Development of a Novel Nanotechnology-Based Vaccine Adjuvant and Delivery System for Systemic and Mucosal Immunity

Mohammed Ali Bakkari
South Dakota State University

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DEVELOPMENT OF A NOVEL NANOTECHNOLOGY-BASED VACCINE ADJUVANT AND DELIVERY SYSTEM FOR SYSTEMIC AND MUCOSAL IMMUNITY

BY

MOHAMMED BAKKARI

A dissertation submitted in partial fulfillment of the requirements for the

Doctor of Philosophy

Major in Pharmaceutical Sciences

South Dakota State University

2019
DEVELOPMENT OF A NOVEL NANOTECHNOLOGY-BASED VACCINE
ADJUVANT AND DELIVERY SYSTEM FOR SYSTEMIC AND MUCOSAL
IMMUNITY.

BY

MOHAMMED BAKKARI

This dissertation is approved as a creditable and independent investigation by a candidate
for the Doctor of Philosophy in Pharmaceutical Sciences degree and is acceptable for
meeting the dissertation requirements for this degree. Acceptance of this does not imply
that the conclusions reached by the candidate are necessarily the conclusions of the major
department.

Hemachand Tummala, Ph.D. Date
Dissertation Advisor

Omathanu Perumal, Ph.D. Date
Head, Department of Pharmaceutical Sciences

Date

Date
THIS DISSERTATION IS DEDICATED TO MY PARENTS AND FAMILY
ACKNOWLEDGEMENTS

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<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>APCs</td>
<td>Antigen presenting cells</td>
</tr>
<tr>
<td>BCR</td>
<td>B-cell receptor</td>
</tr>
<tr>
<td>BLP</td>
<td>Bacterium-like particles</td>
</tr>
<tr>
<td>BMDC</td>
<td>Bone-marrow derived dendritic cells</td>
</tr>
<tr>
<td>CpG</td>
<td>Cytosine-phosphate-guanine</td>
</tr>
<tr>
<td>CT</td>
<td>Cholera toxin</td>
</tr>
<tr>
<td>CTL</td>
<td>Cytotoxic T-lymphocytes</td>
</tr>
<tr>
<td>DCs</td>
<td>Dendritic cells</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>DMEM</td>
<td>Dulbecco’s modified eagle’s medium</td>
</tr>
<tr>
<td>DMSO</td>
<td>Dimethyl sulfoxide</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ELISPOT</td>
<td>Enzyme-linked immunosorbent spot</td>
</tr>
<tr>
<td>FAEs</td>
<td>Follicle associated epithelial cells</td>
</tr>
<tr>
<td>HA</td>
<td>Influenza haemagglutinin protein</td>
</tr>
<tr>
<td>IAV</td>
<td>Influenza A virus</td>
</tr>
<tr>
<td>IFN-γ</td>
<td>Interferon gamma</td>
</tr>
<tr>
<td>IgA:</td>
<td>Immunoglobulin A</td>
</tr>
<tr>
<td>IgG:</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IL</td>
<td>Interleukin</td>
</tr>
<tr>
<td>IN</td>
<td>Intranasal</td>
</tr>
<tr>
<td>LAIV</td>
<td>Live attenuated influenza vaccine</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipo-polysaccharide</td>
</tr>
<tr>
<td>MALT</td>
<td>Mucosal associated lymphoid tissues</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Full Form</td>
</tr>
<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>MHC-II</td>
<td>Major histocompatibility complex class II</td>
</tr>
<tr>
<td>MHC-I</td>
<td>Major histocompatibility complex class I</td>
</tr>
<tr>
<td>MPLA</td>
<td>Monophosphoryl Lipid-A</td>
</tr>
<tr>
<td>MPs</td>
<td>Microparticles</td>
</tr>
<tr>
<td>M-cell</td>
<td>Microfold cell</td>
</tr>
<tr>
<td>MPEC</td>
<td>Memory precursor effector cell</td>
</tr>
<tr>
<td>NPs</td>
<td>Nanoparticles</td>
</tr>
<tr>
<td>NALT</td>
<td>Nasopharynx-associated lymphoid tissue</td>
</tr>
<tr>
<td>NK cells</td>
<td>Natural killer cells</td>
</tr>
<tr>
<td>Ova</td>
<td>Ovalbumin</td>
</tr>
<tr>
<td>PAMPs</td>
<td>Pathogen associated molecular patterns</td>
</tr>
<tr>
<td>PBS:</td>
<td>Phosphate buffered saline</td>
</tr>
<tr>
<td>PLGA</td>
<td>Poly(D,L-lactic-co-glycolic acid)</td>
</tr>
<tr>
<td>PVA</td>
<td>Poly(vinyl) alcohol</td>
</tr>
<tr>
<td>RPM1-1640</td>
<td>Roswell park memorial institute 1640</td>
</tr>
<tr>
<td>s.c.</td>
<td>Subcutaneous</td>
</tr>
<tr>
<td>TCRs</td>
<td>T-cell receptors</td>
</tr>
<tr>
<td>Th1</td>
<td>T-helper 1</td>
</tr>
<tr>
<td>Th2</td>
<td>T-helper 2</td>
</tr>
<tr>
<td>Tregs</td>
<td>Regulatory T-cells</td>
</tr>
<tr>
<td>TLR</td>
<td>Toll-like receptor</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3′,5,5′-Tetramethylbenzidine substrate systems</td>
</tr>
<tr>
<td>TNF</td>
<td>Tumor necrosis factor</td>
</tr>
<tr>
<td>PDI</td>
<td>Polydispersity index</td>
</tr>
<tr>
<td>VLP</td>
<td>Virus-like particles</td>
</tr>
<tr>
<td>ZP</td>
<td>Zeta-potential</td>
</tr>
</tbody>
</table>
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ABSTRACT

DEVELOPMENT OF A NOVEL NANOTECHNOLOGY-BASED VACCINE ADJUVANT AND DELIVERY SYSTEM FOR SYSTEMIC AND MUCOSAL IMMUNITY

MOHAMMED BAKKARI

2019

Many serious infections for which there are no existing vaccines, enter by a mucosal route such as HIV, influenza, and tuberculosis, etc. Therefore, designing safe and effective mucosal vaccines that elicit robust immune response at mucosal sites is still a major challenge without a safe mucosal adjuvant. Previously, our laboratory had discovered inulin acetate as a novel toll-like-receptor-4 agonist (adjuvant).

The study in this dissertation explored the application of nanoparticles prepared with inulin acetate (InAc-NPs) as a vaccine adjuvant and delivery system for enhancing mucosal immunity. The rationale behind selecting InAc-NPs is their proven ability to activate strong systemic immunity along with an extensive understanding of their mechanisms of activation.

In chapter-II, we have clearly established, through intranasal vaccinations in mice, that InAc-NPs could generate strong systemic and mucosal antibodies (IgG1, IgG2a, sIgA) and cytokine response that represents both humoral and cellular immunity. InAc-NPs efficiently delivered the antigen into macrophages as well as activated them to
release inflammatory cytokines such as TNF-α, which have been attributed for robust immune response in mice.

In chapter-III, the efficacy of InAc-NPs as a vaccine adjuvant in swine model was established for the first time along with the safety profile in mice. InAc-NPs are proficient in stimulation of swine PBMCs to secrete cytokines such as IL-6, IL12, and IFN-γ. Importantly, InAc-NPs carrying the influenza peptide (M2e) produced higher mucosal and systemic antibodies compared to unadjuvanted antigen in pigs. The study, for the first time, showed InAc as a vaccine adjuvant in pigs that will have significant implications in swine industry and human health.

In chapter-IV, InAc-NPs were explored as a delivery system to carry TLR7 agonist C-563 to its target site in the phagosomes/endosomes of antigen presenting cells. InAc-NPs loaded with C563 provided a unique dual adjuvant and/or a delivery system for vaccines that require robust immune response such as cancer or influenza vaccines.

In conclusion, we have demonstrated the ability of InAc-NPs as a robust vaccine adjuvant and a delivery platform that provides strong systemic and mucosal immunity, which will have significant implications in fighting challenging diseases such as HIV, influenza, cancer, and tuberculosis.
CHAPTER-I

INTRODUCTION
1.1 Vaccines

In the last century, vaccines have made a significant contribution to human health and overall global wellbeing. Vaccination has resulted in the eradication or a dramatic reduction in the number of cases of smallpox, polio, and tetanus [2]. Each year, millions of people of all ages worldwide are saved due to an increase in their awareness of vaccines and immunization. In 2015 alone, 116 million infants received the diphtheria-tetanus-pertussis vaccine and approximately 85% of the world's children received one dose of measles vaccine, according to WHO [3].

A vaccine trains the immune system to recognize a specific pathogenic antigen and exploit the generations of immunological memory for a rapid response and clearance of the related pathogen, without causing clinical disease [4]. Vaccines support the development of a strong immune response against infection and one shot of immunization may last for more than a decade [5]. Therefore, apart from antibiotics, a vaccine represents a highly efficient, life-saving technology in the history of infectious diseases [6]. However, a huge technological gap still exists when attempting to develop a successful vaccine against pandemic diseases, such as influenza, cancer, human immunodeficiency virus (HIV), hepatitis C virus (HCV) and respiratory syncytial virus (RSV). The challenges that face traditional vaccine technologies are related to their efficacy and safety, which therefore deem them inappropriate for some age groups; additionally, they may not be effective for all diseases [7]. Many of the currently used vaccines contain killed or attenuated pathogens. Several vaccines, such as MMR
(Measles, Mumps, Rubella), chicken pox, oral polio, seasonal influenzas, and yellow fever vaccine, contain weakened pathogens. Vaccines which have killed pathogens include Hepatitis A and inactivated polio vaccines. Despite the fact that these marketed vaccines claim to be safe and noninfectious, they may provide only short-lived protection or require frequent doses for further protection, which increases the risk of evoking an infection in immune-compromised recipients [8]. Hence, many current vaccine developments have shifted toward the use of subunit or recombinant vaccines, which offer better safety, as well as fast and effective production. Subunit vaccines mainly contain highly purified antigens, the part of a pathogen that produces protective immunity. However, unlike attenuated vaccines which contain multiple target antigens, subunit vaccines consist of a single antigenic target, thereby generating a lower immune response against a pathogen [7]. In order to boost immunogenicity, subunit vaccines are usually formulated with the addition of immunostimulants or adjuvants, such as aluminum hydroxide [9].

Adjuvants boost the effectiveness of vaccines by improving and sustaining immune responses, reducing the dose of antigen needed, and enhancing the spectrum of the immune response. Adjuvants are usually mixed with an antigen during the production of the vaccine formulations [10]. All vaccines contain some form of adjuvant, whether it is exogenously added or inherently present in the vaccine. However, selecting the right vaccine-adjuvant combination requires a clear understanding of both the protection mechanisms of the antigen and the interaction of the adjuvant with the immune system.
1.2 Vaccine Adjuvants

Adjuvants are substances that are added to vaccines to enhance, modulate or prolong the immunogenicity of the vaccine’s antigen, and thereby, help to boost their protection against the infection[11]. By increasing efficiency, adjuvants reduce the need for multiple doses and broaden the scope of recipients, thus becoming appropriate for both elderly and young patients. In the presence of an adjuvant, protein-based vaccines have become more effective and have been successfully developed as human vaccines[12].

The adjuvant/immunopotentiation effect can be achieved, not only by the addition of an adjuvant or immunopotentiator, [13] but also by modulating the context of antigen delivery using different delivery systems, such as liposomes and emulsions (Fig. 1.1).
Figure 1.1. Adjuvants mechanisms of action [14].

Table 1.1: Commonly reported Vaccine Adjuvants and Delivery systems

<table>
<thead>
<tr>
<th>Antigen delivery systems</th>
<th>Immune potentiators/Adjuvants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alum.</td>
<td>MPL and synthetic derivatives.</td>
</tr>
<tr>
<td>Calcium phosphate</td>
<td>Saponins.</td>
</tr>
<tr>
<td>Tyrosine.</td>
<td>Alternative bacterial component – LPS</td>
</tr>
<tr>
<td>Liposomes.</td>
<td>dsRNA.</td>
</tr>
<tr>
<td>Virosoles.</td>
<td>MDP and derivatives.</td>
</tr>
<tr>
<td>ISCOMS</td>
<td>Lipopeptides</td>
</tr>
<tr>
<td>Virus-like particles</td>
<td>Viral components – flagellin etc</td>
</tr>
<tr>
<td>Microparticles/nanoparticles</td>
<td></td>
</tr>
</tbody>
</table>
Typically, pathogens stimulate the innate immune system through their interaction with a specific group of receptors in antigen presenting cells (APCs), called pattern recognition receptors (PRRs). PRRs recognize highly conserved molecular motifs associated with microbes/pathogens, called the pathogen-associated molecular patterns (PAMPs)[12]. The PRRs include nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), C-type lectin receptors (CLR) and Toll-like receptors (TLR). PRRs can also recognize the materials released from surrounding cells due to disruptive triggers, which are called damage-associated molecule patterns (DAMPs). In many studies, isolated PAMPs or similar molecules have been shown to augment the immune response when combined or conjugated with the desired antigen[6]. Thus, many new generation vaccine adjuvants are targeted for PPR agonists or motifs [Table 1.1]. Of all the PRRs, TLRs are gaining popularity as vaccine adjuvants due to their high efficacy.

1.3 Innate Immunity: Toll-like Receptors (TLRs)

Mammalian immunity consists of two essential subsystems, innate and adaptive immunity, which work cooperatively to achieve comprehensive protection from numerous pathogens and toxins. Initially, innate immunity was considered a naïve first-line defense system that provided non-specific antimicrobial activity and involvement in the early signaling required for subsequent adaptive immune responses [15]. Nevertheless, recent studies have revealed that the innate immune system has a major role in determining and shaping the adaptive immune response to invading pathogens.
The downstream signaling and the recurrence of adaptive immune cells are much
predicated upon the level and nature of the preliminary signals apparent in pathogen
recognition by innate immune cells following infection and vaccination [16].

PRRs are responsible for recognizing the invading organisms and determining
whether a specific, continuous response and protection will supervene. The Toll-like
receptors (TLRs) family is one of the well-known PRRs, which are expressed on APCs,
innate immune cells (macrophages, B cells, and dendrites), and several epithelial cells
which are involved in the downstream signaling pathways of functional relevance to
immune activation and tolerance [13]. TLRs detect the highly constitutive and conserved
pathogen structures, PAMPs [14], or damage associated molecule patterns (DAMP).
Examples of PAMPs which can be recognized by TLRs include lipopolysaccharides
(LPS), lipopeptides, single-stranded RNA, double-stranded RNA, flagellin and a CpG
motif-containing DNA (Fig. 1.2).

Upon recognition of PAMPs or DAMPs by their specified TLRs, APCs produce
the inflammatory cytokines and costimulatory signals necessary for an up-regulation of
adaptive immune responses [17]. At the periphery site, dendritic cells or macrophages
capture the recognized pathogens and process them to allow a proper presentation to
adaptive immune cells, such as T-cells or B-cells. Phagocytosis of the pathogen leads to
activation and maturation of APCs, which then immigrate to nearby lymph organs to
interact with adaptive T- and B-cells [18]. B-cells can recognize the intact antigen in
soluble form, whereas the interaction of APCs with T-cells is a very sophisticated process
using a cellular complex called Major Histocompatibility complex (MHC) on its surface [19].

**Figure 1.1:** Signaling pathways of TLRs [20]
APCs express MHC class I and class II; their role in antigen presentation depends on the nature of the pathogens. Typically, extracellular infections (e.g. most bacterial infections) are presented by MHC class II molecules to the T-helper (Th) cells, whereas intracellular antigens (e.g. viral infections, tumors) are presented by MHC class I molecules to the cytotoxic T-cells (CTLs), which can directly eliminate the infected cells [21]. Along with antigen presentation, APCs express co-stimulatory signals, release various cytokines to activate natural killer (NK) cells (innate immune response) and amplify T-cell and B-cell effector functions (adaptive immune responses) [22].

Therefore, the type of MHC presentation partially influences the pathogen clearance strategies initiated by the adaptive immunity system, whether a humoral or cellular response. When antigens present through MHCs, several co-stimulatory molecules are expressed and cytokines are released, simultaneously driving T-cells to differentiate into diverse T-cell types, such as T-helper 1 (Th1) and T-helper 2 (Th2). The helper T-cells play a significant role in directing the activities of the immune system [23]. Th1 is related to the cytotoxic activity component of T-cells. Differentiated Th1-cells release cytokines, such as interferon (IFN)-γ, interleukin (IL)-2 and tumor necrosis factor (TNF)-β, which help to coordinate cytotoxic T-cell (CTL) activity as a component of cellular immune response. In contrast, the Th2-response (humoral response) stimulates the release of antibodies, such as IgG1, IgA and IgE by B-Cells [24]. TLRs play a critical role in communicating between the innate and adaptive immune systems. Therefore, targeting TLRs has become of considerable interest in the development of vaccine adjuvants in the recent past. A number of vaccine products with TLR-ligands as
vaccine adjuvants are now marketed or in the clinical phase for human use [25] [Table 1.2].

