. Consequently, there is increasing interest in designing systems that make use of only natural materials and processing conditions that do not affect DNA integrity. Recently, Liu et al.[86] developed an oral delivery system consisting of alginate-coated chitosan/DNA nanoparticles to provide protection against breast cancer metastasis. The coating of alginate, a natural polymer derived from brown seaweed, provided protection to the chitosan/DNA nanoparticles in low pH conditions (i.e. gastric) and were taken up by macrophages and DCs in the intestinal Peyer's patches upon oral administration to mice.[86] Furthermore, this vaccination strategy inhibited tumor growth and increased survival in an orthotopic 4T1 breast cancer model.

Another natural biomaterial that has recently gained interest for gene delivery applications is zein. Zein is the major prolamine, or storage protein, from corn comprising 45–60% of the total corn protein. The presence of polar and non-polar amino acids allows zein molecules to self-assemble into a variety of structures including nano- and microparticles as well as uniform films[87–89] and allows zein to interact with and encapsulate a variety of hydrophobic and hydrophilic compounds including vitamins,[90] essential oils,[91] antiparasitic drugs[92] and in our previous work, DNA.[93] Due to its inherent biocompatibility and biodegradability as well as its ability to self-assemble to form particles and coatings, zein has already been employed in pharmaceutical tableting, specifically for oral delivery applications. Recently, our group has developed an oral delivery system consisting of zein microparticles encapsulating chitosan/DNA nanoparticle cores for enhanced delivery of DNA vaccines to APCs in the intestinal lamina propria. The zein microparticles, due to their resistance to aqueous acidic environments and gastric enzymes, were used to encapsulate and protect the chitosan/DNA nanoparticles from dissolution and degradation in the gastric environment (data not shown). These particles demonstrate that future design of biomaterials for oral DNA vaccine delivery will require much consideration for the various requirements of the different GI tract compartments.

## Conclusions

Vaccination has led to great improvements in overall world health and has served to greatly reduce the prevalence of infectious diseases. However, vaccines for rapidly mutating and emerging diseases often fail to elicit complete protective immune responses. DNA-based vaccine strategies present several advantages over traditional protein-based vaccines. First, DNA vaccines result in the intracellular production of the target antigen and subsequent presentation to the immune system. In turn, a more balanced T-and B-cell response is generated, which ultimately gives rise to populations of resident memory T cells important in fighting mutating viral infections. Second, DNA vaccines allow for rapid, large-scale production of antigen-specific vaccines and eliminate the need for cold chain storage and transportation, making them suited for rapidly emerging, pandemic diseases.

In order to realize the great potential of DNA vaccines and produce clinically relevant vaccine strategies, there remains the need for development of proper delivery platforms combined with appropriate delivery routes that achieve efficient transfection of immune cells. Biomaterial-based delivery systems based on micro- and nanoparticles that encapsulate and protect DNA vaccines represent the most promising strategy for DNA vaccination. Microparticulate delivery systems allow for passive targeting to APCs through size exclusion and can promote sustained presentation of DNA to cells through degradation and release of encapsulated vaccines. Nanoparticles offer increased internalization, overall greater transfection efficiency, and the ability to increase uptake across mucosal surfaces. Moreover, selection of the appropriate biomaterial can lead to increased immune stimulation and activation by triggering innate immune response receptors. Finally, selecting materials with the appropriate properties to achieve efficient delivery via administration routes that are not only conducive to high patient compliance but also generate systemic and local, mucosal immunity can lead to more effective protective humoral and cellular immune responses. With continued material development to increase delivery efficiency and immunogenicity, DNA vaccines will offer a promising alternative to traditional vaccination strategies.

