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Polymeric Nanovaccine Delivery System for Influenza Vaccine

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POLYMERIC NANOVACCINE DELIVERY SYSTEM FOR INFLUENZA VACCINE

BY

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DISSERTATION ACCEPTANCE PAGE

Chaitanya K. Valiveti

This dissertation is approved as a creditable and independent investigation by a candidate for the Doctor of Philosophy 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.

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THIS DISSERTATION IS DEDICATED TO MY PARENTS AND FAMILY

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LIST OF ABBREVIATIONS

APCs	Antigen-presenting cells
CD	Cluster of differentiation
CpG	Cytosine-phosphate-guanine
CSF2	Colony-stimulating factor 2
CT	Cholera toxin
CTB	Cholera toxin B subunit
CTL	Cytotoxic T-lymphocytes
CXCR3	Chemokine receptor 3
DCs	Dendritic cells
DMEM	Dulbecco's modified eagle's medium
ELISA	Enzyme-linked immunosorbent assay
FLT3L	FMS-like tyrosine kinase 3 ligands
HA	Influenza haemagglutinin protein
HIV	Human immunodeficiency virus
IgA	Immunoglobulin A
IgG	Immunoglobulin G
IL	Interleukin
INF	Interferons
Inf-A	Peptide from Influenza A nucleoprotein (a.a. 366-374)
LPS	Lipo-polysaccharide
M2e	Ectodomain of influenza A M2 protein

M-cell	Microfold cell
MHC-I	Major histocompatibility complex-I
MHC-II	Major histocompatibility complex-II
MPLA	Monophosphoryl Lipid-A
NK-cells	Natural killer cells
NPs	Nanoparticles
Ova	Ovalbumin
PAMP's	Pathogen associated molecular patterns
PBS	Phosphate buffered saline
PC	Phosphorylcholine
PDI	Polydispersity index
PLGA	Poly (D,L-lactic-co-glycolic acid)
PRR's	Pattern recognition receptors
PVA	Poly(vinyl) alcohol
RSV	Respiratory syncytial virus
s.c.	Subcutaneous
SPR	Surface plasmon resonance
Th1	T-helper 1
Th2	T-helper 2
TLR	Toll-like receptor
TMB	3,3′,5,5′-Tetramethylbenzidine substrate systems
TNF	Tumor necrosis factor
ZP	Zeta-potential

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ABSTRACT

POLYMERIC NANOVACCINE DELIVERY SYSTEM FOR INFLUENZA VACCINE

CHAITANYA K. VALIVETI

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Vaccines are the most efficient and cost-effective method for preventing illnesses caused by infectious pathogens. Even though the great success of vaccines over decades, the development of safe and robust vaccines is still essential for emerging new pathogens, re-evolving old pathogens, and improving the insufficient protection given by existing vaccines. One of the most critical strategies for developing effective new vaccines is selecting and using a suitable adjuvant or immune stimulant. Immunologic adjuvants are essential for improving vaccine potency by enhancing the immune response of vaccine antigens. The amount of antigen could be spared with improved potency, especially during mass vaccinations in pandemics. In the past, our laboratory had discovered a plant-based novel toll-like-receptor-4 agonist (adjuvant), inulin acetate (InAc), and showed that a particulate delivery system using InAc is a potent vaccine delivery system that produces strong humoral and cell-mediated immunity, which was tested in mouse models.

The study in this dissertation investigated the application of nanoparticles prepared with inulin acetate nanoparticles (InAc-NPs) for dual functionality: as a delivery system and vaccine adjuvant for enhancing mucosal and systemic immunity in mice and pigs. The rationale behind selecting InAc-NPs is their established ability to stimulate strong systemic immunity and a clear understanding of their activation mechanisms.

In chapter II, we have established through subcutaneous vaccinations in swine for the first time that inulin acetate nanoparticles (InAc-NPs) could generate high levels of systemic antibodies (IgG) by using influenza antigens the extracellular domain of matrix protein 2 (M2e), and the influenza virus's surface membrane protein, Hemagglutinin (HA) protein. InAC-NPs, as a vaccine delivery system, protected the antigen from degradation during the storage and efficiently delivered it to swine macrophages (in-vitro). The antibodies induced by InAc-NPs have a strong affinity and avidity to bind to HA. These antibodies potentially prevent the virus from entering the host cell. The study introduced inulin acetate (InAc) as a vaccine adjuvant in swine for subcutaneous vaccine delivery.

Chapter III, for the first time, established the efficacy of InAc-NPs as a vaccine delivery system in a mouse for oral vaccines using influenza peptide (Inf-A) as a model antigen. Importantly, InAc-NPs carrying the Inf-A produced higher mucosal and systemic antibodies than unadjuvanted antigens in mice. InAc-NPs activated mouse macrophages to secrete pro-inflammatory cytokines such as Interleukin-6 (IL-6) and macrophage activation marker nitric oxide (NO).