Table 1.2: Major examples for current TLRs adjuvants formulations that clinically tested or licensed. Adapted from reference [11, 26]

<table>
<thead>
<tr>
<th>Adjuvant</th>
<th>Classes</th>
<th>Outcome</th>
<th>Study phase</th>
</tr>
</thead>
<tbody>
<tr>
<td>CpG</td>
<td>TLR9 agonist-oligonucleotides</td>
<td>Enhance antibodies titers against the infectious pathogen, Th1 type immunity and CD8 T-cell mediated immunity in Melanoma vaccine therapy.</td>
<td>Phase III</td>
</tr>
<tr>
<td>Flagellin</td>
<td>TLR5 agonist - flagellin linked antigen</td>
<td>Enhance internalization of flagellin linked antigen, stimulate TNF-a production and Th1 &amp; Th2 immunity</td>
<td>Phase I</td>
</tr>
<tr>
<td>Polyl: C</td>
<td>TLR3 agonist - double standard RNA analogues.</td>
<td>Antibodies titer enhancement for nasal Influenza vaccination , Th1 type immunity and CD8 T-cell mediate immunity</td>
<td>Phase I</td>
</tr>
<tr>
<td>AS03</td>
<td>TLR4 – H5N1 and H1N1 influenza antigens.</td>
<td>Improve antibodies responses and cell mediated immunity.</td>
<td>Licensed - GSK</td>
</tr>
<tr>
<td>AS04</td>
<td>TLR4 (MPL) - Alum, HBV and HPV</td>
<td>Enhance seroprotection nearly 100 % and increase humeral and cellular immunity.</td>
<td>Licensed - GSK (Cervarix)</td>
</tr>
<tr>
<td>Imiquimod</td>
<td>TLR7/8 – Prostatic peptides</td>
<td>Imiquimod enhanced Ag specific antibodies and CD4 responses in cancer patients.</td>
<td>Phase I, II</td>
</tr>
<tr>
<td>Pam3Cys</td>
<td>TLR2- OspA - Lyme</td>
<td>Elicit high Ag-specific IgG antibodies protection (90%).</td>
<td>Phase II, III</td>
</tr>
</tbody>
</table>
1.4 Mucosal Immunity

Mucosal membranes cover the surfaces of the gastrointestinal, respiratory, and urogenital tracts. These membranes are the entry points for many dangerous pathogens and cause diseases such as influenza, tuberculosis, acquired immune deficiency syndrome (AIDS), herpes, diarrhea, and pneumonia [27]. The immune system, when challenged by a pathogen via the mucosal route, responds by activating the systemic and/or mucosal defense systems. A systemic response occurs via antibodies that are secreted into the bloodstream. A mucosal defense response occurs through secretory immunoglobulin type A (sIgA) antibodies that are secreted into the mucosal tissues [28]. Mucosal tissues are considered a first line immunity defense, which has led to a notable focus on mucosal vaccines in recent years. Conventional vaccines induce a poor mucosal immune response. The more effective way to induce a mucosal immunity response is to administer the vaccines directly to mucosal membranes. Mucosal vaccines are capable of inducing both systemic and mucosal protection responses against pathogens [29]. Unfortunately, the development of a mucosal vaccine has to overcome several challenges, which requires a better understanding of mucosal immunity [30]. These challenges include poor immune response, development of tolerance, limited antigen uptake, and safety due to the sensitivity of the mucosal surface.

Understanding mucosal immunity must begin with the study of the epithelial lining of mucosal membranes. This is the location of the majority of immune cells, including APCs, lymph nodes, and other immune components [31]. The lymph nodes form a pocket of tissue for lymphocytes in a structure called mucosal associated
lymphoid tissues (MALT), which are located below the mucosal surface. Although the MALT compartment function is separate from the systemic immune system, their structures are similar. MALT is populated by different types of lymphocytes, such as dendritic cells (DCs), and B- and T-lymphocytes. MALT has common structures that are relevant to mucosal membrane location, such as the gut-associated lymphoid tissues (GALT), bronchial-associated lymphoid tissues (BALT) and the nasal-associated lymphoid tissue (NALT) [32]. GALT includes specialized structures called Peyer’s patches, located in the small intestine. Overall, MALT structures cover approximately 80% of all immunoglobulin-producing cells in the human body [33]. MALT protects mucosal surfaces from pathogen colonialization, prevents the harm of commensal bacteria and elicits tolerance against consumable soluble substances. Therefore, MALT is exposed to a large number of antigens on a daily basis. Lumina propria are considered effector sites where sIgA antibodies are released, and where NKs and macrophages operate against pathogens [34]. Antigens are either taken up by assimilative epithelial cells or through specialized epithelial cells called microfold cells (M-cells). M-cells are located in Peyer’s patches and facilitate antigen transport across the epithelium [35]. M-cells were discovered in 1974 by Owen and Jones, who revealed their underlying role by having them sample exposed antigens (Fig.1.3). M-cells deliver dangerous antigens to the APCs of the innate immune system, mainly through DCs, B-cells, and macrophages [36].
Figure 1.3. Antigen sampling at mucosal surfaces: a collaboration of epithelial cells and dendritic cells.[37]

Distinguishing neutral antigens from pathogenic antigens in the mucosal membrane requires keen recognition of the danger signals, such as pathogen associated molecular patterns (PAMP) by PRRs. The recognition of pathogens in mucosal surfaces involves the uptake of foreign substances at the epithelium by DCs or the transport through M-cells or goblet cells to get underneath APCs for further processing and maturation [38]. Innate immune cells at mucosal membranes express different PRRs. It is thought that NOD1 is predominately expressed in the innate cells of lungs and intestines. Toll-like receptor 4 (TLR4) is thought to be predominantly expressed in the respiratory area and vaginal tract, whereas TLR7 is expressed in the human intestinal mucosa [29].
APCs are activated through PPRs in response to danger signals. APCs release costimulatory molecules and present processed antigens to adaptive B- and T-cells or they may be transported to local lymph nodes for maturation and further activation [39]. The presentation of peptide fragments through major histocompatibility complex class I or II (MHC-I or MHC-II) molecules on the APC surface, which display the antigenic material for homing CD 4+ or CD 8+ T-cells, depending on the type of antigen. After B and T adaptive cells are activated, they migrate through the lymphatic system to mucosal sites where they differentiate into effector and memory cells [40]. The activated mucosal B-cell response to the second exposure to an antigen reacts by producing sIgA at the mucosal membrane. sIgA is the only antibody to resist protease degradation in the mucosal environment and survives for a long period [41]. sIgA is the dominant antibody secreted in the mucosal fluids of the intestine, internasal mucosa, and saliva. It is a dimer of two monomeric IgA molecules that are covalently linked by a joining chain (J chain) molecule. The molecules bond together with a component molecule produced by mucosal epithelial cells, which help with the resistance of protease cleavages [42]. The protective role of sIgA at the mucosal surfaces gives it the ability to neutralize pathogens and inhibit them from penetrating through and adhering to the mucosa. This protection role is demonstrated by its ability to decrease the attachment of influenza virus and inhibit virus internalization [43]. sIgA also suppresses the proliferation, colonization, and entrance of pathogens across the epithelium [44].
1.5 Mucosal Vaccines

The main goals of mucosal immunization are to generate an adaptive response at the site of pathogen invasion and to prevent infections or diseases by activating humoral mucosal defense (sIgA) and systemic humoral and cellular responses. The local immune response depends on the vaccine's route of administration. Intranasal vaccination leads to a strong mucosal response in the respiratory tract [36]. Interestingly, some mucosal vaccines, such as Kennel Cough vaccine, can generate a response within 48 to 72 hrs.; this indicates a possible use for fast control of endemic infections[45]. After mucosal membrane exposure to the antigen and the activation of memory T-cells, a portion of the memory T-cells migrates to mucosal effector non-lymphoid tissues. These cell populations, called resident memory T-cells, produce a fast effector function and provide high protection against infection when re-exposure occurs [46]. For mucosal therapeutic vaccination against local lung tumors, local delivery of the antigen has been shown to induce local effector cytotoxic T-lymphocytes (CTLs) by local DCs through upregulation of different T-cell subtypes (CD49a, CD103, and CD8þ) in the lung parenchyma [47]. Only the intranasal route was able to trigger local DCs and cause infiltration of the tumor by CD8, thus causing tumor regression. The common mucosal response, which is signified by the immune response in multiple mucosal tissues, is one of the highlighted advantages of mucosal vaccines. Mucosal immunization routes, such as oral, rectal, and intranasal, can encourage general mucosal immune responses at distant effector sites. In fact, respiratory immunizations induce antibody responses in a broad variety of tissues, including saliva and the urogenital tract (Fig. 1.4).
In contrast, oral immunization induced a more restricted mucosal response, which may be due to their restricted homing receptor profile. [43]. In addition to these advantages, mucosal vaccination has also improved the cost, safety, and physical and psychological comfort of vaccine administration. However, despite the advanced features that mucosal vaccination can add to modern vaccines, only a few licensed mucosal vaccines are available now, due to the challenges of generating a strong immune activation by this route.

Notably, most of the licensed mucosal vaccines are composed of weakened viruses or whole pathogen materials. Recently, subunit vaccines have emerged as an alternative, potential approach. Subunit antigens include soluble or recombinant proteins, which are weak immunogens. Therefore, the development of subunit mucosal vaccines has evolved to ensure a suitable delivery or adjuvant system, aiming to augment the immune response, reduce the toxicity risk and improve the cost over conventional vaccines [48] [Table 1.3]. The route of administration, along with the appropriate adjuvant and delivery system, is targeted for better antigen delivery and greater stimulation of APCs. Large particle forms of antigens or synthesized nanoparticles have been reported to possess an immune-stimulating feature, as they have pathogen-like forms and can be immunogenic by themselves or with the incorporation of PAMPS [49].
Figure 1.4. Antibody response in mucosal immune system [50].
Table 1.3: Licensed Mucosal Vaccine

<table>
<thead>
<tr>
<th>Infections.</th>
<th>Antigens.</th>
<th>Route of Administration.</th>
</tr>
</thead>
<tbody>
<tr>
<td>S. Typhi</td>
<td>Live-attenuated S.</td>
<td>Oral</td>
</tr>
<tr>
<td>Poliovirus.</td>
<td>Live-attenuated poliovirus.</td>
<td>Oral</td>
</tr>
<tr>
<td>Rotavirus.</td>
<td>Live reassortant rotavirus, G1, G2, G3.</td>
<td>Oral</td>
</tr>
<tr>
<td></td>
<td>Live-attenuated human virus G1p.</td>
<td></td>
</tr>
<tr>
<td>Cholera</td>
<td>Killed whole-cell O1, O139, and recombinant CTB subunit.</td>
<td>Oral</td>
</tr>
</tbody>
</table>

1.6 Strategies to Induce Mucosal Immune Responses

1.6.1 Local Administration

The stimulation of APCs at the mucosal sites, including oral, internasal, or intratracheal sites, promote T-cell homing at the same site. The oral and nasal routes are the most suitable to trigger the cellular machinery which initiates IgA and induces an adaptive effector response in mucosal tissues. For example, the oral vaccines against pathogens such as *S. Typhi and Poliovirus* have been available for decades and have generated efficient and adequate immune protection for individuals [51]. Another example is the immunization of children using the intranasal formulation of the influenza vaccine, FluMist, which results in greater protective immunity compared to the intramuscular route [52]. The aerosol administration of live-attuned Edmonston-Zagreb measles vaccine generates high serum antibodies and mucosal IgA titers when compared to the subcutaneous route [53]. Intranasal vaccination with an influenza virus, in combination
with the outer membrane of Neisseria meningitis, resulted in a significant increase in serum and nasal wash antibodies with no observed toxicity [33].

### 1.6.2 Mucosal Adjuvants

Although most known parenteral vaccine adjuvants use augmented systemic antibody cellular immune response, most of them are not as effective as mucosal adjuvants [6]. Continuous efforts are underway to develop adjuvants for subunit vaccines that trigger cellular events which involve IgA secretion or induction of adaptive effector cells at mucosal sites [54]. Despite the fact that local delivery of mucosal vaccines has a better potential to induce mucosal immunity due to their low immunogenicity, immune enhancers are a necessary part of the mucosal vaccines. This can be achieved through an integrated adjuvant system where the system provides improved retention at the site of administration, better antigen delivery, as well as immune activation through specific PRRs [55]. Delivering vaccines through a particulate system offers efficient delivery and protection of the antigen cargo in a harsh environment. Bioengineered cationic nanoparticles showed increased adherence to the epithelial cells at the nasal mucosa; this format was shown to be significantly effective in boosting immune responses [31]. The fabricated biocompatible particles and biodegradable polymer are more suited to uptake by Peyer’s patches or APCs at the mucosal sites. Furthermore, some of the nonmicrobial products (e.g., oil emulsions, liposomes or nanoparticles) act as mucosal adjuvants by delivering antigens or by causing mucosal damage or disruptive stress leading to the release of DAMPs at the site of vaccination. DAMPs adjuvants, such as hydroxypropyl–
B-cyclodextrin, work in the nasal mucosae and cause epithelial damage at high concentrations, release the DNA of hosting cells and induce Th1 [48]. For example, the Endocrine™ adjuvant also works by inducing cell death and by releasing RNA at local mucosal cell sites after nasal administration, which suggests DAMPs stimulation pathways [56]. Exogenous mucosal adjuvants are not limited to DAMPs molecules, but also include a wide range of discovered PAMPs, which have been extensively studied as systemic and mucosal vaccine adjuvants. A number of these molecules have successfully made it to the market. Examples of PAMPs include bacterial toxins, CpG motifs, monophosphoryl lipid A, saponins and live vectors.

_Bacterial toxins and their derivatives_

Nontoxic derivatives of the cholera toxin (CT) and Escherichia coli (LT) have been intensively applied as adjuvants to induce mucosal immunity. They showed the capability to induce strong systemic and mucosal antibody immune responses and evoke cellular immunity in vaccinated models [57]. As a mucosal adjuvant, CT derivatives have demonstrated strong responses through intranasal administration; however, in the murine model, toxicity in the olfactory nerve was observed. Therefore, the development of mutant LT, in the form of LT-k3, was used as a safe and potent adjuvant for intranasal co-administration of recombinant measles virus nucleoprotein [58]. Despite the lower toxicity of mutant forms of CT and LT, the toxicity of others toxins as mucosal adjuvants, remain uncertain and safety is still is a major concern [59].
Monophosphoryl lipid-A (MPLA)

MPLA is a detoxified derivative of LPS from Salmonella Minnesota R595, Gram-negative bacterium. It is a TLR4 agonist that proved to induce mucosal immunity when administrated by mucosal routes. MPLA has been introduced into various formulations and marketed in combination with Alum (AS04) in the commercial prophylactic vaccine, Cervarix [48]. To induce mucosal immunity, intravaginal administration of MPLA and saponin formulations was shown to enhance systemic and mucosal immunity in an HIV vaccine study with macaques [60]. Furthermore, MPLA induces a strong systemic response after intranasal vaccination of mice, with the addition of a cationic liposome-hyaluronic acid hybrid nanoparticle and the antigen Yersinia pestis [61]. In addition, a comparison study was done with MPLA mucosal adjuvant and the two potent mucosal adjuvants, CpG and the B subunit of Escherichia coli (LTB), for use as a pulmonary vaccination with Mycobacterium tuberculosis. The results reported that MPLA generated a Th-17 type response by increasing IL-17A levels in the lungs and spleen [39].

CpG motifs

The oligodeoxynucleotide (OND), containing unmethylated cytosine-phosphate-guanine motifs (CpG), is a well-known TLR9 agonist, which stimulates CD4+ Th1 and CD8+ CTL responses and induces protective immune responses such as those triggered by bacterial DNA. CpG has been used intranasally with anthrax lethal toxin and produced promising data as a mucosal adjuvant. It induced high levels of Ag-specific mucosal sIgA and serum IgG2a antibodies against anthrax lethal toxin [28]. Moreover, CpG was tested in a
pneumococcal vaccination. Nasal administration of a plasmid encoding the cDNA of the Flt3 ligand and CpG adjuvant improved mucosal protection [62]. Additionally, mucosal administration of CpG adjuvants is believed to be associated with less toxicity. In in vivo studies, CpG-ODN demonstrated a potent Th1 type immune response. The administration of CpG ODN via the lung route for treatment of allergies has tested safe in primates and humans [63].

Saponins

Saponins are glycosides containing a hydrophobic backbone of a triterpenoid structure linked to a carbohydrate chain [64]. Saponins were found to induce strong immune responses against antigens, including cytotoxic T-cell responses [65]. The saponin component, Quil A, has the ability to interact with the membrane cholesterol, disrupt the intact barrier and form pores which increase the antigen uptake to induce strong immunity. When it is administrated orally in the form of a lipophilic immune-stimulating complex (ISCOMs), the saponin adjuvant Quil A reported a significant systemic immune response, including Th1, Th2 and protective secretory IgA antibodies [66]. Another study focused on a semi-synthetic derivative of the saponin adjuvant, GPI-0100, used as a mucosal adjuvant for influenza subunit vaccines with different mucosal routes. Strong mucosal antibody response in the respiratory tract was observed with intrapulmonary and intranasal vaccination. In addition to the protective mucosal response, GPI-0100 elicited strong systemic immune responses [67].
Live vectors

Bacterial carrier systems, such as live bacteria vectors and lactic acid bacteria systems, have grown rapidly as DNA vaccine adjuvants through mucosal routes, including intranasal, oral, or intravaginal routes [68]. For example, vectors derived from salmonella have been used as vaccine cargo to deliver antigens and to elicit mucosal and systemic levels of protective antigens in appropriate lymphoid inductive sites. Most of the live vector systems have been reported to induce both antibody and T helper responses against recombinant antigens [69]. Additionally, these vectors have also been utilized to develop therapeutic cancer vaccines that deliver tumor-associated molecules, such as cancer antigens, RNAs or DNA.