**Authors' contributions:** All authors contributed to the writing and editing of this manuscript.

#### ACKNOWLEDGEMENTS

The Nebraska Research Initiative, University of Nebraska-Lincoln Tobacco Settlement Funds, National Science Foundation (CBET-1254415), Center for Nanohybrid Functional Materials (NSF EPS-1004094), American Heart Association (#10SDG2640217), the University of Nebraska Foundation (Layman Funds), National Institute of General Medical Sciences of the National Institutes of Health (P20GM104320), the Crohn's and Colitis Foundation of America (#3578), and the Nebraska Corn Board, UNL Research Council Interdisciplinary Seed Grant, UNL Research Council-Tobacco Settlement Funds Biomedical Seed Grant and USDA CSREES-Nebraska [NEB-21-146 and NEB-26-211] are acknowledged for funding this work.

#### DECLARATION OF CONFLICTING INTERESTS

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

#### REFERENCES

1. Walters AA, Krastev C, Hill AVS, Milicic A. Next generation vaccines: single-dose encapsulated vaccines for improved global immunisation coverage and efficacy. *J Pharm Pharmacol* 2015;**67**:400–8
2. Kim SH, Lee KY, Jang YS. Mucosal immune system and M cell-targeting strategies for oral mucosal vaccination. *Immune Netw* 2012;**12**:165–75
3. Nguyen DN, Green JJ, Chan JM, Langer R, Anderson DG. Polymeric materials for gene delivery and DNA vaccination. *Adv Mater* 2009;**21**:847–67
4. Josefsberg JO, Buckland B. Vaccine process technology. *Biotechnol Bioeng* 2012;**109**:1443–60
5. Kumru OS, Joshi SB, Smith DE, Middaugh CR, Prusik T, Volkin DB. Vaccine instability in the cold chain: mechanisms, analysis and formulation strategies. *Biologicals* 2014;**42**:237–59
6. Langer B, Renner M, Scherer J, Schüle S, Cichutek K. Safety assessment of biolistic DNA vaccination. In: Sudowe S, Reske-Kunz AB (eds). *Biolistic DNA delivery*. New York: Humana Press, 2013, pp. 371–88
7. Liu MA. DNA vaccines: an historical perspective and view to the future. *Immunol Rev* 2011;**239**:62–84
8. Deering RP, Kommareddy S, Ulmer JB, Brito LA, Geall AJ. Nucleic acid vaccines: prospects for non-viral delivery of mRNA vaccines. *Expert Opin Drug Deliv* 2014;**11**:885–99
9. Kutzler MA, Weiner DB. DNA vaccines: ready for prime time? *Nat Rev Genet* 2008;**9**:776–88
10. Stachyra A, Gora-Sochacka A, Sirko A. DNA vaccines against influenza. *Acta Biochim Pol* 2014;**61**:515–22
11. Ingolotti M, Kawalekar O, Shedlock DJ, Muthumani K, Weiner DB. DNA vaccines for targeting bacterial infections. *Expert Rev Vaccines* 2010;**9**:747–63
12. Carvalho JA, Rodgers J, Atouguia J, Prazeres DM, Monteiro GA. DNA vaccines: a rational design against parasitic diseases. *Expert Rev Vaccines* 2010;**9**:175–91
13. Anderson RJ, Schneider J. Plasmid DNA and viral vector-based vaccines for the treatment of cancer. *Vaccine* 2007;**25**(Suppl 2): B24–34
14. van der Bruggen P, Traversari C, Chomez P, Lurquin C, De Plaen E, Van den Eynde B, Knuth A, Boon T. A gene encoding an antigen recognized by cytolytic T lymphocytes on a human melanoma. *Science* 1991;**254**:1643–7
15. Yang B, Jeang J, Yang A, Wu TC, Hung CF. DNA vaccine for cancer immunotherapy. *Hum Vaccines Immunother* 2014;**10**:3153–64
16. O'Hagan DT, Singh M, Ulmer JB. Microparticles for the delivery of DNA vaccines. *Immunol Rev* 2004;**199**:191–200
17. Prabha S, Zhou W-Z, Panyam J, Labhasetwar V. Size-dependency of nanoparticle-mediated gene transfection: studies with fractionated nanoparticles. *Int J Pharm* 2002;**244**:105–15
18. Chen K, Cerutti A. Vaccination strategies to promote mucosal antibody responses. *Immunity* 2010;**33**:479–91
19. Page DT, Cudmore S. Innovations in oral gene delivery: challenges and potentials. *Drug Discov Today* 2001;**6**:92–101