In conclusion, we have demonstrated the capability of InAc-NPs as a robust vaccine delivery and adjuvant platform for parenteral and oral vaccines that offer strong systemic and mucosal immunity, which will have substantial implications in fighting several viral diseases in humans and animals (pigs) in future.

CHAPTER I

INTRODUCTION

1.1. Components of the immune system

Two components of the immune system play critical roles in fighting against infection. These are innate and adaptive immune responses. The process induces long-lasting immunity against pathogens. The innate immune response reacts in a non-specific or broadly specific manner, whereas the adaptive immune response reacts in a highly specific manner (1). The innate immune response uses physical/chemical barriers and cells involved in inflammation and phagocytosis to prevent the initial phase of pathogen invasion. Physical barriers include skin and mucous secreted on the mucosal layer, whereas examples of chemical barriers are chemicals such as bactericidal peptides and enzymes secreted by the body's own cells, which also contribute to pathogen elimination (2, 3). The innate immune response also utilizes soluble proteins such as complements to target pathogen lysis and immune cells such as neutrophils, macrophages, natural killer cells (NK-cells), and dendritic cells to cause pathogen destruction. The process of phagocytosis utilizes the pattern recognition receptors (PRRs) in phagocytes for recognition and binding to pathogen-associated molecular patterns (PAMPs) located on the pathogen cell exterior parts. Alternatively, PRRs also recognize opsonized pathogens. Common opsonins include complements and antibodies. The process of phagocytosis leads to the internalization of the pathogens and fusion with lysosomes, where the pathogens are degraded by the lysosomal enzymes (4, 5).

The adaptive immune response is an acquired immune response where both T and B cells contribute equally to immune response. The hallmarks of adaptive immune response include self/nonself-recognition, antigenic specificity, diversity, memory response, and division of labor among B and T lymphocytes (6-8).

The ability of adaptive immune response to produce the diversified population of antibodies, each specific to a given antigen at the initial exposure, along with the production of memory cells, contributes efficiently to the total elimination of pathogens when the body is exposed to the same pathogen second time. The main participants in the adaptive immune responses are the B lymphocytes which contribute to humoral immunity, and T lymphocytes which contribute to cellular immunity. For the adaptive immune response to occur, professional antigen-presenting cells are involved in the processing and presenting of the antigen by major histocompatibility complex (MHC I and MHC II) molecules. The internalized antigen gets into endocytic compartments of dendritic cells, macrophages, which are presented to helper T cells as peptides to MHC II molecules. The endogenously produced antigens in the cell's cytosolic compartment are presented to CD8+ T cells as peptides to MHC I molecules, thus letting the CD8+ lymphocytes recognize and eliminate virally infected cells. (9, 10). This process causes signal transduction and releases cytokines such as IL-4, IL-2, IL-5, and IFN-γ by T cells. These cytokines act on B and T cells to drive cell differentiation and proliferation, leading to the generation of activated effector T cells (Th1 response) and memory B cells (Th2 response).

Plasma cells secrete a large amount of antibodies to eliminate pathogens and are short-span; the memory B cells are stored in the body for a more extended period (11). Upon second exposure of the body to the pathogens, the memory cells are differentiated into plasma cells capable of secreting antibodies in large amounts. The cytokines released by the helper T cells also augment the cytotoxic T cell function. Thus, B cells produce a humoral response; cytotoxic T cells mediate the cellular responses contribute to the adaptive immune response, and help eliminate pathogens (7, 12-14).

1.2. Vaccine

Vaccination is administering an antigenic component to stimulate an immune response to protect the body against infectious diseases (15). Vaccine preparations may contain a weakened form of the pathogen (bacteria, viruses, etc.) or protein or a toxin isolated from the organism. Vaccines induce cellular and humoral responses that are part of adaptive immune responses and prevent sickness from infection by pathogens. Ever since the introduction of the smallpox vaccine by Edward Jenner in 1798, several vaccines have been developed for a variety of infectious diseases. The vaccines developed for humans include vaccines against Rotavirus, Hepatitis B, Diphtheria, Pertussis, Pneumococcus, Polio, Influenza, Measles, Haemophilus influenza type b, Meningococcus, and Sars-cov-2 virus to name few. Infectious diseases also affect animals such as pigs, dogs, cats, sheep, chickens, fish, and cows. One of the pioneers who contributed to the development of animal vaccines was Louis Pasteur. For example, between 1880 and 1885, he developed two necessary vaccines: chicken cholera and rabies. The typical veterinary vaccines in use today include Influenza, Porcine Circovirus Type 2, Pseudorabies, and Rabies.

Vaccines, when administered, activate the immune system to protect against infectious diseases. The vital component of most vaccines is one or more proteins that produce immune responses and offer protection. Some vaccines contain polysaccharide antigens that can stimulate immune responses to prevent bacterial infections. The typical examples in this category are pneumonia and meningitis caused by staphylococcus pneumonia.