1.7 Mucosal Vaccine Development: Challenges

Despite continuous efforts in the development of mucosal vaccines, only a few have been approved for use in the market, in contrast to the many registered injectable vaccines. The key challenge has been the low immunogenicity of antigens when delivered to mucosal surfaces due to their poor accessibility to local immune cells under the nasal epithelial barrier (Fig. 1.3). The natural protection barrier created by mucus production at the mucosal membrane and its transport through cilia, which are meant to keep pathogens away from the epithelium, limits antigen retention and transportation across the epithelium. Moreover, the high molecular weights of the antigens hamper their transport across the epithelium to reach the lamina propria where the majority of the local immune cells reside. Antigen degradation at the mucosal surface also contributes to poor antigen
delivery. This is a concerning problem for unprotected protein, particularly in the gastrointestinal tract. Further difficulties appear with the tolerance nature of mucosal immune response surfaces.

To address those problems, advanced vaccine delivery systems that have the potential for immune activation are required. As mentioned before, the rational design for a vaccine system should have safe and efficient adjuvants, as well as an appropriate delivery system to improve antigen protection, increase antigen uptake and enhance the immune response to the antigen.

1.8 Next-Generation Mucosal vaccine Strategies

1.8.1 Nano-Vaccine Delivery Systems

Drug delivery systems which are based on polymeric or lipid particles are part of many licensed pharmaceutical products. However, very few licensed vaccines are based on particulate systems [70]. Polymeric particulate systems have several advantages for use in vaccine delivery, which include their ability to be recognized and taken up by APCs due to their particulate nature, their versatility to modulate the particle size and shape, and their charge to suit the target in the immune system [71]. The size range of various reported vaccine delivery systems is described in Figure 1.5.

It has been suggested that smaller polymeric particles produce higher antibody titers than larger particles, due to the higher amount of antigen adsorption. One of the
well-known vaccines nano-particulate delivery systems is PLGA polymer-based nanoparticles. For example, PLGA particles encapsulating ovalbumin and CpG ODN were found to have better uptake by DCs and good up-regulation of CD86 and MHC class I molecules on BMDCs, when compared to particles >300nm [70]. Additionally, polymers can also be used as self-adjuvating delivery systems, therefore increasing the resident time and delivering efficient antigens or epitopes to immune cells at mucosal membrane [72]. Thus, the polymeric nanoparticle became particularly attractive because it was found to stimulate better antigen uptake by APCs [72]. In addition to providing antigen protection by facilitating antigen transport to immune cells, the key factor in nanoparticulate delivery systems is boosting immune response. Similarly, researchers are showing a growing interest in using natural polymers based on polysaccharide to prepare nanoparticle adjuvants. Particles have been prepared from polymer-like chitosan, pullulan, and inulin [31]. For instance, chitosan-based nanoparticles have gained much popularity for vaccine delivery due to their high safety profile, biocompatibility, and ease of modulating the system [51]. Several studies used chitosan nanoparticles with various vaccines, including DNA and HBV vaccines. Another example of natural polymer-based particles is the inulin-based nanoparticles, which are known as potent adjuvants due to their activation of the complement system via the alternative pathway [52]. AdvaxTM is a commercial nanoparticle adjuvant derived from inulin, which has been shown to enhance the immune response in influenzas and hepatitis B vaccines [42].
Other advantages of nanoparticle systems are their capability to be modulated into a mucoadhesive particle and to enhance the uptake of antigens by nasal epithelial cells and/or M-cells. In addition, targeting M-cells may improve the uptake and transport of antigens to the sub-epithelial region of the NALT and subsequent secondary lymphoid organs. Apart from lymphatic distribution, many studies have supported the utility of nanoscale-modified material to transport and facilitate the move molecules or antigens through the gastrointestinal tract or respiratory route.
1.8.2 Targeted Adjuvants: TLR Agonists

The most striking feature of next generation vaccine delivery components is their immunogenicity and their ability to attract APCs[73]. Throughout the examples mentioned earlier, the recent advances in successful mucosal adjuvant candidates were oriented toward using APC receptor ligands or improving delivery systems. Thus, the current trends are to achieve better immune responses based on mimicking pathogens by delivering vaccines through the particulate system using immune-attracting properties. TLR targeting is an activation feature that can be fabricated into the particulate system with micro- or nano-sized particles, representing viral and bacterial shapes, as described below.

1.9 TLRs in Mucosal Immunity

TLRs are expressed by many cell types in the mucosal tracts, including epithelial cells, monocytes, macrophages, and dendritic cells [74]. TLRs (e.g. TLR2, TLR3, TLR4, and TLR7/8) play an essential role in maintaining the integrity of the epithelial cells and controlling the mucosal immune system [75]. TLRs influence the initiation, maintenance, and progression of inflammatory diseases. TLR receptors also perform a strong role in innate immune signaling by maintaining the right balance of the microbiota in the mucosal compartment [76]. The expressions of TLRs in the mucosal system vary, influenced by diseases, bacteria or dysregulation of the immune responses [74]. The nature of the adaptive immune responses at the mucosal membrane also shows unique features, including preferential secretion of IgA and a large population of T-helper 17
(Th17) cells, a CD4+ T-cells subset (characterized by the production of highly inflammatory cytokine), and interleukin-17. Although Th17 is not TLR dependent, TLR signaling involves either induction of mucosal protection, IgA responses, or an adaptive immune response which influences Th1 and Th2 responses [77]. Several potent TLR ligands have been studied as adjuvants for mucosal immunizations in different models and through different routes of mucosal administration. Examples of those adjuvants include FSL-1 (TLR2/6), poly I:C (TLR3), MPLA (TLR4), CpG-B (TLR9), Pam3CSK4 (TLR1/2), and R848 (TLR7/8) [78].

1.10 Inulin Acetate is a Polymer-based TLR4 Agonist

Inulin is a natural fructan classified as a dietary fiber, in which an unbranched chain of fructose moieties is linked to terminal glucose [79]. Inulin was introduced to the pharmaceutical industries as a stabilizer for drug or protein formulations, peptide-based drugs and vaccine formulations [80-83]. Inulin can exist in both water-soluble and water-insoluble forms, depending on the procedures used to precipitate it. The insoluble forms of inulin (gama and delta) have been shown to activate the immune system through alternate complement pathways [79].

Recently, our group has shown that hydrophobic acetylated inulin (inulin acetate, InAc) stimulates the innate immune system through the activation of TLR4 on APCs. However, the parent compound inulin failed to activate TLR4 [84]. Furthermore, InAc did not activate the alternate complement pathway, similar to insoluble inulin [84]. The TLR4 agonistic activity of these polymers was established in multiple immune cells
(microglials, dendritic cells, and PBMCs) by various genetic and pharmacological approaches.

The investigational study by our team has shown that acetylated inulin (inulin acetate) particles activate the innate immune system, thus acting as a potent vaccine adjuvant [84]. Inulin Acetate is synthesized as a polymeric immune active compound with TLR4 agonistic activity. It has the ability to stimulate multiple immune cells, such as in microglial, dendritic, peripheral blood mononuclear and exclusive TLR4 expressed cell epithelial cells [84].

1.1 Pathogenic-Mimicking Delivery System prepared using InAc as a Biomaterial

By using InAc as an immune-active polymer, our laboratory has rationally engineered a “Pathogen-Mimicking Vaccine Delivery System” (PMVDS) that could potentially encapsulate multiple antigens [85]. The uniqueness of our PMVDS is that it is both an efficient vaccine delivery system, similar to nanoparticles, and a vaccine adjuvant due to its ability to activate TLR4. The polymers and PMVDS particles were thoroughly characterized by a myriad of physicochemical techniques. The effect of the size of the particles and the dose of an antigen and adjuvant on immune activation was studied in mice. The safety of PMVDS was assessed using cytotoxicity, skin histochemistry and in-vivo imaging techniques. The robustness of PMVDS in preventing and treating diseases was investigated on influenza and melanoma mouse models.

The first study to examine the InAc particulate-based PMVDS reported the ability of such a system to target antigen-presenting cells, such as dendritic cells. [85]. PMVDS
offered a polymeric vaccine adjuvant delivery system that activated the innate immune system via TLR4 and produced a high humoral and cellular response. A nanoparticle model using InAc as the polymer to target immune cells also showed advanced results, compared to PLGA nanoparticles. It showed efficient encapsulation of the antigen and potent activation of multiple immune cells, including DCs and primary swine and human cells [86]. The uniqueness of the PMVDS technology is its combination as both vaccine adjuvant and delivery system. InAc particles were able to enhance immune response when injected along with an antigen (Ova) [85]. However, encapsulation of the antigen in InAc particles in the form of PMVDS advanced the humoral response significantly and generated significant cytotoxic T-cell activity against cancer cells. The advanced feature of PMVDS over other adjuvant systems is attributed to the utilities of the polymeric particulate system as a delivery system. Thereby, it offers the advantage of delivering both an antigen and an adjuvant to the same cell, which has been shown to be critical in determining the adaptive immune response [87] (Fig. 1.6).

In addition to the vaccine delivery system and the protection of the antigen, PMVDS could be used to deliver other interested molecules to immune cells, such as small molecule-based vaccine adjuvants (chapter IV). One of the advantages of PMVDS technology is the affordability of the raw materials and the ease of preparation, which is essential when translating lab discoveries into field/clinical applications.

Being a particulate delivery system, PMVDS offers several future opportunities, such as tunable physical properties, the prospect of encapsulation, the controlled delivery of multiple antigens, and simultaneous incorporation of other immune-stimulatory factors
or PAMPs. There is further potential for InAc particles to target M-cells for mucosal immunity. M-cells represent a critical portal for mucosal drug and vaccine delivery. Several studies showed that delivery through M-cells could be achieved using particulate delivery vehicles, such as PLGA or liposomes. The delivery through M-cells can vary, depending on the properties of the delivery system [88].

Figure 1.6. PMVDS adjuvant and vaccine delivery systems [85].

Based on previous findings, we hypothesize that PMVDS could be an important candidate for mucosal vaccine delivery due to its modulating particulate features, targeting properties and immune potentiation nature.

In this dissertation, the ability of InAc nanoparticles to stimulate both systemic and mucosal immunity after intranasal administration was investigated in mice and pigs
using model antigen ovalbumin and influenza viral subunit antigen M2e peptide

(Chapter-II& III). Further more, InAc-NPs were utilized to deliver small molecular-based TLR7 agonist C-563 along with the antigen as a multifunctional vaccine delivery system (Chapter IV) to activate robust systemic and mucosal immunity.
Chapter-II

Novel Immune Active Nanoparticles as Intranasal Vaccine Delivery System for Inducing Systemic and Mucosal Immunity.
2.1. Introduction

Nasal vaccination often elicits mucosal immune responses in the respiratory tract, which is important in preventing numerous respiratory infections such as influenza and bacterial pneumonia. In contrast to injectable vaccines, intranasal vaccinations have critical ability to provide antibody-mediated protection at the respiratory mucosal site of pathogen entry[89]. In addition, they exhibit common protection at various distal mucosal tissues such as intestinal and urogenital tracts. Therefore, intranasal vaccination has been an attractive preventive strategy for not only respiratory infections but also genitourinary infections[54]. Despite these advantages, producing a protective immunity against several mucosal diseases is still a challenge that often requires large doses. This may be due to the instability of antigen at the mucosal surface or lack of suitable mucosal vaccine adjuvant[90].

The effective strategies are utilizing live attenuated organisms for nasal vaccination, and a good example is a seasonal influenza virus vaccine (FluMist®). However, FluMist is limited to only individuals with strong immunity (2–49 years of age), leaving the most susceptible populations such as infants and the elderly [91]. Moreover, as a live attenuated vaccine, FluMist has the high unpredictability of protection against different strains of the same pathogen and has a potential risk of adverse side effects. These challenges shift the focus on developing subunit intranasal vaccines[48]. However, subunit antigens are not highly immunogenic and often require a potent vaccine adjuvant and special delivery system to boost the immune stimulation. The current vaccine adjuvant
discovery research is mainly focused on pathogen recognition receptor (PRRs) present on professional antigens presenting cells (APCs) as targets, such as a Toll-Like receptor (TLRs) or Nod-Like Receptors (NLRs). TLR ligands mimic pathogen-associated molecular patterns (PAMPs) and initiate signaling for a strong adaptive immune response [26]. Of several TLRs discovered, TLR4 has been a major target of vaccine researchers for both systemic and mucosal immunity. The first FDA-approved TLR4-based adjuvant is AS04 in human papillomavirus vaccine, Cervarix. It contains alum and MPLA (Monophosphorylate lipid-A), a TLR4 ligand as active ingredients [54],[92]. In addition to immune adjuvants, several particulate vaccine delivery systems have been reported based on encapsulating immune stimulant and subunit antigen in the form of nanoparticles or microparticles for improved immune stimulation [92, 93]. Particulate delivery system provides several advantages including, increased antigen stability by protecting it from degradation from both exogenous and endogenous factors, and improved immune stimulation by increasing the uptake of antigen by APCs. Thereby, particulate vaccines greatly enhance the efficacy of the intranasally administered vaccines [94],[95].

In a search for novel vaccine adjuvants, our laboratory previously reported the discovery of a polymeric toll-like receptor-4 (TLR4) agonist, inulin acetate (InAc) that activates TLR4 on several APCs including, microglial, dendritic, and peripheral blood mononuclear cells from various species [84-86]. InAc was synthesized from a plant polysaccharide inulin[85]. Furthermore, we have prepared both nanoparticle- and microparticle-based pathogen-mimicking vaccine delivery systems (PMVDS) using bioactive InAc as a polymer to improve antigen delivery to APCs and induce a higher
immune response [84, 85]. A particulate-PMVDs stimulated both humoral and cellular immune responses against the encapsulated antigen when delivered through subcutaneous or intradermal routes. However, through parenteral routes tested, InAc-based vaccines failed to stimulate mucosal immunity.

In this chapter, we evaluated the ability of InAc-based nano-vaccine delivery system (InAc-NPs) as a versatile platform for mucosal vaccination through intranasal delivery. InAc-NPs with around 250 nm in diameter were prepared with ovalbumin as an antigen. InAc-NPs as a delivery system activated mouse macrophage cells to release inflammatory cytokines, however, failed to stimulate macrophages from mice deficient in TLR4 receptors, which further affirms the TLR4 agonistic properties of the delivery system. When administered intranasally to mice, immune-active InAc-NPs produced very strong systemic (IgG1 and IgG2a) and mucosal antibody response (sIgA) against the encapsulated antigen. Strong secretory-IgA (sIgA) antibody titers were recorded in various mucosal tissues such as lungs, intestine and nasal-associated lymphoid tissue (NALTs). InAc-NPs provide a unique immune-active delivery system as a platform technology for various mucosal vaccines.

2.2. Materials and Methods

2.2.1. Materials

Inulin was purchased from MP Biomedicals Solon, OH, USA. The poly (lactic-co-glycolic acid) (PLGA) was purchased from Sigma-Aldrich, St Louis, MO, USA. Monophosphoryl lipid A (MPLA) and Lipopolysaccharide-RS (LPS-RS) was purchased
from Invivogen, San Diego, CA, USA. Macrophage cells (NR-9456 & NR-9458) were obtained through BEI Resources, ATCC, Manassas, VA, USA. Mouse macrophages were cultured in RPMI glucose medium (Thermofisher Scientific, USA), supplemented with antibiotics (penicillin/streptomycin) and 10% fetal bovine serum (FBS). MPLA and endotoxin free ovalbumin (Ova) was purchased from Invivogen, Sandiego, CA, USA. All other chemicals were purchased from Fisher Scientific, Pittsburgh, PA, USA.

2.2.2. Synthesis of inulin acetate and preparation of nanoparticles

Inulin acetate (InAc) was synthesized by acetyllating hydroxyl groups of inulin using acetic anhydride and characterized by Fourier transform infrared (FTIR) and proton NMR spectroscopy as described previously [84, 85]. Inulin acetate nanoparticles (InAc-NPs) were prepared with and without the antigen ovalbumin by double (w/o/w) emulsion followed by solvent evaporation technique as described in earlier manuscript[86]. Briefly, the primary emulsion was prepared with an aqueous phase of 10 mM phosphate buffer (PB, pH7.4) containing 2% (w/v) Pluronic F-68 solution as a surfactant, with/without Ova. The oil phase was prepared by dissolving InAc (100 mg) in dichloromethane (DCM). The aqueous solution was added drop by drop to the polymer solution to form a primary (w/o) emulsion. Then, the formed emulsion was added drop-wise into water containing 0.5% (w/v) polyvinyl alcohol. The mixture was sonicated with an ultrasonicator probe for 5 min, 10 seconds on - 1 second off cycle, using a Q500 Sonicator (Qsonica, CT). To evaporate DCM, the final emulsion was stirred for 12 hrs., and the precipitated particles were collected via centrifugation at 50,000xg for 30 min at
4°C and lyophilized. PLGA nanoparticles (PLGA-NPs) were prepared by a similar method by using PLGA instead of InAc[84]. The nanoparticles prepared without antigen are called blank-nanoparticles.

2.2.3. Size and zeta potential

Size and zeta potential of nanoparticles (NPs) were measured by a dynamic light scattering (DLS) technique (Malvern Zeta-Sizer, Malvern Ltd, MA, USA). The NPs were re-suspended in a filter-sterilized citrate buffer (10 mM, pH 7.4), and diluted using filter sterilized water before recording particle size and z-potential.