20. Neutra MR, Kozlowski PA. Mucosal vaccines: the promise and the challenge. *Nat Rev Immunol* 2006;**6**:148–58
21. Kiyono H, Fukuyama S. NALT versus PEYER'S-patch-mediated mucosal immunity. *Nat Rev Immunol* 2004;**4**:699–710
22. Barbon CM, Baker L, Lajoie C, Ramstedt U, Hedley ML, Luby TM. In vivo electroporation enhances the potency of poly-lactide co-glycolide (PLG) plasmid DNA immunization. *Vaccine* 2010;**28**:7852–64
23. Zhao K, Li GX, Jin YY, Wei HX, Sun QS, Huang TT, Wang YF, Tong GZ. Preparation and immunological effectiveness of a Swine influenza DNA vaccine encapsulated in PLGA microspheres. *J Microencapsul* 2010;**27**:178–86
24. Gao S, Zhao N, Amer S, Qian M, Lv M, Zhao Y, Su X, Cao J, He H, Zhao B. Protective efficacy of PLGA microspheres loaded with divalent DNA vaccine encoding the OmpA gene of Aeromonas veronii and the hly gene of Aeromonas hydrophila in mice. *Vaccine* 2013;**31**:5754–9
25. Eratalay A, Coskun-Ari FF, Oner F, Ozcengiz E. In vitro and in vivo evaluations of PLGA microsphere vaccine formulations containing pDNA coexpressing hepatitis B surface antigen and interleukin-2. *J Microencapsul* 2010;**27**:48–56
26. Matijevic M, Hedley ML, Urban RG, Chicz RM, Lajoie C, Luby TM. Immunization with a poly (lactide co-glycolide) encapsulated plasmid DNA expressing antigenic regions of HPV 16 and 18 results in an increase in the precursor frequency of T cells that respond to epitopes from HPV 16, 18, 6 and 11. *Cell Immunol* 2011;**270**:62–9
27. Singh M, Briones M, Ott G, O'Hagan D. Cationic microparticles: a potent delivery system for DNA vaccines. *Proc Natl Acad Sci USA* 2000;**97**:811–6
28. Reddy KS, Rashmi BR, Dechamma HJ, Gopalakrishna S, Banumathi N, Suryanarayana VVS, Reddy GR. Cationic microparticle poly(d,l-lactide-co-glycolide)-coated DNA vaccination induces a long-term immune response against foot and mouth disease in guinea pigs. *J Gene Med* 2012;**14**:348–52
29. Lee PW, Hsu SH, Tsai JS, Chen FR, Huang PJ, Ke CJ, Liao ZX, Hsiao CW, Lin HJ, Sung HW. Multifunctional core-shell polymeric nanoparticles for transdermal DNA delivery and epidermal Langerhans cells tracking. *Biomaterials* 2010;**31**:2425–34
30. Shuaibu MN, Cherif MS, Kurosaki T, Helegbe GK, Kikuchi M, Yanagi T, Sasaki H, Hirayama K. Effect of nanoparticle coating on the immunogenicity of plasmid DNA vaccine encoding P. yoelii MSP-1 C-terminal. *Vaccine* 2011;**29**:3239–47
31. Gold MJ, Antignano F, Hughes MR, Zaph C, McNagny KM. Dendritic-cell expression of Ship1 regulates Th2 immunity to helminth infection in mice. *Eur J Immunol* 2015;**46**:122–30
32. Uto T, Toyama M, Nishi Y, Akagi T, Shima F, Akashi M, Baba M. Uptake of biodegradable poly($\gamma$-glutamic acid) nanoparticles and antigen presentation by dendritic cells in vivo. *Results Immunol* 2013;**3**:1–9
33. Koping-Hoggard M, Tubulekas I, Guan H, Edwards K, Nilsson M, Varum KM, Artursson P. Chitosan as a nonviral gene delivery system. Structure-property relationships and characteristics compared with polyethylenimine in vitro and after lung administration in vivo. *Gene Ther* 2001;**8**:1108–21
34. Csaba N, Koping-Hoggard M, Alonso MJ. Ionically crosslinked chitosan/tripolyphosphate nanoparticles for oligonucleotide and plasmid DNA delivery. *Int J Pharm* 2009;**382**:205–14
35. Liu WG, Zhang X, Sun SJ, Sun GJ, Yao KD, Liang DC, Guo G, Zhang JY. N-alkylated chitosan as a potential nonviral vector for gene transfection. *Bioconjug Chem* 2003;**14**:782–9
36. Lee D, Lockey R, Mohapatra S. Folate receptor-mediated cancer cell specific gene delivery using folic acid-conjugated oligochitosans. *J Nanosci Nanotechnol* 2006;**6**:2860–6
37. Lu HD, Zhao HQ, Wang K, Lv LL. Novel hyaluronic acid-chitosan nanoparticles as non-viral gene delivery vectors targeting osteoarthritis. *Int J Pharm* 2011;**420**:358–65