2.2.4. Scanning electron microscopy (SEM)

The shape and size of InAc-NPs were evaluated using a scanning electron microscope (SEM Model S-3400N, Hitachi, Japan). For the preparation of samples, lyophilized powder free from cryoprotectant was mounted on a metal holder using conductive double-sided tape. The particles were sputter coated with a 10-nm gold layer before analysis. The micrographs were captured at a beam voltage of 5 kV, and 50,000×g magnifications were used for imaging, with a working distance of 5-15 mm and a spot size of three. The ImageJ software was used to measure the average diameter of the particles, with the average diameter representing at least 100 particles.

2.2.5. Determination of Ova loading

The amount of antigen encapsulated into the nanoparticles was calculated as described elsewhere[84]. A measured amount of Ova containing InAc-NPs was added to acetone
and incubated at 37 °C for 4 hrs. As the acetone dissolved the InAc polymers, the Ova was precipitated, which was collected by centrifuging at 10,000 xg for 30 min. The collected pellet was dissolved in 1% sodium dodecyl sulfate (SDS) solution. The Ova in SDS solution was quantified by a Pierce™ Bicinchoninic acid (BCA) Protein Assay Kit (Thermo Fisher Scientific Inc., Waltham, MA, USA), according to manufacturer’s instructions. The Ova loading (n = 3–4) was reported as μg of Ova present per mg of InAc-NPs (w/w).

2.2.6. Determination of endotoxin levels

The endotoxin levels in the final preparation were determined using the ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit from GenScript (Piscataway, NJ), following the manufacturer's instructions. All formulations used in this study have a low detection limit of endotoxins as per the United States Pharmacopeia for parenteral administration [96].

2.2.7. In-vitro antigen release studies

To study the release kinetics of antigen from InAc-NPs, Ova loaded InAc-NPs (10 mg) were dispersed in 10 ml of 10 mM PB (pH 7.4) and stirred at 100 RPM at 37 °C using an orbital shaker. At predetermined time intervals, an aliquot of 500 μl was withdrawn and centrifuged at 20,000g to collect the supernatant. The amount of Ova released in the supernatant was determined by the Micro-BCA method after filtering through a 0.2 μm filter.
2.2.8. TLR4 activation assay

The effect of InAc-NPs on activating TLR4 was studied using mouse macrophages (0) from wild-type and TLR4 knock out mice[15]. Triplicate wells containing macrophages were incubated with different concentrations of blank InAc-NPs without antigen (50, 100 and 250 μg/ml) or PLGA-NPs (250 μg/ml) for 48 hrs. A well-known TLR4 agonist MPLA (2 μg/ml) was used as a positive control. The activation of macrophages was quantified by determining the levels of TNF-α in the culture supernatants using TNF-α ELISA Ready-SET-Go Kit (e-Bioscience, San Diego, CA). The concentration of TNF-α was calculated based on a standard curve generated with mouse recombinant TNF-α. The TNF-α levels from untreated cells were considered as a background[97].

2.2.9. Cellular uptake of InAc-NPs in the macrophage

The cellular internalization or uptake of InAc-NPs loaded with fluorescently labeled Ova (FITC-Ova) was studied using mouse macrophage cells. Macrophages were cultured on glass coverslips for 24 hrs. for attachment. Subsequently, the cells were treated for 1 hr with the following agents: no treatment (only media), FITC-Ova as a solution (25 µg/ml) and FITC-Ova delivered through InAc-NPs. The cells were washed with cold 50 mM phosphate buffered saline, pH 7.4 (PBS) for 3 times followed by fixing with 4% w/v paraformaldehyde in PBS. Cellular uptake of FITC-Ova was observed under a fluorescence microscope.
2.2.10. Mice Immunization

The experiments involved using animals were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC) of the South Dakota State University. Male BALB/C mice (n = 4-5 per group, 6–8 weeks old) purchased from The Jackson Laboratory (Bar Harbor, ME) were used for all experiments. Immunizations were performed under light isoflurane anesthesia. Mice were immunized intranasally (i.n) with 10-15 μl of vaccine formulations per nostril using a micropipette. The antigen Ova (20μg / dose) was administered as a solution in saline or through PLGA-NPs or InAc-NPs (~2 mg of NPs) as a delivery system. Primary immunization (day 1) was followed by two booster doses with 2-weeks apart. Blood was collected on days 14, 28, and 42. The serum was separated using Microtainer serum separator (BD Biosciences, San Jose, CA) and stored at -20 °C until further analysis. On day 42, lung fluid (20-30 μl) was collected from euthanized mice after carefully administrating 50 μl PBS with protease inhibitors into the lungs through the trachea. The intestinal washes were collected after the entire small intestines were surgically removed from euthanized mice and perfused with 10 ml PBS with protease inhibitors. The supernatants from the intestinal washes were collected after centrifugation at 5000 xg at 4°C. The lung and intestinal washes were stored at −20°C until further use.
2.2.11. Nasopharynx-associated lymphoid tissue *ex-vivo* culture

On day 42 after the primary immunization, the Nasopharynx-associated lymphoid tissues (NALT) palates were surgically removed from each mouse under aseptic conditions. Each individual palate was intensively washed with a complete medium consisting of RPMI-1640 supplemented with 10% fetal bovine serum, 100 μg/ml streptomycin, 100 UI/ml penicillin, and 50 μg/ml gentamicin. The plates were cultured for 36 hrs. in 48-well plates (Becton Dickinson, Franklin Lakes, NJ) containing 500 μl of the fresh complete medium at 37°C with 5% CO₂. Supernatants were collected from each NALT culture, centrifuged to remove fragments and stored at −20°C until further analysis [98].

1. NALTs were surgically removed from euthanized mice.
2. After proper washing, NALTs were incubated in medium at 37 °C, for 36 hrs.
3. Sample were collected by centrifuging supernatants at 5000 xg, sample were analyzed for sIgA using indirect ELISA.

Figure 2.1. Ex-vivo culture for Nasal Associated Lymphatic Tissues.

2.2.12. Splenocyte proliferation assay and Cytokine analysis: *ex-vivo*

Single cell suspensions of the splenocytes were prepared from the spleens of immunized mice, as described previously [85]. The splenocytes (1 × 10⁶ cells/well) were seeded in triplicate into a 96-well plate, either with Ova (100 μg/ml) or with concanavalin A (5
µg/ml; Con A) as a mitogen. After 3 days of incubation at 37°C (5% CO₂, 95% humidity), the plates were centrifuged for 10 min at 4000 xg to pellet the cells, and 200 µl of supernatant was collected for the analysis of secreted cytokines. Cell proliferation was assessed in the same plates by adding 50 µl of 3-(4, 5-dimethylthiazol-2-yl-2, 5-diphenyltetrazolium bromide) (MTT) assay as described in earlier manuscripts [85]. Stimulation indices (SI) were calculated by dividing the mean OD₅₄₀ of Con A, or Ova treated cells by the mean OD₅₄₀ of untreated cells containing the only medium.

2.2.13. Cytokine analysis

Sandwich-ELISA assay was used to measure the cytokines in the supernatants of the splenocytes culture. The assay was performed using a Mouse Th1/Th2 ELISA Ready-SET-Go Kit (e-Bioscience, San Diego, CA) as per the kit instructions[99].

2.2.14. Statistical analysis

Results were expressed as mean ± standard deviation (SD). The data were analyzed using Instant Graph Pad software (CA), and the significant differences in the mean were tested by Student's t-test or two-way ANOVA followed by Bonferroni's or Dunnett's post-hoc multiple comparison tests for statistical significance. P<0.05 was considered a statistically significant difference unless specified under figure legends.
2.3. Results and Discussion

A strong immune activation at the mucosal surface serves as a first line of defense against the entry of mucosal pathogens. A vast majority of human vaccines are administered parenterally, which often fail to provide protection at the mucosal surface. Due to the paucity in the availability of efficient technologies, there are only very few mucosal vaccines available in the market. Therefore, the development of a safe and effective mucosal vaccine adjuvant/delivery system has been the priority for non-replicating or subunit-based vaccines. In a previous study, our laboratory had demonstrated that a novel immune active vaccine delivery system (microparticles) prepared with a bioactive polymer, InAc significantly increased the antibody and cell-mediated immune response to an intradermal and suncutaneously injected vaccine[84, 85]. In this report, InAc-NPs-based nanovaccine delivery system is established for its ability to elicit strong mucosal and systemic antibody response after intranasal vaccination. The activation of TLR4 by InAc-NPs was further confirmed using wild-type and TLR4-knockout macrophage cells. The improved antigen delivery and immunostimulatory properties (TLR4 agonist) of InAc-NPs contributed to strong antibody response produced by InAc-NPs-based delivery system.

2.3.1. Physicochemical characterization of InAc-NPs-based vaccine delivery system

As described previously, InAc polymer was prepared by acetylating the hydroxyl functional groups of fructose/glucose subunits of inulin by using acetic anhydride under
inert gas [85]. The formation of InAc and the batch-to-batch consistency were assessed by $^1$H-NMR spectroscopy and Fourier transform infrared (FTIR) spectroscopy [84]. In the previous manuscript, the role of the presence of an acetyl function groups and the high hydrophobicity of InAc in activating TLRs have been discussed [85]. Using InAc as a major polymer, nanoparticles were prepared by encapsulating ovalbumin as an antigen using a double-emulsion solvent-evaporation method. The InAc-NPs were characterized by their size, shape, charge, antigen loading, and presence/absence of endotoxins. The size of NPs was around 245 nm in average diameter with a slightly negative charge (-0.62) as determined by dynamic light scattering (DLS) (Fig.2.2A-B). Further, scanning electron microscopy (SEM) images revealed InAc-NPs as spherical particles with an average diameter of 253 ± 5.2 nm (Fig. 2.2C). The nanoscale features of InAc particles aimed to enhance the intimate interaction with epithelia at nasal mucosa, facilitate the uptake of the antigen by APCs and improve the delivery of the antigen to the lymphoid tissue for enhancing immune stimulation [100]. The preceding research has shown that nanoparticles are preferentially phagocytosed by APCs and elicit stronger cytokine release compared to microparticles [101, 102] [102]. In this context, the delivery system performs a major role in augmenting nasal mucosal immunity. Nanoparticles have been explored as nasal or pulmonary vaccine carriers mainly due to their potential to diffuse through the mucosa, their avoidance of degradation, and their ability to co-deliver both adjuvants and antigens [103]. In addition, due to their size range, NPs are trapped in the mucin matrix, which further promotes their interaction with the nasal epithelium [54,
104]. Taken together, we hypothesize the InAc-NPs will be suitable as an intranasal vaccine delivery system.

Figure 2.2. Characterization of inulin acetate based nanoparticles used for intranasal vaccine delivery. (A) Particle size distribution measured using DLS as percent intensity. (B) Surface charge (ζ-potential) distribution. (C) Scanning electron microscopy showing inulin acetate nanoparticles with spherical morphology.

Encapsulating proteins into a hydrophobic matrix such as InAc or PLGA is a challenging task. The double emulsion-based method provided better loading of antigen (µg of protein/mg of final formulation) in InAc particles than nanoprecipitation as shown previously [85]. Around 22 ± 4.57 µg and 20 ± 8.13 of ovalbumin was loaded for every milligram of InAc-NPs and PLGA-NPs, respectively. The antigen, Ova was released from InAc-NPs in a sustained pattern over 16 days, after around 20 % of the encapsulated antigen was released immediately within 30 min of incubation (Burst release) [Fig.2.3].
This dual release pattern of antigen is considered advantageous for vaccines [105] as it provides fast and continuous antigen stimulation[106].

![Figure 2.3. In-vitro release kinetics of Ova from InAc-NPs.](image)

InAc-NPs (1 mg/ml) were dispersed in 0.1 M PBS, pH 7.4 at ~100 RPM. Ova concentration in the supernatant solution at different time points was measured by using BCA assay (n=3).

### Table 2.1: Characterization of InAc-NPs and PLGA-NPs

<table>
<thead>
<tr>
<th>Parameters</th>
<th>InAc-NPs</th>
<th>PLGA-NPs</th>
</tr>
</thead>
<tbody>
<tr>
<td>Size</td>
<td>245 ± 5.49 nm</td>
<td>250 ± 2.14 nm</td>
</tr>
<tr>
<td>Loading</td>
<td>22 ± 4.57</td>
<td>20 ± 8.13</td>
</tr>
<tr>
<td>Zeta-potential</td>
<td>-0.62</td>
<td>-2.82</td>
</tr>
</tbody>
</table>

#### 2.3.2. InAc-NPs activate macrophages via TLR4 stimulation

To evaluate InAc-NPs stimulation of macrophage to overcome the tolerance state at the nasal mucosal surface, an in-vitro cytokines release study was conducted with murine macrophages. The mouse macrophages significantly released TNF-α, a potent cytokine,
when treated with InAc-NPs or with a known TLR4 agonist monophosphoryl lipid-A (MPLA) [Fig. 2.4A]. In contrast, at the same concentration of InAc-NPs, PLGA-NPs (inert polymer) failed to stimulate mouse macrophages [Fig. 2.4B]. However, the activation and the secretion of TNF-α by macrophages in response to InAc-NPs was abrogated when the cells were pre-treated with LPS-RS a TLR4 antagonist or macrophages from the TLR4 (-/-) mice were used. The observations from the above pharmacological and genetic methods conclude that the InAc-NPs based delivery system (without any antigen) activates APCs (macrophages) through the stimulation of TLR4 on their surface. However, the role of other TLRs such as TLR2 in InAc-dependent activation of other types of APCs in an intact animal cannot be ruled out. Because of the high expression of TLRs, including TLR4 on mucosal surfaces, their activation has a tight and dynamic influence on regulating pathogen invasions and elimination. Our previous studies demonstrated the immune stimulation properties of InAc on dendritic cell and microglia [84, 107], which led us to examine its effect on macrophage activation in this manuscript to include the gamut of APCs.

As an antigen presenting cell with highly phagocytic capability, macrophages play a key role in bacterial recognition and elimination at nasal mucosal sites[108]. The macrophages present in the upper respiratory tract along with other lymphocytes mobilize to the nasal mucosa when responding to antigen stimuli [6]. Despite their abundance in mucosal surfaces, macrophages in the steady state lack the ability to induce proinflammatory cytokines and work to maintain the mucosal hemostasis from an excessive immune response. Moreover, macrophage plays a key role in producing
regulatory T cells and induces tolerance[108]. Stimulating macrophages with appropriate PRR ligand is critical in driving the protective immune response. The observation that InAc particles (TLR4 ligand) activate macrophages and various other types APCs further supports its design for intranasal vaccine delivery system [85].

2.3.3. InAc-NPs were efficiently recognized and internalized by APCs

An ideal feature of a vaccine delivery system is its ability to target immune cells and deliver an adequate antigen to trigger an immune response. In addition to activating

![Figure 2.4: TLR4 selective activation of InAc-NPs](image)

Figure 2.4. TLR4 selective activation of InAc-NPs. Macrophages from wild-type mice (Ø WT) and TLR knockout mice (Ø TLR4−/−) were activated for 48 h with LPS (1 µg/ml), MPLA (2.5 µg/ml) or blank-NPs prepared with InAc or PLGA (250 µg/ml). The activation was assessed by measuring the concentration of TNF-α in the culture supernatant after 48 hrs. To inhibit TLR4 (panel A), TLR4 antagonist; LPS-RS (60 ng/ml) was added to the cells one hour prior to their activation. One-way ANOVA followed by Dunnett's post-hoc multiple comparison tests indicates the significant values of p ≤0.001 between the following groups: Panel A: PLGA vs InAc; InAc vs InAc+LPS-RS; PLGA vs PLGA+LPS-RS, Pane B: treatments of Ø WT cells vs respective treatments of Ø TLR4 (−/−) cells.
APCs, the ability of InAc-NPs in delivering the antigen to macrophages was tested using fluorescently labeled antigen. The NPs were prepared with InAc or PLGA as polymers (Table 2.1) and FITC-ovalbumin (20:100) as an antigen. InAc-NPs were more efficient than PLGA-NPs in delivering antigen to macrophages [Fig. 2.5]. These results are in accordance with our previous observation that InAc-microparticles (1.428 µm in average diameter) delivered significantly high antigen to dendritic cells[85]. Many current studies of particulate vaccine delivery systems are designed to enhance cellular uptake with an emphasis on particle fabrications, physiochemical characterizations, particle geometries or the use of conjugated ligands [109] [71, 110]. Indeed, nanoparticle morphology, size and surface charge are known to influence nanoparticle biological activity. In most approaches, however, the chosen particulate system is a vehicle prepared with inert materials such as PLGA or chitosan [71].

Despite having similar physicochemical properties (size, charge, and hydrophobicity) [Fig. 2.2], InAc-NPs demonstrated higher antigen delivery than PLGA-NPs. This further emphasizes the role of TLR4 interaction in particle/vaccine recognition. In conclusion, InAc particles have proven as an efficient antigen delivery system[84].
2.3.4. InAc-NPs generate strong serum antibody titers in mice

Male BALB/C mice were immunized with InAc-NPs loaded with Ova (20 μg per dose) thrice with 2-week intervals between doses. The immune response generated by the InAc-NPs was compared to PLGA-NPs or PBS as antigen delivery systems. InAc-NPs as a delivery system consistently produced higher antibody titers than PLGA-NPs after primary and booster immunizations [Fig. 2.6]. After 2nd booster, InAc-NPs produced very strong serum IgG1 antibody titers (226,115) compared to PLGA (3500) or PBS (2600) as a delivery vehicle (p<0.001 compared to PLGA group). Similarly, the IgG2a titers were also robust with InAc-NPs (100,653) compared to PLGA-NPs (5247) or PBS (1564) as a delivery system. InAc-NPs stimulated ~65-fold and 19-fold higher serum IgG1 and
IgG2a titers, respectively as compared to PLGA-NPs as a delivery system. The data further signifies the importance of the adjuvant properties InAc-NPs [Fig. 2.4] in addition to their ability as a delivery system [Fig. 2.5] in generating strong antibody-response.

Previously, intradermal or subcutaneous immunization with InAc-particles generated a very high serum IgG1 and IgG2a levels after only one booster injection. Nevertheless, intranasal vaccination with InAc-NPs exhibited similar IgG1 and IgG2 titers only after the second booster dose, indicating that three doses may be required to achieve the full effect. Despite producing robust serum humoral response with a smaller number of doses, InAc-particles previously failed to produce detectable levels of mucosal antibodies (IgA) with parenteral routes [85, 86]. However, in this manuscript, the intranasal route was selected for its ease of accessibility, presence of high density of APC cells, and importantly, for their ability to produce both systemic and mucosal immunity[93] [94]. Similar to parenteral immunizations, InAc-NPs produced signals for the activation of both humoral and cell-mediated immune responses as indicated by high IgG1 and IgG2a antibody titers, which is further established with the type of cytokines released [Fig.2.7]
The cytokine response was assessed using ex-vivo cultures of splenocytes prepared from immunized mice [Fig. 2.7] three weeks after the second booster dose. In an ex-vivo culture, the splenocytes were challenged with Ova, and the amount of secreted cytokines representing Th1 (IL-2 and IFN-γ) and Th2 (IL-4 and IL-10) -type immune responses were quantified in the culture medium. [Fig. 2.7A&B]. The splenocytes collected from the mice immunized with InAc-NPs produced significantly higher levels of both Th1- and Th2-type cytokines as compared to splenocytes from the mice.

**Figure 2.6: Serum OVA-specific IgG-1 & IgG2a titers.** Mice (n = 4-5 per group) were administrated intranasal with Ova (20 µg) alone or loaded in InAc nanoparticles (250 µg) or PLGA on days 1, 14 and 28. Sera were collected at 2 weeks after the primary, 2nd and 3rd immunizations for analysis of antibody titers using indirect ELISA. The titer is the common end serum dilution at which the absorbance is more than average absorbance plus two standard deviations of the PBS control. * Indicates that the results are statistically significant at p < 0.05 as compared to PLGA group using one way ANOVA followed by Bonferroni’s multiple comparison test.
immunized with PLGA-NPs or PBS as a vehicle (at p <0.01; Fig. 2.7). Besides, the splenocytes from mice vaccinated with InAc-NPs showed a high-level proliferative response to the antigen challenge when compared to splenocytes originated from the mice vaccinated with Ova in PBS or in PLGA-NPs [Fig.2.8], which is an indirect indication of a strong memory response. Similar polarization pattern was observed previously with InAc particles using intradermal/subcutaneous routes. The activated cell-mediated immunity was strong enough to significantly reduce or prevent tumor progression and metastasis [85]. Taken together, the first proof-of-concept study in mice indicates that InAc-NPs could stimulate strong systemic antibody response even after intranasal immunization.
Figure 2.7. The release of cellular (A) and humoral (B) cytokines in an ex-vivo challenge of splenocytes. Splenocytes were prepared from mice immunized with ovalbumin in PBS (OVA) or loaded In-Ac-NPs or PLGA-NPs and cultured for 72 hrs. in the presence of Ova (100 µg/ml). After 72 hrs., the concentration of cytokines in the culture supernatant was measured using sandwich-ELISA. * Indicates results are statistically significant as compared to PLGA-Ova immunized group at P < 0.001 using student t-test.
In Ac-NPs boost Ova-specific mucosal IgA titers in mice

The major goal of this study is to generate strong immune responses both systemic (humoral & cellular) and mucosal responses at lung and nasal sites, the primary sites of several respiratory infections. Therefore, we have used three mucosal sites to quantify the mucosal immune response as Ova specific sIgA titers: bronchial alveolar lavage fluid (Lung washes); intestinal fluids and NALT cultures. Protection at the mucosal surface is often correlated with secretory immunoglobulin-A (sIgA) antibodies which, along with

Figure 2.8 Splenocyte proliferative responses to Ova and Con A. Splenocytes were prepared from mice immunized with Ova alone, Ova loaded inulin acetate nanoparticles or PLGA, and cultured for 72 hrs. in the presence of Con A (2.5 µg/ml) or Ova (100 µg/ml) or RPMI. Splenocyte proliferation was measured by the MTT assay and shown as a stimulation index (SI). SI = the absorbance value for treated cultures divided by the absorbance value for non-stimulated cultures. * Indicates results are statistically significant as compared to Ova immunized group in the same mitogen treated group (P < 0.001) using student t-test.
other innate defense mechanisms, protect from attacking pathogens[56]. The prime inductive site for mucosal immunity in the nasopharyngeal tract in rodents is the nasal-associated lymphoid tissue (NALT) [98]. Several studies have reported that NALT works similar to gut-associated tissue to stimulate the common mucosal immune system [104]. Murine NALT may be a functional analog to human tonsils. The bronchial alveolar lavage fluid and the intestinal secretions were used to determine the common mucosal responses at distinct sites. The IgA titers secreted into the culture medium were significantly higher from an ex-vivo cultured NALT tissue collected from the mice immunized with InAc-NPs as a delivery system compared tissue from the mice immunized with PBS or PLGA-NPs as vehicles [Fig. 2.1, Fig. 2.9]. The titers of NALT from the mice immunized with InAc-NPs have higher sIgA titers (15885), compared to NALT tissues from other groups (>30-fold). It is generally accepted that mucosal immunity produced after nasal vaccination is not restricted to the upper respiratory tract, as sIgA antibodies can also be detected in other mucosal secretions due to the complex interaction of immune components from various mucosal tissues. Mucosal immunity, mostly nasopharyngeal immunity, constitutes the major component of the immunological humoral and cell-arbitrated responses in the lower and upper respiratory. Provoking the antibodies protection in pulmonary surfaces lead to better eradication and protection of respiratory infections[91]. In this regard, InAc-NPs showed higher levels of secretory IgA antibody in the lung secretions two weeks after 2nd booster immunization (Avg. titers 11,008 ± 1677).
Furthermore, sIgA was highly elevated in intestines of InAc-NPs immunized mice (Fig. 2.9). The immune response in remote organs suggests that InAc-NPs was able to evoke common mucosal immunity in mice, which is an important element of pathogens clearance mechanism for broader protection [111]. Nasal immunization has been proved to reflect distance mucosal responses at different effector mucosal sites, such as in saliva and gastrointestinal tract [112]. Although common mucosal responses have almost exclusively been reported for S-IgA antibody responses, it is expected that T cells can also be provoked at distance sites [113]. Mucosal immunity is first line protection against mucosal transmitted pathogens such as influenza.

**Figure 2.9. OVA-specific sIgA-Titers.** Mice (n = 4-5 per group) were administrated intranasal with Ova (20 µg) alone or loaded in InAc nanoparticle (250 µg) on days 1, 14 and 28. NALTs were ex-vivo cultured after 2 weeks after 3rd immunizations, Lung and intestines wash were performed at the endpoint. IgA analysis of antibody titers using indirect ELISA. * indicates that the results are statistically significant at p < 0.05 as compared to PLGA group using one way-ANOVA followed by Bonferroni’s multiple comparison test.
Several strategies have been reported including directly conjugating TLR ligands to protein antigens or co-encapsulating immunostimulatory agents and proteins hydrophobic polymeric particles. They have been proven to induce effective immunity as a nasal vaccine antigen delivery system by mimicking the structure and immunological processing of actual pathogens. However, InAc-NPs is a unique system that polymeric delivery vehicle and immunopotentiator in one go, that allow more versatility and modularity in design vaccines adjuvant and delivery system that suit the needs. InAc-NPS produced strong humoral and systemic immune responses including mucosal immunity against the encapsulated antigen when administered intranasally, which confirm complete cross-protection against lethal viruses attacks[114]. Humoral response in the mucosal tissue is critical to prevent the entry of the respiratory pathogens such as influenza. The entry site of respiratory viruses are not limited to the respiratory mucosal lining, but infection can occur in other mucosal surfaces. Therefore the production of humoral response at different mucosal sites offers a great advantage in preventing communicable diseases [115].
2.4. Conclusions

Overall, this first-time proof-of-concept study showed a novel mucosal vaccine delivery system prepared with InAc as a biomaterial to stimulate both mucosal and systemic immune response. The multidisciplinary features of this technology is oriented on using immune-active polymers to prepare the nanoparticles delivery system that mimics naturally occurring pathogens in both size and immune activation. InAc-NPs has been consistently reported to activate both humoral and cellular immune responses. The InAc-based vaccine delivery technology offers a versatile platform for delivering nasal mucosal vaccinations against several viral and bacterial pathogens.
Chapter-III:

Development of a Novel Mucosal Vaccine Adjuvant and Delivery System for Influenza.
3.1. Introduction

As one of the most prevalent respiratory diseases, influenza is responsible for substantial mortality and morbidity worldwide causing upwards of 500,000 deaths each year (WHO, 2014). Influenza epidemics cost billions of dollars in healthcare services [116] [117]. The pathogenesis of influenza infection is mediated by a subset of RNA viruses within the orthomyxoviridae family [118]. Immunization currently proves to be the most efficient method of preventing influenza [119]. Commercially available vaccines are primarily based on inactivated influenza viruses [120]. Influenza vaccines in research are usually based on a whole inactivated or dead virus, protein subunits, split vaccines, and virosomes [121]. Of these type different types of antigens, subunit vaccines are the most commonly researched on account of their acceptable safety profile [122].

Influenza subunit vaccines are based on immunogenic proteins or peptides derived from its viral structure [Fig. 3.1] [123]. These include haemagglutinin protein (HA), a transmembrane glycoprotein present on the surface of the virus, and a peptide from the extracellular domain of influenza Matrix 2 protein (M2e), a component of the viral membrane [124]. Although subunit vaccines are deemed safe, they are poorly immunogenic [125, 126]. Subunit vaccines yield a moderate humoral (Th2-type) response and fail to induce clinically relevant cellular immunity (Th1-type) including the
generation of CD8+ cytotoxic T lymphocytes (CTL) against the virus [127]. However, to attain complete elimination and prevention of influenza infection, activation of both cellular and humoral immune responses is required [128-131]. The induction of potent cellular and humoral immunity by subunit antigens can be achieved by the incorporation of a potent vaccine adjuvant in the formulation (Chapter-I; Table 1.2). Several adjuvants have been evaluated for influenza vaccines [118, 132]; however, their clinical utility is limited either due to their high toxicity or their inability to induce both cellular and humoral immunity [133-135]. These necessitate an urgent need for the development of safe and potent vaccine adjuvants that elicit both humoral and cellular immunity [136].

Previously, our laboratory has investigated the ability of antigen-loaded Inulin Acetate (InAc) microparticles as a vaccine adjuvant (TLR4 agonist) and delivery system to induce complete immune response (cellular and humoral) against the influenza subunit antigens, HA and M2e [1]. The results in mice demonstrated significant capabilities of InAc microparticles to protect from H1N1-influenza virus infection. The immune responses against the virus were evaluated via antibody titers in the serum, hemagglutinin inhibition, virus neutralizing antibody titers, and secretion of Th1-type and Th2-type cytokines [Fig. 3.1, Fig. 3.2]. Taken together, a pathogen-mimicking vaccine delivery system prepared with a TLR4 agonist (InAc) as material with influenza subunit antigens provided both humoral and cellular immunity. The above-unpublished study is performed in mice using the subcutaneous route with InAc-microparticles.
Figure 3.2. HA and M2e specific IgG-total antibody titers in immunized mice serum. Mice (n = 4-5 per group) were injected subcutaneously with unadjuvanted HA (10 µg) or HA loaded in InAc microparticles, unadjuvanted M2e (10 µg) or M2e loaded in InAc microparticles on days 1 and 21 as primary and booster doses, respectively. The data represents mean ± standard deviation (n=4-5). (adapted from [1])
Figure 3.3. Cytokine response of splenocytes prepared from the immunized mice when challenged with the respective antigen. Splenocytes from the mice immunized with InAc microparticles loaded with HA or M2e secreted very high levels of cytokines representing both Th1 and Th2 type immune responses. (adapted from [1])
To this end, this current study investigated the ability of Inulin Acetate (InAc) nanoparticles as a delivery system to induce a complete immune response against the influenza subunit antigen (M2e) in a swine model. The long-term goal of the project is to generate a universal influenza vaccine that applies to both human uses and for the swine industry. This study is different from the previous investigations from our laboratory in three aspects: a) the use of swine model, b) comparing the intranasal vs. subcutaneous routes, and c) use of nanoparticles instead of microparticles. The rationale for the proposed changes is described below. Swine model is more clinically relevant for influenza than mouse model because of the conserved immunological components between pigs and human both in terms of structure and function. Also, the clinical manifestation of the disease symptoms are similar between pigs and humans, and importantly, the swine influenza virus can be transmittable to humans suggesting overlapping infectious mechanisms[114, 137]. Furthermore, the study in swine not only addresses a serious influenza threat to food safety and human/farmer health but also directly relevant to the productivity of today's swine farms improving the economics of the farmers [138].

The intranasal route was selected to test whether InAc-particles produce mucosal immunity in larger animal species (swine) similar to previous observation mice (Chapter-II). It is important for a new novel vaccine candidate to undergo preclinical testing in two animal models (one rodent and one non-rodent) before advancing to clinical phase [139]. However, in adjuvant research, the success of an adjuvant in one animal species cannot be extended to another species until proven. Therefore, it is necessary to investigate in
pigs separately. Previously, Kumar et al. have shown that InAc-microparticles produce a depot at the injection site. InAc-NPs were selected over microparticles in this study due to their potential ability to be cleared from the injection site because the formation of a depot at the injection site or injection site lesions adversely affect patient compliance in humans and meat quality in the swine industry.

The results from this study demonstrated that InAc-NPs prepared with M2e as an antigen are around 300 nm in size, and the antigen was released in a sustained manner. Importantly, InAc-NPs based M2e vaccine generated a strongly systemic and mucosal humoral immune response when administrated intranasally or subcutaneously in pigs. The data for the first time indicates the ability of InAc-NPs as a platform technology for vaccine delivery for pigs.

3.2. Materials and Methods

3.2.1. Materials and Animals

The M2e peptide (MSLLTEVETPTRNEWECRCSDSSD) was purchased from GenScript USA Inc. (Piscataway, NJ). Inulin, MP Biomedicals™ (Cat:198971) and all other chemicals were purchased from Fisher Scientific (Pittsburgh, PA, USA). Goat anti-porcine IgG -HRP conjugated secondary antibodies (Cat:6050-05) were purchased from Southern Biotech (Birmingham, Alabama, USA). Goat anti-porcine IgA secondary antibodies (A100-102P) were purchased from Bethyl laboratories, (Montgomery, TX). Piglets (Yorkshire) purchase from Commercial Farm Herds (Midwest Swine Research,
3.2.2 Vaccine formulation: InAc-NPs with M2e as antigens

Antigen (M2e) loading into InAc-NPs was achieved by double (w/o/w) emulsion solvent evaporation technique as described in Chapter-II with minor modifications. M2e antigen (2.5 mg) was incorporated in the aqueous phase and added dropwise to the organic phase containing for 100 mg of InAc during the first emulsification step. For the preparation of InAc-NPs loaded with a near-IR dye carbocyanine-DiOC18, the dye was added to the organic phase along with the polymer. The lyophilized InAc-NPs containing the antigen or the dye were kept at 4 °C before administration.

2.3. Measurement of antigen loading of InAc nanoparticles

The amount of peptide (M2e) encapsulated in InAc nanoparticles was determined by using high-performance liquid chromatography (HPLC) as described previously[1]. The M2e peptide was extracted by dispersing 10 mg of InAc-NPs in 500 μl of acetone, followed by vortexing, and centrifugation at 14,000 xg for 15 min at 4°C. InAc dissolved in acetone, whereas the peptide precipitated. The peptide in the pellet was collected and dissolved in 200 μl of 1 % sodium dodecyl sulfate (SDS) solution. The amount of peptide present in the SDS solution was quantified using reverse phase HPLC with a C18 column (5 μm, 250 mm × 4.6 mm) AGILENT™. The mobile phase consisted of buffer A (0.05% TFA and 2% acetonitrile in water) and buffer B (0.05% TFA in acetonitrile-water (90:10, v/v) and the flow rate (0.2 ml/min). A linear gradient was performed by
increasing the amount of buffer-B from the initial 20% to 50% [140]. The concentration of peptide in the SDS solution was further confirmed using micro-BCA assay as described in Chapter-II.