38. Zhang J, Tang C, Yin C. Galactosylated trimethyl chitosan-cysteine nanoparticles loaded with Map4k4 siRNA for targeting activated macrophages. *Biomaterials* 2013;**34**:3667–77
39. Bueter CL, Lee CK, Rathinam VAK, Healy GJ, Taron CH, Specht CA, Levitz SM. Chitosan but not chitin activates the inflammasome by a mechanism dependent upon phagocytosis. *J Biol Chem* 2011;**286**:35447–55
40. Umthong S, Buaklin A, Jacquet A, Sangjun N, Kerdkaew R, Patarakul K, Palaga T. Immunogenicity of a DNA and recombinant protein vaccine combining LipL32 and Loa22 for leptospirosis using chitosan as a delivery system. *J Microbiol Biotechnol* 2015;**25**:526–36
41. Chai DF, Yue Y, Xu W, Dong CS, Xiong SD. Mucosal co-immunization with AIM2 enhances protective SIgA response and increases prophylactic efficacy of chitosan-DNA vaccine against coxsackievirus B3-induced myocarditis. *Hum Vaccines Immunother* 2014;**10**:1284–94
42. Xu W, Shen Y, Jiang ZG, Wang Y, Chu YW, Xiong SD. Intranasal delivery of chitosan-DNA vaccine generates mucosal SIgA and anti-CVB3 protection. *Vaccine* 2004;**22**:3603–12
43. Sawaengsak C, Mori Y, Yamanishi K, Srimanote P, Chaicumpa W, Mitrevej A, Sinchaipanid N. Intranasal chitosan-DNA vaccines that protect across influenza virus subtypes. *Int J Pharma* 2014;**473**:113–25
44. Tahamtan A, Ghaemi A, Gorji A, Kalhor HR, Sajadian A, Tabarraei A, Moradi A, Atyabi F, Kelishadi M. Antitumor effect of therapeutic HPV DNA vaccines with chitosan-based nanodelivery systems. *J Biomed Sci* 2014;**21**:10
45. Zhao K, Shi XM, Zhao Y, Wei HX, Sun QS, Huang TT, Zhang XY, Wang YF. Preparation and immunological effectiveness of a swine influenza DNA vaccine encapsulated in chitosan nanoparticles. *Vaccine* 2011;**29**:8549–56
46. Nanda RK, Hajam IA, Edao BM, Ramya K, Rajangam M, Sekar SC, Ganesh K, Bhanuprakash V, Kishore S. Immunological evaluation of mannosylated chitosan nanoparticles based foot and mouth disease virus DNA vaccine, pVAC FMDV VP1-OmpA in guinea pigs. *Biologicals* 2014;**42**:153–9
47. Peng YX, Yao WJ, Wang B, Zong L. Mannosylated chitosan nanoparticles based macrophage-targeting gene delivery system enhanced cellular uptake and improved transfection efficiency. *J Nanosci Nanotechnol* 2015;**15**:2619–27
48. Yao WJ, Peng YX, Du MZ, Luo J, Zong L. Preventative vaccine-loaded mannosylated chitosan nanoparticles intended for nasal mucosal delivery enhance immune responses and potent tumor immunity. *Mol Pharm* 2013;**10**:2904–14
49. Layek B, Lipp L, Singh J. APC targeted micelle for enhanced intradermal delivery of hepatitis B DNA vaccine. *J Control Release* 2015;**207**:143–53