3.2.4. Activation of swine peripheral blood mononuclear cell (PBMCs)

Preparation and activation of PBMCs: Peripheral blood (~10 ml) was collected from three-month-old pigs using heparinized tubes (BD Vacutainer, Franklin Lakes, NJ) and the swine PBMCs were prepared as described previously using percoll gradient centrifugation [84]. For the activation, the PBMCs (1×10^6 cells/well of a 6-well plate) in triplicate were incubated for 12 hrs. at 37°C with 5% CO₂ before treating with 200 µg/ml of nanoparticles prepared with PLGA or InAc as polymers for 24 hrs. After the treatment, the PBMCs were collected by centrifugation (1000 x g for 15 min) for RNA isolation.

cDNA synthesis and gene expression analysis: The total RNA from PBMCs was extracted using the RNaseasy mini kit. (Qiagen, Valencia, CA). The RNA was analyzed using Nanodrop ND-1000 and 1% agarose gel electrophoresis. All RNA samples showed the A260/A280 ratio around 2.00 while 1% agarose gel electrophoresis showed two distinct bands as 28S and 18S. The RNA was converted to cDNA using the First-Strand cDNA Synthesis kit (Thermo Scientific, Waltham, MA) and used as templates in the following real-time polymerase chain reactions (RT-PCR) using SYBR green dye [84, 86]. Ribosomal protein large subunit 4 (RPL4) gene was used as an internal control.
The forward primers used were:

**IL6**-AGATGCCAAAGCTGATGC; **TNF-α**-GGGGTCCTTGGGTTTGGATT

**IL12**-AATCTCTCAACCACCTCCCA; **RPL4**-GGCGTAAAGCTGCTACCCTC

The reverse primers include:

**IL6**-ACAAGACCGGTGGTGATTCTCA; **TNF-α**-TTGGAACCCAAGCTTCCCTG

**IL12**-GGCAACTCTCATTGGGCT; **RPL4**-GGATCTCTGGGCTTTTCAAGATT.

The RT-PCR conditions include initial heating at 95 ºC for 10 mins, followed by 45 cycles of 95 ºC for 15 sec and 60 ºC for 40 sec. The relative gene expression of IL-6 with internal control RPL4 was calculated. The fold change in gene expression is represented. All PCRs were performed in triplicates. All PCRs were performed in triplicates [85, 86].

3.2.5. Safety of InAc-NPs

3.2.5.1. In-vitro cytotoxicity: The cytotoxicity of InAc-NPs was investigated by quantifying the viability of murine dendritic cells (DC2.4 cells) using (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT assay). Briefly, DC2.4 cells (5000/well in 96-well plate) were treated with InAc-NPs at concentrations of 0 to 500 µg/ml for 48 h. Subsequently, the cells were treated with the MTT solution (0.5 mg/ml; 4 hrs.) followed by dissolving the tetrazolium crystals using DMSO (150 µl/well;
1 hr.). The absorbance was then measured at 570 nm and 650 nm [141]. The cell viability was compared with the untreated cells.

3.2.5.2. Skin toxicity: The skin toxicity of InAc-NPs or InAc-microparticles (2 mg) or Complete Freund's Adjuvant (CFA) at the injection site was evaluated by assessing the gross structural damage 21 days after the subcutaneous (s.c.) injection in male BALB/C mice using hematoxylin and eosin (H&E) staining.

3.2.5.3. Injection-site clearance and safety of InAc-NPs: Eight-week-old BALB/C mice were injected into the footpad subcutaneously with one milligram of InAc-NPs loaded with carbocyanine-DiOC18. The retention time of InAc-NPs at the injection site was determined by imaging with Bruker-Xtreme in-vivo imager at excitation/emission wavelengths of 750/780 nm [86].

3.2.6. Immunization study

Four weeks old piglets (n=5 per group) purchased from (Midwest Research Swine, Gibbon, MN) were immunized subcutaneously (s.c.) and intranasally on day 1 and 21 as primary and booster doses, respectively. Treatment groups consisted of M2e in saline or InAc-M2e in saline (25 µg antigen per dose). Sera were collected two weeks after prime and booster for analysis of M2e-specific total IgG titers by indirect ELISA as previously described in chapter-II. ELISA plates (Fisher thermo-scientific) were coated with M2e (1 µg/well) in 50 mM carbonate buffer (pH 9.6). Results are expressed as serum IgG titers. Bronchoalveolar fluids (BALFs) were collected from animals after necropsy (3 weeks after 2nd booster) by introducing 50 ml of DMEM medium containing antibiotics (100 U/ml penicillin and 0.1 mg/ml streptomycin) into the lung of each pig, massaged gently
and recollected. BALFs were further centrifuged at 2000 xg for 10 min. All supernatants were stored at -20 °C until analysis.

3.3. Results and Discussion

3.3.1. Physicochemical analysis of antigen-loaded InAc nanoparticles

The InAc particles with M2e antigen were around 315 nm in average diameter. Around 4.03 μg of M2e peptide was loaded inside a milligram of InAC-NPs (Table 3.1).

<table>
<thead>
<tr>
<th>Particle Size</th>
<th>315 ± 0.54</th>
</tr>
</thead>
<tbody>
<tr>
<td>Zeta-Potential</td>
<td>-0.6 ± 0.18</td>
</tr>
<tr>
<td>Polydispersity index</td>
<td>0.38</td>
</tr>
<tr>
<td>Loading of the antigen (M2e peptide) (µg/mg)</td>
<td>4.03 ± 0.62</td>
</tr>
</tbody>
</table>

- Data represent the mean ± standard deviation (n=3). Loading is defined as µg of antigen per mg of InAc nanoparticles.

3.3.2. Safety and clearance of InAc-NPs from the injection site

Previously, it has been shown that microparticles prepared with InAc as the polymer formed a depot at the injection site (unpublished data). Due to the nanoscale size of the InAc-NPs, they are hypothesized to be cleared from the injection site by the lymphatic system without forming a depot[142]. In swine/meat industry, lesions or having a vaccine depot at the injection site are not acceptable. Therefore, it was important to investigate the safety and clearance of InAc-NPs from the injection site in mice before advancing to swine immunization.
Multiple approaches were used to assess the safety of InAc-NPs. In-vitro cytotoxicity studies indicate that InAc-NPs did not significantly alter the viability of mouse dendritic cells (DCs) up to a concentration of 500 μg/ml [Fig. 3.4A]. In mice studies, injecting 2 mg of InAc-NPs did not show any depot formation or tissue damage at the injection site as observed through histochemistry sections (H&E staining) of the skin at the site of injection. However, InAc microparticles and CFA showed a depot formation at the injection site with infiltrated immune cells. Furthermore, CFA caused distinct tissue damage at the site of injection [Fig.3.4B]. As hypothesized, InAc-NPs were cleared from the injection site within 36 hrs of injection as shown by the fluorescence images of the mice after injecting near-IR dye loaded InAc-NPs (Fig. 3.4C), which further endorses the observation from the histopathology skin sections. Injection at the footpad is a common method of studying the clearance by the lymphatic system. The above data (Fig. 3.4A-C) suggests that InAc-NPs are safe to administer as a vaccine delivery system [143]

3.3.3. Effect of InAc-NPs on the activation of swine PBMCs

The efficiency and the safety of InAc-NPs as a bioactive vaccine delivery system are well established through this study (Chapter-II; Fig. 3.4) and previous studies[86]. In adjuvant research, the success of an adjuvant in one animal species cannot be extended to another species until proven because of the variation of the PRRs in both structure and functionality between species [144]. For example, a chemotherapeutic drug Taxol activate murine TLR4, however, failed as an agonist for human TLR4[145]. Similarly,
human TLR4 responds to lipid-A but doesn’t get activated by lipid-IVa, whereas both lipids function as agonists for mouse TLR4 [146]. Therefore, before, advancing with expensive animal studies using swine model, we tested the ability of InAc-NPs, for the first time, to activate swine PBMCs. PBMCs have been used as a surrogate model for an immune function or inflammation studies as they contain lymphocytes, monocytes, and dendritic cells. Due to their vital involvement in almost any immune development, it very important to examine their responses to any proposed adjuvant system.
Figure 3.4. A) In-vitro cellular toxicity of InAc-NPs. Murine dendritic cells (DC2.4) were treated with InAc-NPs (0-500 μg/ml) for 48 h. The relative cell viability was determined by using MTT assay with respect to untreated cells (n = 3-4). * indicates that the results are significant at p ≤ 0.05 vs. untreated cells using one-way ANOVA followed by Bonferroni's multiple comparison tests. B) Skin toxicity of InAc-NPs. Histology images of mouse skin sections stained with H&E at the site of injection shows that InAc-NPs did not form a depot or caused any gross structural damage at the injection site. C) In-vivo clearance of InAc-NPs from the injection site. Mice were injected with 1 mg of InAc-NPs loaded with near-IR dye on the right foot pad. The clearance of vaccine from the injection site was studied by imaging the mice at different time points after injection till 30 hrs.
As a novel TLR4 agonist, InAc-NPs were able to activate swine PBMCs to release critical cytokines (IL-6, IL-12, and TNF-α) necessary for immune activation as shown by quantified RNA transcripts using real-time-PCR. Nanoparticles prepared with an inert polymer PLGA were used as a control. InAc-NPs enhanced the expression of IL-6, IL-12, and TNF-α by approximately 10, 140 and 12 folds, respectively as compared to medium or PLGA-NPs (Fig. 3.5). Cytokine such as IFN-γ, TNF-α, IL-12, and IL-6 are important signals for immune activation. For example, The IFN-γ is one of the main cytokines that represents cell-mediated immunity, which is also an important factor that controls the antibody isotype switching to IgG2a [147, 148]. The significant release of these cytokines indicates the ability of InAc-NPs to activate swine originated immune cells [149].
Figure 3.5. Activation of swine peripheral blood mononuclear cells (PBMCs) Swine PBMCs were incubated with following formulations (i) Medium (ii) PLGA particles (200 µg/ml) or (iii) InAc particles (200 µg/ml) for 24 hrs. The activation of PBMCs after treatment was measured by determining the level of transcription genes of IL12 (A), IL6 (B) and TNF-α (C), using real-time PCR. Fold change in mRNA levels was calculated with respect to the levels of non-stimulated (medium treated) cells. *indicates that results are statistically significant as compared to medium treated cells. Data are expressed as mean ± standard deviation of triplicate determinations.
3.3.4. Swine immunization with M2e antigen delivered through InAc-NPs

Once it is confirmed that InAc-NPs can activate swine immune cells ex-vivo (Fig. 3.5), in-vivo immunization studies were conducted to assess the potential adjuvant activity of InAc-NPs using M2e as an antigen. The M2e sequence was a consensus sequence from multiple swine influenza type-A viruses[150]. The M2e peptide (20 µg) was delivered either in sterile saline or loaded in InAc-NPs through the subcutaneous and intranasal route as primary and booster doses. Two weeks after each immunization, serum antibody titers (total IgG) specific for M2e were measured using ELISA. InAc-NPs as a delivery system produced 20-fold higher total IgG titers in the serum compared to saline after subcutaneous booster immunization (Fig. 3.6). Similarly, intranasal administration of M2e-encapsulated in InAc-NPs (M2e-InAc NPs) has augmented the titers of M2e-specific IgG antibody 4-fold more than intranasal M2e in saline (Fig. 3.7). Production of very high titers through intranasal vaccination is significant for a weak antigen like M2e because of poor retention of the formulations in the nasal cavity of pigs due to the anatomical structure of the nasal area in the pig and frequent exhale reflection mechanisms. However, the strong results indicate that the thermo-gel supportive formulations may have contributed to an improved activity along with the adjuvant effects of the InAc-NPs as a TLR4 agonist. Although M2e protein is considered as a weak immunogen, several studies have shown that it is effective in inducing cross-protection after intranasal and systemic immunization in mice with appropriate adjuvant(s) or delivery systems[151].
Figure 3.6. **M2e specific serum IgG-total antibody titers in subcutaneously immunized pigs.** Pigs (n = 5) were injected subcutaneously with M2e encapsulated InAc-NPs or with unadjuvanted M2e in saline (25 µg) on days 1 and 21. Commercial adjuvant Addvax® was used as a positive control. Sera were collected at 2 weeks after the primary and booster immunizations for the analysis of IgG titers against M2e peptide using indirect-ELISA. Data was analyzed by one-way ANOVA followed by Mann-Whitney test. Data was considered significant at p-value <0.05 in comparison to M2e in saline. The Y-axis was plotted on log10-scale.
Figure 3.7. M2e specific serum IgG-total antibody titers in intranasally immunized pigs. Pigs (n = 5 per group) were injected intranasally with M2e encapsulated InAc-NPs or with unadjuvanted M2e (25 µg) on days 1 and 21 using 1 ml syringe connected to atomizers. Sera were collected at 2 weeks after the primary and booster immunizations for analysis of IgG titers against M2e peptide using indirect ELISA. Data was analyzed by one-way ANOVA followed by Mann-Whitney test. Data was considered significant at p-value <0.05 in comparison to M2e in saline.
**Figure 3.8. M2e specific sIgA antibody titers in immunized pigs.** Pigs (n = 5 per group) were intranasally or subcutaneously immunized with M2e (25 µg) through InAc-NPs or in saline on days 1 and 21. BALFs were collected 3 weeks after booster immunization. sIgA titers against M2e in the BLAF fluid were quantified using indirect-ELISA. Data represents mean ± standard deviation (n=4-5). Data was analyzed with one-way ANOVA followed by Mann-Whitney test. ** represents that the data was significant at p<0.05 vs M2e in saline.
3.3.5. M2e-InAc NPs induce of H1N1-specific sIgA antibody response in the lungs

Beside the increase of M2e-specific serum IgG titers in pigs immunized with InAc-NPs, local antibodies at the major site of infection that recognized M2e were also detected in BAL fluid collected from pigs after challenge with H1N1 influenza virus. We observed higher M2e-specific IgA titers in BALF from the pigs vaccinated with InAc-NPs as an adjuvant compared with the pigs immunized without an adjuvant. The increased response was observed for both intranasal and subcutaneous groups (Fig. 3.7 A and B). Mucosal M2e-specific IgA antibodies found in the lungs are vital for clinical protection of the local airways from influenza virus infection [114]. Mucosal M2e-specific IgA antibodies in the BAL fluids were detected as early as five days after virus exposure, which suggest a significant role that local sIgA plays in defending against M2e induced disease. The induction of high levels of serum IgG and lung IgA against a conserved antigen such as M2e as shown in this study along with HA as antigen will provide a greater opportunity for protection from homologous and heterologous influenza virus infection [152].

Viral diseases are a serious threat not only to the productivity of today's swine farms but also to food safety, and human/farmer health[153]. The major limitations in today's animal vaccines against viral diseases are antigenic drift, lack of potency to stimulate cellular immune responses, lesions at the injection site, instability and the high cost of the formulation. These challenges could be addressed with an appropriate vaccine adjuvant as identified in this study (InAc-NPs).
3.4. Conclusions

In conclusion, these results show that prime and boost vaccination with M2e-InAc NPs elicits antigen-specific mucosal and systemic immune responses to an influenza virus antigen in a large animal model such as swine. The current study for the first time showed that a polymer-based TLR4 agonist InAc could activate the pig immune system to generate strong humoral and mucosal immunity. This study has created a pathway for more extensive studies in our laboratory, that are currently undergoing to generate strong systemic and mucosal antibodies against HA and M2e peptides. The findings of this work will not only pave the way to significantly improved pig influenza vaccines but also provide a new platform technology for other viral vaccine formulations for both human application and swine industry.
Chapter-IV
A Multifunctional Vaccine Delivery System with Dual Adjuvants: TLR4 and TLR7 Agonists
4.1. Introduction

Recently, protein subunit and nucleotide-based vaccines have become more popular than live attenuated or killed vaccines, due to their enhanced safety profile, ease of manipulation and production through recombinant DNA technology, and their utility against cancers using cancer specific antigens[154]. However, subunit vaccines have low immunogenicity and require co-administration of an immune-stimulant/adjuvant [77]. The modern vaccine adjuvant development is focused on activating the innate immune system through the discovery of ligands for pattern recognition receptors (PRRs). Toll-like receptors (TLRs) represent one of the most popular groups of PRRs and their agonists have been explored as vaccine adjuvants [155]. TLRs are highly expressed on antigen-presenting cells, such as macrophages and dendritic cells, which play a critical role in communicating to the adaptive immune system to direct the type and magnitude of the vaccine or pathogen response. TLR-agonists have been developed as vaccine adjuvants against both infectious diseases and cancer, with several of them currently in clinical use [26, 156, 157].

Thirteen TLRs have been discovered in mammals so far, with 10 TLRs (TLR1-10) identified in the human genome [16]. Of all the TLR agonists, TLR4, TLR3, TLR7/8, and TLR9 ligands have been extensively explored in vaccine research mainly due to their ability to stimulate cell-mediated immunity[26]. TLR4 agonists such as monophosphoryl lipid-A (MPLA) have been explored as immune stimulants in various vaccines. One of the licensed and marketed vaccines Cervarix, a prophylactic vaccine for cervical cancer consists of MPLA and alum in the form of AS04 as a vaccine adjuvant[48]. Recently, several TLR7 agonists including Resiquimod (R848) and Imiquimod (R837) have been
explored for cancer immunotherapy due to their ability to activate cell-mediated
immunity[43, 158]. Substantial interest has been shown in using the synergistic effect of
the combination of TLR agonists to enhance immunogenicity and the efficacy of vaccines
esp. against cancers[159] [26]. For instance, co-administration of MPLA and CpG-rich
oligonucleotide (CpG, a TLR9 agonist) has been shown to activate APCs via two
different pathways, with a synergistic effect that boosts cellular and humoral immune
response [92, 160]. The combination of TLRs 7, 8 and TLR9 agonists has been shown to
augment CTL and NK tumor cell infiltration, eradicate large tumor masses and launch
long-term protective immunity [161]. In choosing an appropriate delivery system for a
combination of TLR agonists, it is critical to consider the localization of TLRS within
APCs. A set of TLRs is located on the surface of APCs (E.g. TLRs-2,4,5, etc.), while
another set is at the membrane of endosomes/phagosomes (TLRs-7,8, 9, etc.). Therefore,
for endosomal TLRs, there is need to deliver the ligands to the target site, which is inside
endosomes or phagosomes.

A combination of TLR agonists can be efficiently delivered to APCs by using a
polymeric particulate delivery system, along with the antigen(s) [26]. The advantages of
using polymeric particles for co-delivering a combination of adjuvants include lower
clearance from the injection site, sustained stimulation for efficient APC activation, and
priming the same APC with both the adjuvantss to activate multiple signaling pathways
[55]. In addition, particulate delivery systems can deliver multiple antigens along with
adjuvants, to phagocytic cells such as APCs more efficiently than a physical combination.
This is due to the inherent function of APCs as phagocytic cells to recognize and engulf
particles. Particulate systems can also be modulated to required size and shape to enhance the immune recognition [162, 163].

Previously, our laboratory reported on the discovery of a novel TLR4 ligand, inulin acetate (InAc), which is a modified polysaccharide [84, 86]. A pathogen-like particulate vaccine delivery system (PMVDS) was designed, taking advantage of immune activating and the hydrophobic polymeric nature of InAc [85]. PMVDS is a functional vaccine delivery system with the inherent property of activating TLRs on APCs. Furthermore, it is highly efficient in delivering the antigen to the phagosomes/endosomes of APCs due to its particulate nature and its interaction with surface TLR4 [164, 165]. In comparison to PLGA particles with similar physicochemical properties, InAc particles delivered 6.2 times more antigen to dendritic cells [85]. Simultaneously, Dr. Ferguson group has recently reported several small molecular-based synthetic TLR7/8 ligands, including the compound C-563, a TLR7-specific ligand. TLR7 is present in the endosomes; therefore, its agonists need to be delivered to the endosomes for receptor activation. We hypothesized that by delivering C-563 through InAc nanoparticles (InAc-NPs), C-523 can be delivered to endosomes very efficiently and importantly, activate multiple pathways for strong immune activation.

In this study, aTLR7 agonist C-563 was encapsulated into InAc-NPs (a TLR4 agonist) (InAc-563 NPs). The efficiency of antigen delivery and immune stimulant properties of InAc-563 NPs were investigated using murine macrophages from wild-type and TLR4 and TLR7 knock out mice. Immunization studies in mice confirm the synergistic effects of the dual targeting by InAc-563 NPs, as indicated by strong antibodies titers in the serum and cytokine response in the ex-vivo splenocyte culture that
represents signals for both humoral and cell-mediated immunity. InAc-563 NPs provided a plat form technology both as a potent vaccine adjuvant and as a delivery system for antigen and/or other endosomal TLR ligands.

4.2. Materials and Methods

4.2.1 Materials

Soluble inulin with an average molecular weight of 5 KDa was purchased from MP Biomedicals LLC, Santa Ana, CA, USA. Poly (lactic-co-glycolic acid) PLGA was purchased from Sigma-Aldrich, St Louis, MO, USA. A small molecule imidazoquinoline analog (C563) was obtained from Ferguson Lab, University of Minnesota, Minneapolis. Imiquimod (R837) and Monophosphoryl lipid A (MPLA) were purchased from Invivogen, San Diego, CA, USA. All other chemicals were purchased from Fisher Scientific, Pittsburgh, PA, USA.

4.2.2 Cell lines and mice

Macrophage cells from Wild-type (NR-9460), TLR7 knockout (NR-915634) and TLR4 knockout (NR-9458) mice were obtained through BEI Resources, ATCC, Manassas, VA, USA. The macrophages were plated in complete RPMI-1640 medium (Thermofisher Scientific, USA), supplemented with antibiotics (penicillin/streptomycin) and 10% fetal bovine serum (FBS) in flat-bottom 24-well plates at a concentration of 0.5 x 10^5 cells/ml/well unless specified elsewhere. They were cultured in a 5% CO_2 incubator at 37°C. BALB/c mice were purchased from Jackson’s Labs (Maine, USA) and experiments were performed at 6–12 weeks of age. All experiments were performed following The
4.2.3 Preparation of Inulin Acetate-563 nanoparticles (InAc-563 NPs)

Inulin acetate nanoparticles carrying C563 (InAc-563 NPs) and both C563 and ovalbumin (InAc-563-Ova) were prepared using double (w/o/w) emulsion solvent evaporation techniques as described elsewhere (Chapter-II). Briefly, an aqueous phase of the primary emulsion was prepared with a 10 mM phosphate buffer (PB, pH7.4) containing 2% (w/v) Pluronic F-68 solution as a surfactant, with or without Ova. The oil phase was prepared by dissolving InAc (100 mg) and C-563 (5 mg) in 5 ml of dichloromethane (DCM). The aqueous solution was added drop by drop to the DCM-polymeric mixture to form a primary (w/o) emulsion, which was subsequently added drop-wise into 45 ml of water containing 0.5% (w/v) polyvinyl alcohol while stirring at 200 RPM. To evaporate DCM, the final emulsion was stirred for 12 hrs., and the precipitated particles were collected via centrifugation at 50,000 g for 30 min at 4°C. The particles were washed twice, re-suspended in 100 mM of citrate buffer, pH of 7.4 and lyophilized with mannitol (20% w/w) (VirTis, Gardiner, NY).

4.2.4 Size and zeta potential

As described previously, the size and zeta potential were measured by the dynamic light scattering (DLS) technique using Malvern Zeta-Sizer, Malvern Ltd, MA, USA. InAc-563 was re-suspended in a filter-sterilized citrate buffer (10 mM, pH 7.4) and diluted using filtered sterilized water before recording particle size and z-potential.
4.2.2 Scanning electron microscopy (SEM)

The shape and size of InAc-563 NPs were evaluated using a scanning electron microscope (SEM) (Model S-3400N, Hitachi, Japan) (Engineering Department, SDSU). For the preparation of the sample, the lyophilized powder, free of cryo-protectant, was mounted on the metal holder using conductive double-sided tape. The particles were sputter coated with a 10-nm gold layer before analysis. The micrographs were captured at a beam voltage of 5 kV, and 50,000X magnification with a working distance of 5-15 mm and a spot size of three. ImageJ software was used to measure the average diameter of the particles representing at least 100 particles. Refer to Chapter-II for additional details.

4.2.3 Determination of the loading of C-563 and the antigen in InAc-563 Ova

The amount of C-563 compound loaded into the InAc nanoparticles was measured using high-performance liquid chromatography (HPLC) (Water Coulter). DMSO was added to 1 mg of nanoparticles to extract the C-563 from the InAc-563 particles, then quantified using HPLC. The separation was achieved using a 40:60 mixture of ammonium acetate buffer (pH 4-5) and acetonitrile as the mobile phase, with a flow rate of 1 ml/min through an Eclipse C-18 reverse-phase HPLC column (Agilent, 4.6 150 mm, particle size 4 mm). The C-563 was detected and quantified using a UV detector at 254 nm. For measurement of antigen (Ova) loading, 1 mg of InAc-563 NP was dissolved in 500 µl of acetone. As InAc-563 NP dissolve in the acetone, Ova was precipitated and then collected in 1% sodium dodecyl sulfate (SDS). The collected Ova was quantified by a Pierce™ Bicinchoninic acid assay (BCA), refer to Chapter-II for further details.
4.2.4 **In-vitro drug release studies for C-563**

In-vitro release of C-563 from InAc-563 NPs was determined using a dialysis technique. InAc-563 nanoparticles (~ 10 mg/ml) were suspended in PBS (pH 7.4) and incubated in a shaker (New Brunswick Scientific) at 37°C, 100 RPM. At predetermined time points, a 500 µl of the suspension was collected and centrifuged at 10,000 xg to collect the soluble supernatant portion. Collected samples were filtered through 0.2 µM filter and then lyophilized. The C-563 was dissolved in 200 µl DMSO and quantified by using the HPLC, as described above.

4.2.5 **In-vitro immune-activation**

Murine macrophages (from wild-type, TLR4, and TLR7 specific knockout mice) were seeded in 12-well plates (0.5 x 106 cells/well) for 48 hrs in complete RPMI medium, Macrophages were incubated with InAc-563 NP (250 µg/ml/well) for 24 h. Imiquimod (5µg/ml/well) and MPLA (2 µg/ml/well) were used as positive controls and media alone was used as a negative control. The culture supernatants were collected after centrifuging the plates at 4000 xg. The 100-µl aliquots were analyzed in triplicate for the levels of TNF-α using TNF-α ELISA Ready-SET-Go Kit (e-Bioscience, San Diego, CA) as instructed by the kit. TNF-α concentrations were calculated based on a standard curve that was generated by using purified mouse recombinant TNF-α. The TNF-α level in the untreated wells was considered a background[85, 166].
4.2.6 In-vivo immunization in mice

Male BALB/c mice (n = 4–5 per group, 6–8 weeks old) were immunized through a subcutaneous route (s.c) by injecting 100 μl of vaccine formulations (50 μl per site) using a 25G needle with the following groups:

i) PBS (50 mM Phosphate buffered saline, pH 7.2)
ii) Ova (20 μg per mouse)
iii) Ova (20 μg) encapsulated inside InAc-563
iv) Ova (20 μg) physically mixed with InAc-563 NPs.

All the formulations were injected in a sterile vehicle containing 20 % Pluronic F-127 gel in PBS. Mice were administered with booster dose three weeks after the initial immunization. A blood samples were collected three weeks after the primary and booster immunization and sera were isolated using Microtainer serum separator (BD Biosciences, San Jose, CA). The sera were stored at -20°C until further analysis.

4.2.10 Enzyme-linked Immunosorbent Assay (ELISA)

The 96-well plates were coated with 1 μg/ml of Ova in a carbonate buffer (pH 9.6) for overnight. The wells were blocked with a PBS containing 1% skim milk after washing the uncoated antigen. The sera from immunized mice were incubated on the antigen-coated wells overnight at various dilutions. After washing three times with PBS, goat anti-mouse total IgG or IgG2a conjugated with horseradish peroxidase (Southern Biotech) were added at 1:7000 dilution and incubated for 1 hr at room temperature. After five more washings with the PBS, the plates were incubated with a TMB (3,3′,5,5′-
Tetramethylbenzidine) substrate for 5-7 min. The reaction was stopped with 50 µl of 1 N H₂SO₄, and then the absorbance was measured at 450 nm. Antibody titers were calculated as dilutions of the serum at which the OD value is equivalent two standard deviations above the average OD value of the serum from PBS immunized mice (Cut-off OD). Anti-Ova titers were represented as the highest dilution giving the OD value over the cut-off OD value.

4.2.11 Splenocyte Activation assay: ex-vivo

T-cell proliferation assay was used to determine the generation of memory T-cells in immunized mice after being challenged with the antigen in ex-vivo conditions as described previously[85]. Briefly, single cell suspensions of the splenocytes were prepared from the spleens of immunized mice in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin, 1 mM sodium pyruvate and 50 µM 2-mercaptoethanol. The erythrocytes were eliminated using 0.1 M NH₄Cl lyses buffer, and the viability of resultant splenocytes was assessed by trypan blue exclusion using a Cellometer (Nexcelom Bioscience, Lawrence, MA). The splenocytes (1 × 10⁶ cells/well) seeded in triplicate into a 96-well plate were challenged with Ova (100 µg/ml) or with concanavalin A (5 µg/ml; Con A; Sigma) as a mitogen. Splenocytes incubated with medium alone were used as a negative control. After three days of incubation at 37° C (5% CO₂, 95% humidity), the plates were centrifuged for 10 min at 1000 xg to pellet the cells, and 200 µl of supernatant was collected for the analysis of secreted cytokines.
4.2.12 Cytokine analysis

Sandwich-ELISA assay was used to measure the cytokines in the supernatants of the splenocytes culture. The assay was performed using a Mouse Th1/Th2 ELISA Ready-SET-Go Kit (e-Bioscience, San Diego, CA). Th1-type (interleukin-2 [IL-2], gamma interferon [IFN-γ]) and Th2-type (IL-4 and IL-10) cytokines were measured by comparing them with the standard curves generated from murine recombinant cytokines (IL-2, IL-4, IL-10, and IFN-γ) after linear regression analysis [99].

4.2.13 Determination of endotoxin levels

The endotoxin levels in the final preparation were determined using the ToxinSensor™ Chromogenic LAL Endotoxin Assay Kit from GenScript (Piscataway, NJ), following the manufacturer's instructions. All formulations used in this study have a low detection limit of endotoxins as per the United States Pharmacopeia for parenteral administration [96].

4.2.14 Statistical analysis

Results were expressed as mean ± standard error (SD) unless specified. All presented data were analyzed with Instant Graph Pad software (CA) and tested by Student's *t*-test one-way or two-way ANOVA, for the analysis of variance, as required. Additionally, Bonferroni's or Dunnett's post-hoc multiple comparison tests for statistical significance were performed and P<0.05 was considered a statistically significant difference unless otherwise mentioned in the figure legends.
4.3 Results and Discussion

The limited availability of safe vaccine adjuvants in the market warrants the discovery of new vaccine adjuvants targeted towards specific innate immune signaling pathway such as TLR or NLR pathways. Several TLR agonists, esp. against TLR4, TLR7 and TLR 9, have been investigated for the design of modern vaccines against viral diseases and cancer[16] [167-169]. Previously, we demonstrated that an immune active nano-vaccine delivery system prepared with a TLR4 agonist (InAc) as a polymer (InAc-NPs) significantly increased the serum antibody titers and cell-mediated immune response against an injected antigen [85]. In previous studies, co-injection of antigen with InAc particles failed to significantly activate the immune system as an adjuvant [Chapter-II]. It was necessary to encapsulate the antigen inside InAc-particles to produce a robust humoral and cellular immune response. In this chapter, we examined the efficiency of a novel combination of TLR4 (InAc) and TLR7 agonists (C-563) as an adjuvant and a vaccine delivery system using both in-vitro and in-vivo models. A small molecular TLR7 agonist, C-563, was encapsulated in InAc-NPs to deliver them together (InAc-563 NPs).

4.3.1 Physicochemical characterization of InAc-563 NPs

The InAc-563 NPs were characterized by their size, shape, charge, antigen loading, loading of C-563, and endotoxin level. HPLC analysis showed that 3.5 µg ± 0.24 of C-563 was encapsulated per 1 mg of nanoparticles. The loading of C-563 into InAc-NPs was around three times higher than the level reported for similar compounds with PLGA-NPs [170]. The NPs had an average diameter of approximately 289 nm with a slightly negative charge (-0.62), as determined by dynamic light scattering (DLS) (Fig.4.1.A-C).
Further, scanning electron microscopy (SEM) images revealed spherical particles with an average diameter of $280 \pm 6.2$ nm (Fig. 4.1 B) [Table 4.1]. Nanoscale structures of InAc-563 particles were meant to increase the contact with APCs at the site of injection, facilitate the uptake of the antigens and improve the delivery to the lymphoid tissue for proper bio-distribution [100]. The literature review in Chapter-I clearly established the advantages of nanoparticles for vaccine delivery, as APCs preferentially phagocytose them and elicit stronger cytokine release compared to microparticles [156] [102]. In this regard, the nanoparticle delivery system has also been shown to augment mucosal immunity after intranasal vaccination [Chapters I & II]. Polymeric nanoparticles encapsulating different TLR agonists have been investigated for improved immune activation. PLGA nanoparticles are the most commonly used carriers for TLR agonists. For example, PLGA particles encapsulating a TLR9 agonist (CpG) resulted in greater DC uptake, maturation and T-cell activation [103]. However, the versatility of inulin acetate nanoparticles as carriers emerges from their inherent ability to activate the innate immune system through TLR4 [86] and superior ability as an antigen delivery system[85].

Since inulin acetate targets TLR4 on the cell membrane of the immune cells, InAc-NPs significantly facilitate cargo uptake into the phagosomes/endosomes of APCs, thereby expected to delivery C-563 to the site of TLR7 localization. In addition to carrying the TLR7 agonists, InAc-NPs were also loaded with the antigen. Antigen loading was determined by precipitating the antigen in acetone, extracting it using a surfactant (SDS) and quantifying it by BCA assay. Approximately $21.0 \pm 0.47 \mu g$ of ovalbumin was loaded for every milligram of InAc-563 NPs, which is consistent with previous reports[86] [85]. The NPs are expected to deliver the antigen efficiently to the
APCs of the innate immune system, a major target site for vaccines [171]. The particle uptake by APCs was influenced by particle size, antigen loading and exposure time [110]. Despite being hydrophobic, InAc-563-NPs efficiently encapsulated both the protein antigen (Ova) and the small molecular TLR agonist, C-563 (Table 3.1). Previously, we demonstrated that the antigen encapsulated inside InAc particles was released in a sustained pattern over days (Chapter-II, Fig.2.3) [84].

![Figure 4.1](image_url)

**Figure 4.1. Characterization of InAc-563 nanoparticles.** (A) Particle size distribution measured using DLS as percent intensity. (B) Scanning electron microscopy image showing InAc nanoparticles with spherical morphology. (C) Surface charge (ζ-potential) distribution.
Similar to the release of antigen, the encapsulated C-563 was also released from the InAc-NPs in a steady and sustained manner. Although 20-30% of the antigen was released within the first 30 minutes (burst release), possibly due to a surface-adsorbed antigen, such a dual release pattern was not observed with C-563 (Fig. 4.2). Zero percent of the C-536 was released within the first 30 min, while approximately 1.33% of total encapsulated C-563 was released in the first 2 hrs. of incubation (Fig. 3.2).