50. Felgner PL, Gadek TR, Holm M, Roman R, Chan HW, Wenz M, Northrop JP, Ringold GM, Danielsen M. Lipofection: a highly efficient, lipid-mediated DNA-transfection procedure. *Proc Natl Acad Sci USA* 1987;**84**:7413–7
51. Lonez C, Bessodes M, Scherman D, Vandenbranden M, Escriou V, Ruysschaert J-M. Cationic lipid nanocarriers activate toll-like receptor 2 and NLRP3 inflammasome pathways. *Nanomedicine* 2014;**10**:775–82
52. Srinivas R, Garu A, Moku G, Agawane SB, Chaudhuri A. A long-lasting dendritic cell DNA vaccination system using lysinylated amphiphiles with mannose-mimicking head-groups. *Biomaterials* 2012;**33**:6220–9
53. Perche F, Benvegnu T, Berchel M, Lebegue L, Pichon C, Jaffres PA, Midoux P. Enhancement of dendritic cells transfection in vivo and of vaccination against B16F10 melanoma with mannosylated histidylated lipopolyplexes loaded with tumor antigen messenger RNA. *Nanomedicine* 2011;**7**:445–53
54. Wang J, Zhu RR, Gao B, Wu B, Li K, Sun XY, Liu H, Wang SL. The enhanced immune response of hepatitis B virus DNA vaccine using SiO2@LDH nanoparticles as an adjuvant. *Biomaterials* 2014;**35**:466–78
55. Li A, Qin L, Wang W, Zhu R, Yu Y, Liu H, Wang S. The use of layered double hydroxides as DNA vaccine delivery vector for enhancement of anti-melanoma immune response. *Biomaterials* 2011;**32**:469–77
56. Doroud D, Zahedifard F, Vatanara A, Najafabadi AR, Taslimi Y, Vahabpour R, Torkashvand F, Vaziri B, Rafati S. Delivery of a cocktail DNA vaccine encoding cysteine proteinases type I, II and III with solid lipid nanoparticles potentiate protective immunity against Leishmania major infection. *J Control Release* 2011;**153**:154–62
57. Vangasseri DP, Cui Z, Chen W, Hokey DA, Falo LD Jr, Huang L. Immunostimulation of dendritic cells by cationic liposomes. *Mol Membr Biol* 2006;**23**:385–95
58. Kim NW, Lee MS, Kim KR, Lee JE, Lee K, Park JS, Matsumoto Y, Jo DG, Lee H, Lee DS, Jeong JH. Polyplex-releasing microneedles for enhanced cutaneous delivery of DNA vaccine. *J Control Release* 2014;**179**:11–7
59. Kim YC, Yoo DG, Compans RW, Kang SM, Prausnitz MR. Cross-protection by co-immunization with influenza hemagglutinin DNA and inactivated virus vaccine using coated microneedles. *J Control Release* 2013;**172**:579–88
60. Kines RC, Zarnitsyn V, Johnson TR, Pang YYS, Corbett KS, Nicewonger JD, Gangopadhyay A, Chen M, Liu J, Prausnitz MR, Schiller JT, Graham BS. Vaccination with human papillomavirus pseudovirus-encapsidated plasmids targeted to skin using microneedles. *Plos One* 2015;**10**:e0120797
61. Glenn GM, Kenney RT, Ellingsworth LR, Frech SA, Hammond SA, Zoeteweij JP. Transcutaneous immunization and immunostimulant strategies: capitalizing on the immunocompetence of the skin. *Expert Rev Vaccines* 2003;**2**:253–67
62. Hong X, Wei L, Wu F, Wu Z, Chen L, Liu Z, Yuan W. Dissolving and biodegradable microneedle technologies for transdermal sustained delivery of drug and vaccine. *Drug Des Devel Ther* 2013;**7**:945–52
63. Hu Y, Xu B, Xu J, Shou D, Liu E, Gao J, Liang W, Huang Y. Microneedle-assisted dendritic cell-targeted nanoparticles for transcutaneous DNA immunization. *Polym Chem* 2015;**6**:373–9
64. DeMuth PC, Min YJ, Huang B, Kramer JA, Miller AD, Barouch DH, Hammond PT, Irvine DJ. Polymer multilayer tattooing for enhanced DNA vaccination. *Nat Mater* 2013;**12**:367–76
65. Levine MM. IDEAL'' vaccines for resource poor settings. *Vaccine* 2011;**29**(Suppl 4): D116–25
66. O'Neill MJ, Bourre L, Melgar S, O'Driscoll CM. Intestinal delivery of non-viral gene therapeutics: physiological barriers and preclinical models. *Drug Discov Today* 2011;**16**:203–18
67. Bivas-Benita M, Laloup M, Versteyhe S, Dewit J, Braekeleer JD, Jongert E, Borchard G. Generation of toxoplasma gondii GRA1 protein and DNA vaccine loaded chitosan particles: preparation, characterization, and preliminary in vivo studies. *Int J Pharma* 2003;**266**:17–27
68. Jones DH, Corris S, McDonald S, Clegg JCS, Farrar GH. Poly(DL-lactide-co-glycolide)-encapsulated plasmid DNA elicits systemic and mucosal antibody responses to encoded protein after oral administration. *Vaccine* 1997;**15**:814–7
69. Wang DA, Xu J, Feng YH, Liu Y, McHenga SSS, Shan FP, Sasaki J, Lu CL. Liposomal oral DNA vaccine (mycobacterium DNA) elicits immune response. *Vaccine* 2010;**28**:3134–42
70. Jain S, Singh P, Mishra V, Vyas SP. Mannosylated niosomes as adjuvant–carrier system for oral genetic immunization against Hepatitis B. *Immunol Lett* 2005;**101**:41–9
71. Forrest ML, Koerber JT, Pack DW. A degradable polyethylenimine derivative with low toxicity for highly efficient gene delivery. *Bioconjug Chem* 2003;**14**:934–40
72. Lynn DM, Langer R. Degradable poly(beta-amino esters): synthesis, characterization, and self-assembly with plasmid DNA. *J Am Chem Soc* 2000;**122**:10761–8
73. Kurisawa M, Yokoyama M, Okano T. Gene expression control by temperature with thermo-responsive polymeric gene carriers. *J Control Release* 2000;**69**:127–37
74. Beloor J, Ramakrishna S, Nam K, Choi CS, Kim J, Kim SH, Cho HJ, Shin H, Kim H, Kim SW, Lee SK, Kumar P. Effective gene delivery into human stem cells with a cell-targeting peptide-modified bioreducible polymer. *Small* 2015;**11**:2069–79
75. Hashida M, Takemura S, Nishikawa M, Takakura Y. Targeted delivery of plasmid DNA complexed with galactosylated poly(L-lysine). *J Control Release* 1998;**53**:301–10
76. Asthana GS, Asthana A, Kohli DV, Vyas SP. Mannosylated chitosan nanoparticles for delivery of antisense oligonucleotides for macrophage targeting. *Biomed Res Int* 2014;**17**:526391.