Table 4.1: Physiochemical characterization of InAc-563-Ova.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Particle Size (diameter)</td>
<td>289 ± 0.61 nm</td>
</tr>
<tr>
<td>Zeta-potential</td>
<td>-0.71</td>
</tr>
<tr>
<td>Polydispersity index</td>
<td>0.31</td>
</tr>
<tr>
<td>Loading of C-563 (µg/mg NP)</td>
<td>3.5</td>
</tr>
<tr>
<td>Loading of antigen (Ova)(µg/mg NP)</td>
<td>21 ± 0.48</td>
</tr>
</tbody>
</table>

Data represent the mean ± standard deviation (n=3). Loading indicates the amount of the substance (µg) present in one milligram of the nanoparticles (NP)
For the activation of APCs, murine macrophages were treated with blank InAc-563 NPs without the antigen. The activation was assessed by measuring the secreted cytokine TNF-α. InAc-563 NPs activated murine macrophages similar to other TLR agonists such as MPLA and imiquimod (Fig. 4.3A). This data is consistent with our previous reported work, where InAc particles activated several other APCs, including dendritic cells and microglial cells [84, 86] (Chapter-II, Fig. 2.4). However, the same concentration of InAc-563 NPs failed to activate macrophages lacking functional TLR4 receptor, indicating that the presence of TLR4 is necessary for the activity of the InAc delivery system as an immune-stimulant. MPLA, a known TLR4 agonist showed similar pattern as InAc-particles (Fig. 2.4). Despite the presence of TLR7 agonist (C-563), InAc-563 NPs failed to stimulate macrophages from TLR4−/−, which suggests that C-563 may not be delivered
to phagosomes, the target site, in significant amounts by InAc-NPs in the absence of TLR4. This is a confirmation of our previous reports that InAc activates APCs through TLR4 receptors using LPS-RS, a pharmacological antagonist of TLR4 (Chapter-II, Fig. 2.4).

Interestingly, InAc-563 NPs have partially stimulated macrophages from the TLR7 knockout mice (TLR7−/−) whereas imiquimod (TLR7 agonist) completely failed to activate TLR7 knockout cells. The data indicate that the polymer InAc used in the preparation of InAC-563 NPs may have contributed to the activation of TLR7−/− cells through surface TLR4. However, the role of other TLRs cannot be ruled out (Fig.4.3B). Taken together, the above data suggest that InAc-563-NPs have a dual activity that activates both TLR4 and TLR7 receptors, which could complement in-vivo immune stimulation as a multifunctional vaccine delivery system. The combination of synthetic small molecular ligands of TLR4 (MPLA) and TLR7 has been shown to generate long-lived cellular and humoral immunity[172]. The above combination has been shown to work through Myd88-dependent signaling pathway[172]. The previous report from our laboratory established that InAc-particles also function through Myd88-dependent pathway[84]. Combining two TLR ligands within a formulation not only allow dose-sparing of antigen and adjuvant but also enables broad protection.

Macrophages and other APCs show high expression of TLRs, either on their surfaces or in the endosomes in the cytoplasm (Chapter-I); thus, their activation has a strong and dynamic influence on regulating pathogen invasions and their clearance[55]. In a separate study, C-563 and other related compounds demonstrated specific targeting of TLR7/8 on bone marrow-derived dendritic cells[173]. Our previous studies have
revealed the immunostimulant properties of InAc particles on dendritic cell, microglia and macrophage cells (Chapter-II). In addition, InAc particles have been shown to activate peripheral blood mononuclear cell (PBMCs) from the human and porcine origin (Chapter-III). Multiple studies confirmed the efficiency of InAc-NPs in delivering antigens to APCs [84-86] (Chapter-II, Fig.2.5). The presence of C-563 did not interfere with the antigen delivery function of InAc-NPs (Fig. 4.4). InAc-563 NPs may activate APCs through multiple mechanisms: better antigen delivery, persistent antigen delivery inside APCs (antigen persistence), activation of both TLR4 and TLR7, and efficient delivery of C-563 to the target site (endosomes) (Fig. 3.4). Taken together, the in-vitro data suggests that InAc-563-NPs represent both a potent delivery system for antigens and other adjuvants, as well as an immune potentiator.
Figure 4.3. Macrophage activation of InAc-563 NPs. Macrophage cells (θ) originated from wild-type (WT), TLR4 knockout (TLR4−/−) (A), TLR7 knockout (TLR7−/−) (B) mice were incubated with MPLA (2 μg/ml), a known synthetic TLR4 agonist, imiquimod (5 μg/ml), a known synthetic TLR7 agonist, or InAc-563 NPs (250 μg/ml) without antigen. The activation of macrophages was measured by estimating the concentration of TNF-α in the culture supernatant after 24 hrs of incubation. ** indicates results are statistically significant as compared to knockout cells (P < 0.001). The experiments were performed in triplicate.
4.3.4. InAc-563 NPs generates strong serum antibody titers in mice

Once established in vitro that InAc-563 NPs function as both a delivery system and immune activator (macrophages), their efficiency as a vaccine adjuvant was investigated in male BALB/c mice with two doses with a 3-week apart [174]. After three weeks of primary immunization, the Ova loaded inside InAc-563-NPs, and Ova as a physical mixture with InAc-563 NPs produced significantly strong serum antibody titers (IgG1 and IgG2a), compared to the Ova without adjuvant (p<0.001) (Fig. 4.4A). After a booster dose, the Ova-loaded InAc-563 NPs stimulated 150-fold higher serum IgG1 titers compared to Ova alone. The physical mixture of Ova with InAc-563-NPs also produced strong IgG1 titers in the serum (81-fold vs. Ova alone). Similarly, InAc-563 NPs loaded with Ova (67-fold), and the physical mixture with InAc-563 NPs (47-fold) produced strong IgG2a titers in the serum (Fig. 4.5B).

**Figure 4.4 Internalization of InAc-563 NPs by macrophages** Wild-type macrophage cells were incubated with FITC-Ova encapsulated InAc-563 NPs at 37 °C. After 1 hr the cells were extensively washed, fixed in 4% (w/v) paraformaldehyde and observed under a fluorescent microscope. The nucleus was stained with DAPI. Scale bars are equal to 10 µm ( ).
Interestingly, previous administration of InAc particles that were physically mixed with Ova produced significantly lower antibody titers than the current study. Previously, the physical mixture of InAc microparticles with Ova generated just a 2-fold (IgG1) greater response than Ova alone [85]. In this study, with the addition of thermo-gel, InAc-563 NPs were able to generate an 81-fold increase over Ova alone. The explanation for that high response is related to the use of the thermo-gel as an Ova vehicle which allowed the presence of TLR agonists and antigens together within phagosomes of APCs or the addition of C0563 as a dual adjuvant. This determined the efficiency of the antigen presentation by MHCs and critically directed the adaptive immune response [85]. Indeed, by changing the context of antigen presentation, the type of adaptive immune response was altered [85, 86]. Previously, when InAc particles were delivered along with the antigen in saline, the antigen might have diffused out from the injection site faster than the particles, which adversely affect the co-delivery of the antigen and the adjuvant to the same cell. Such co-delivery was achieved in this study, either by encapsulating the antigen within the particles of InAc-563 or by delivering them in the thermo-gel formulation. The thermo-gel formulation is liquid at 4 °C for the ease of injection and assumes a solid matrix form at body temperature to retain the antigen and the adjuvant (InAc-563) for a longer period at the site of the injection, allowing more exposure to the peripheral APCs [174].
The previous reports and Chapter-II noted that intradermal and internasal immunization of InAc-based particles showed robust serum-IgG1 and IgG2a titers, which are attributed to the function of InAc-NPs as both adjuvant and antigen delivery system. The results in this study signify, for the first time, the importance of the adjuvant properties of InAc-563 NPs beyond a delivery system (physical mixture). However, we cannot rule out the additional immune-stimulatory activation through TLR7 signaling contributed by the C-567 in the adjuvant properties observed in this study.

Figure 4.5. Ova-specific antibody titers in the serum. Mice (n = 4 per group) were injected Ova (20 µg) in solution or Ova along with InAc-563 NPs as a physical mixture (InAc-563 NPs + Ova) or Ova loaded inside InAc-563 NPs on days 1 and 21 as primary and booster doses, respectively. Serum samples were collected at 3rd week after the immunizations for analysis of antibody titers (IgG-1, and IgG-2a) using indirect ELISA. The titer is the reciprocal end serum dilution at which the absorbance is more than average absorbance plus two standard deviations from the PBS-immunized mice serum. The data represents mean ± SD, plotted on logarithmic scale.
4.3.5 InAc-563 NPs generate both humoral and cellular cytokines in ex-vivo splenocyte challenge

Many studies have shown the direct relationship between APCs’ response to PAMPs through PRRs, and the activation and expansion of antigen-specific memory T-cells representing both humoral and cellular responses [175]. Mice immune response was further assessed indirectly for the generation of antigen-specific memory T-cells by challenging splenocytes collected from the spleens of immunized mice with the antigen. Splenocytes were challenged ex-vivo cultures with ovalbumin and the resultant release of IL-2, IFN-γ, IL-4 and IL-10 into the supernatant were quantified (Fig.4.6 and Fig.4.7).

The splenocytes collected from the mice immunized with Ova encapsulated in InAc-563 NPs or as a physical mixture generated significantly higher levels of both Th1- and Th2-type cytokines, as compared to splenocytes from the mice immunized with soluble Ova in a thermo-gel (p<0.001). The antibody subclasses indirectly indicate splits in the T-helper cell (Th) response, in which IgG1 is linked to the Th2-type response and IgG2a is linked to the Th1-type response [176]. The results concluded that InAc-563-NPs as an adjuvant and/or a delivery system generated strong humoral and cellular immune responses as evidenced by significantly higher IgG1 and IgG2a antibodies (Fig.3.4) and the induction of cytokines represents both cellular (IL-2 and IFN-γ) and humoral (IL-4 and IL-10) immune responses.
Figure 4.6. Measurement of Th2-type cytokines (IL-4 and IL-10). Splenocytes were prepared from mice immunized with soluble Ova (Ova), soluble Oval along with blank-InAc-563 NPs or Ova-loaded InAc-563 NPs. The splenocytes were cultured for 72 hrs in presence of Ova (100 µg/ml). After the incubation, supernatant from different treatment groups were collected and the concentration of different IL-4 and IL-10 were measured using sandwich-ELISA. ** indicates results are statistically significant as compared to ova immunized group (P < 0.001).
TLR4 is one of the extensively studied TLRs as a target for vaccine adjuvant discovery. Indeed, a synthetic TLR4 agonist, MPLA in combination with alum (ASO4) is a part of two commercial vaccines Fendrix, a vaccine for hepatitis B, and Cervarix, a vaccine for human papillomavirus [177-180]. MPLA is produced after extensive chemical modification of biologically originated lipopolysaccharides (LPS), which results in large variability from a batch to batch and high cost of the final product. To overcome the above challenge, a polysaccharide polymer based inulin acetate (InAc) was discovered as a TLR4 agonist [84]. A major advantage of InAc is that it can be used as material to prepare a vaccine delivery system (InAc NPs) that can inherently activate

**Figure 4.7. Measurement Th1-type cytokines (IFN-γ and IL-2).** Splenocytes were prepared from mice immunized with Ova form or Ova with InAc-563 NPs ova loaded InAc-563 and cultured for 72 hrs in presence of Ova (100 µg/ml). Supernatant from different treatment groups were collected and the concentration of different IFN- γ and IL-2 were measured using Sandwich-ELISA. ** indicates results are statistically significant as compared to Ova alone immunized group (P < 0.001).
TLR4 on APCs [85]. InAc NPs are based on a modified polysaccharide from plant origin. The procedure is simple to prepare and economical for commercialization.

Of all endosomal TLRs, TLR7/8 are broadly expressed on most of the APCs including plasmacytoid dendritic cells (pDCs) in contrast to TLR9, which is primarily expressed on pDCs. The expression pattern translates into better adjuvanticity for synthetic TLR7/8 agonists compared to TLR9 agonists such as CpG ODNs [181]. However, unformulated small molecular TLR7/8 ligands do not work well as vaccine adjuvants mainly because of their quick diffusion from the injection site, which may cause systemic toxicity, and their inability to reach the target site (endosomes/phagosomes)[182].

Combinations of TLR4 and TLR7 ligands were examined as adjuvants in different models and stages, reporting promising results in preclinical trials esp. against viral diseases and cancer [172, 182]. A TLR4 agonist such as MPLA and TLR7 agonists such as imiquimod are hydrophobic in nature and require a formulation to deliver (Eg. polymeric particles, emulsion, liposomes, etc.). In addition, a particulate formulation (Eg. Nanoparticles, liposomes or micelles) will reduce the diffusion of the inflammatory TLR7 agonists and promote their uptake by APCs such as macrophages [182]. In this study, by encapsulating C-563 inside InAc NPs along with the antigen, we could achieve both the delivery requirement for TLR7 agonist and dual adjuvanticity of activating TLR4 and TLR7.
4.4 Conclusion

The \textit{in-vivo} and \textit{in-vitro} studies suggest that the InAc-563 NPs-based vaccine delivery system, with TLR4/7 ligands, could facilitate strong immune response with or without encapsulation of the subunit antigen. In mice, the delivery of InAc-563 NPs led to a stronger antigen-specific serum antibody response. InAc-563 NPs function as both vaccine adjuvant and a vehicle to deliver C-563 and antigen. This work provides a unique platform technology using InAc-563 nanoparticle as multifunctional vaccine delivery system against challenging diseases such as viral diseases and cancer, where cell-mediated immune activation is necessary.
Summary and Future Studies

Because of recently evolving respiratory pathogens (viral and bacterial), growing antibiotic resistance coupled with rising occurrences of inflammatory mucosal diseases, there is an urgent medical need for discovery of a novel vaccine technology that activates both systemic and mucosal protection. However, there is a serious paucity in the availability of vaccine technologies or vaccine adjuvants to address the above need. The above limitation has hampered the development of successful preventive vaccines against challenging mucosal pathogens such as HIV, HPV, influenzas, tuberculosis, etc.

Towards addressing the above goal, previously, our laboratory reported a “pathogen mimicking vaccine delivery system” (PMVDS) prepared with inulin acetate, a TLR4 agonist as a polymer. PMVDS was successfully tested previously through subcutaneous or intradermal route to generate strong systemic immunity for a skin cancer vaccine (Melanoma) using peptide antigens. However, through parenteral routes of administration failed to produce detectable mucosal immunity.

The main goal of this dissertation is to investigate the potential of InAc based nanoparticles (InAc-NPs) as a mucosal adjuvant and delivery system alone or in combination with other adjuvants. Based on the previous promising results and existing literature, we hypothesize that of nano-PMVDS (InAc-NPs) have potential to generates strong systemic and mucosal immune responses if given intranasally. As in Chapter-II, intranasal delivery of InAc-NPs induced high levels of secretory IgA (sIgA) in the nasal-associated lymphoid tissue (NALT) and IgG1 and IgG2a in the serum with a well-balanced Th1- and Th2-type responses. In addition to antibody secretion at nasal tissue,
other distinct mucosal tissues (lungs and intestine) had also shown high levels of sIgA. The high efficiency of InAc-NPs as a delivery system and adjuvant was supported by various in-vitro mechanistic investigations that showed improved antigen delivery and strong activation of macrophages in TLR4 dependent manner by InAc-NPs.

Once the proof-of-the-concept that InAc-NPs could provide efficient platform delivery system for mucosal vaccines is established using artificial antigen (Ova) in mice, we advanced the concept to test in larger animals like pigs with disease-relevant antigen (influenza-M2e) in chapter-II. InAc-NPs activated pig primary immune cells (PBMCs) to release inflammatory cytokines necessary for vaccine efficiency. Importantly, when immunized through InAc-NPs as a delivery system, a weak antigen like M2e peptide produced higher systemic (20-fold) and mucosal (1.45 fold) antibody response in pigs. This is a preliminary study that signifies the ability of InAc-NPs as an adjuvant and a delivery system in pigs. More studies are underway in our laboratory to advance towards generating a universal influenza vaccine using HA and M2e as antigen and testing them for protection against homologous and heterologous strains of influenza.

In addition, being a TLR4 agonist (adjuvant), InAC-NPs are reported as an efficient targeted vaccine delivery system to deliver the encapsulated antigen (cargo) to the phagosomes of various APCs (macrophages, dendritic cells, and microglials) more efficiently than NPs prepared with an inert polymer PLGA. In chapter-IV, we have tested the ability of InAc-NPs in delivering another TLR7 agonist, C-563 along with the antigen to APCs to generate a strong immune response. InAc-NPs released encapsulated C-563 in a sustained manner. Importantly, InAc-NPs containing C-563 activated murine macrophages in both TLR4 and TLR7 dependent pathways. The C-563 containing InAc-
NPs produced very strong antibody titers in mice and cytokines in ex-vivo cultures that represent both humoral and cellular immunity. Importantly, such robust activity was observed even without encapsulation of the antigen inside the particles, which was shown for the first time for InAc particles. This is very significant in the translation of the technology for the field application as encapsulation procedures usually lead to the loss of significant antigen. Conserving antigen will be critical in pandemic attacks where there is a severe shortage of antigens.

Further, InAc-563-based dual TLR4/7 system will be developed as a potential novel synergistic vaccine adjuvant system for cancer immune therapy. InAc-NPs (a TLR4 agonist) failed to stimulate a strong immune response when delivered orally (data not shown here), may be due to gut tolerance developed to commonly encountered TLR4 ligands in the gut (LPS from E.coli or other microbes). However, with the development of combination technology, InAc-NPs could be used as a delivery system for TLR7/8 agonists in the future oral vaccine development.

In conclusion, the dissertation advanced a new platform technology for mucosal vaccines for both human and animal applications. In addition, a preliminary study was performed to explore InAc-NPs as a delivery system for other TLR agonists, which could have implications in oral vaccine delivery or dual adjuvant delivery for cancer immune therapy.
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