77. Dudhani AR, Kosaraju SL. Bioadhesive chitosan nanoparticles: preparation and characterization. *Carbohydr Polym* 2010;**81**:243–51
78. Sadeghi AMM, Dorkoosh FA, Avadi MR, Weinhold M, Bayat A, Delie F, Gurny R, Larijani B, Rafiee-Tehrani M, Junginger HE. Permeation enhancer effect of chitosan and chitosan derivatives: comparison of formulations as soluble polymers and nanoparticulate systems on insulin absorption in Caco-2 cells. *Eur J Pharma Biopharma* 2008;**70**:270–8
79. Jiang HL, Kang ML, Quan JS, Kang SG, Akaike T, Yoo HS, Cho CS. The potential of mannosylated chitosan microspheres to target macrophage mannose receptors in an adjuvant-delivery system for intranasal immunization. *Biomaterials* 2008;**29**:1931–9
80. Kim TH, Jin H, Kim HW, Cho MH, Cho CS. Mannosylated chitosan nanoparticle-based cytokine gene therapy suppressed cancer growth in BALB/c mice bearing CT-26 carcinoma cells. *Mol Cancer Ther* 2006;**5**:1723–32
81. Oliveira CR, Rezende CMF, Silva MR, Borges OM, Pego AP, Goes AM. Oral vaccination based on DNA-chitosan nanoparticles against Schistosoma mansoni infection. *Sci World J* 2012;**11**:938457
82. Ye T, Yue Y, Fan XM, Dong CS, Xu W, Xiong SD. M cell-targeting strategy facilitates mucosal immune response and enhances protection against CVB3-induced viral myocarditis elicited by chitosan-DNA vaccine. *Vaccine* 2014;**32**:4457–65
83. Bhavsar MD, Amiji MM. Development of novel biodegradable polymeric nanoparticles-in-microsphere formulation for local plasmid DNA delivery in the gastrointestinal tract. *AAPS PharmSciTech* 2008;**9**:288–94
84. Bhowmik T, D'Souza B, Uddin MN, D'Souza MJ. Oral delivery of microparticles containing plasmid DNA encoding hepatitis-B surface antigen. *J Drug Target* 2012;**20**:364–71
85. Channarong S, Chaicumpa W, Sinchaipanid N, Mitrevej A. Development and evaluation of chitosan-coated liposomes for oral DNA vaccine: the improvement of Peyer's patch targeting using a polyplex-loaded liposomes. *AAPS PharmSciTech* 2011;**12**:192–200
86. Liu Z, Lv D, Liu S, Gong JB, Wang D, Xiong M, Chen XN, Xiang R, Tan XY. Alginic acid-coated chitosan nanoparticles loaded with legumain DNA vaccine: effect against breast cancer in mice. *Plos One* 2013;**8**:11
87. Mehta SK, Kaur G, Verma A. Fabrication of plant protein microspheres for encapsulation, stabilization and in vitro release of multiple anti-tuberculosis drugs. *Colloids Surf A Physicochem Eng Asp* 2011;**375**:219–30
88. Zhang Y, Niu Y, Luo Y, Ge M, Yang T, Yu L, Wang Q. Fabrication, characterization and antimicrobial activities of thymol-loaded zein nanoparticles stabilized by sodium caseinate–chitosan hydrochloride double layers. *Food Chem* 2014;**142**:269–75
89. Wang H-J, Lin Z-X, Liu X-M, Sheng S-Y, Wang J-Y. Heparin-loaded zein microsphere film and hemocompatibility. *J Control Release* 2005;**105**:120–31
90. Luo Y, Teng Z, Wang Q. Development of zein nanoparticles coated with carboxymethyl chitosan for encapsulation and controlled release of vitamin D3. *J Agric Food Chem* 2012;**60**:836–43
91. Parris N, Cooke PH, Hicks KB. Encapsulation of essential oils in zein nanospherical particles. *J Agric Food Chem* 2005;**53**:4788–92
92. Liu X, Sun Q, Wang H, Zhang L, Wang J-Y. Microspheres of corn protein, zein, for an ivermectin drug delivery system. *Biomaterials* 2005;**26**:109–15
93. Regier MC, Taylor JD, Borcyk T, Yang Y, Pannier AK. Fabrication and characterization of DNA-loaded zein nanospheres. *J Nanobiotechnol* 2012;**10**:44