1.3. Types of vaccines

Currently, six categories of vaccines are in use today in humans: 1) live attenuated microorganisms; 2) inactivated whole microorganisms; 3) sub-unit vaccines (purified protein, recombinant protein sub-unit, polysaccharide, polysaccharide/protein conjugates); 4) toxoids; 5) DNA and RNA vaccines and 6) viral vector-based vaccines (Fig.1.1). There are apparent differences between live attenuated vaccines and inactivated vaccines. The former may potentially replicate in an uncontrolled manner in people with immunocompromised conditions, such as individuals with immuno-deficiencies, HIV-infected patients, or those treated with immune-suppressive drugs. This possesses some degree of restrictions on their use.

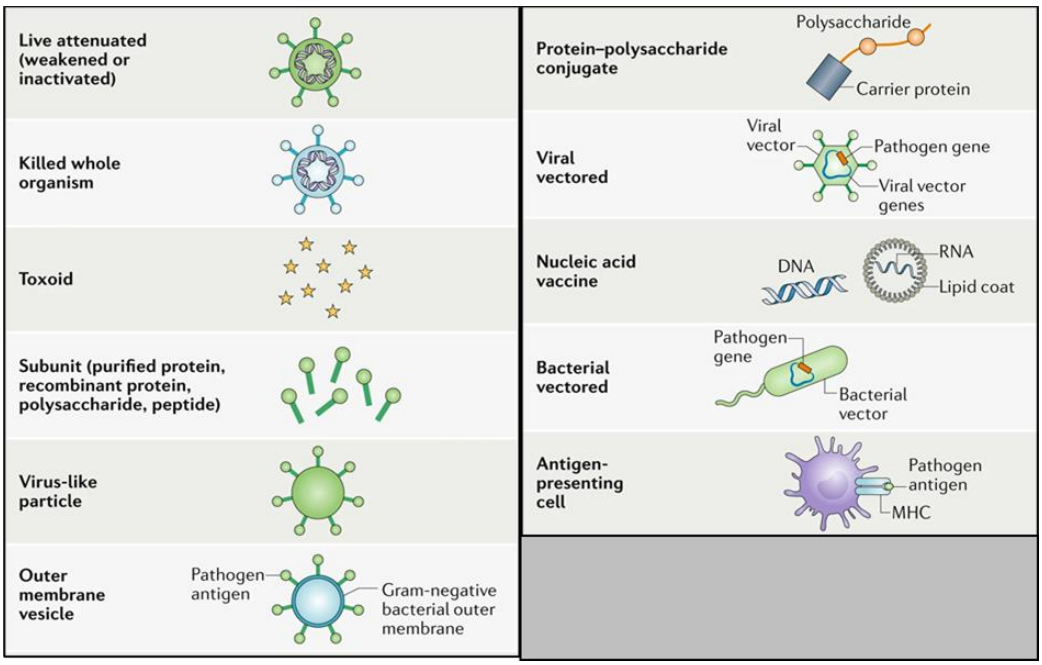


Fig. 1.1. Different types of vaccines. Modified and adopted from the reference (16). Traditionally vaccines are divided as live and non-live to differentiate by their presence of attenuated replicating strains or killed pathogens. In addition to traditional vaccines, new vaccine platforms are emerging.

Attenuated live vaccines include rubella, measles, mumps, rotavirus, and polio. One of the most significant advantages of the live attenuated vaccine is that the microorganisms replicate sufficiently to elicit an immune response without causing significant infectious symptoms. In contrast, inactivated vaccines do not pose any risk to immune-compromised people.

Examples of non-live vaccines include the whole-cell pertussis vaccine and inactivated polio vaccine. These preparations can be made through heat inactivation or chemical treatment. Acellular pertussis vaccine is a purified protein vaccine. Some recombinant protein vaccines include hepatitis B vaccine, and examples of toxoid vaccines include inactivated protein toxins isolated from tetanus and diphtheria bacteria treated with formaldehyde. It is essential to point out that other than whole virus vaccines or any different strategy, it is often administered with an adjuvant to potentiate their immunogenicity before administering to the body. In this regard, a few adjuvants are used routinely in vaccine preparations. Traditional examples of adjuvants are an alum and its salts. However, liposome-based adjuvants and oil-in-water (o/w) emulsions have recently been used. An example of the o/w emulsion is MF59 used in influenza vaccines; another example is AS01 used in shingle and malaria vaccines; ASO4 used in human papilloma vaccines. Although the mechanism of action of adjuvants is not known, it is believed that they provide danger signals mimicking those present in pathogens to the immune cells.

Vaccines may also contain ingredients that act as preservatives. Each excipient has its benefit, the formulation, such as non-ionic surfactants that act as emulsifiers (polysorbate-80) and stabilizers (gelatin or sorbitol). Other components used in the manufacturing of vaccines may also be present in the vaccine as trace amounts. This may

include antibiotics, yeast proteins, egg, formaldehyde/glutaraldehyde, latex, and pH regulators (salts). Generally, these trace amounts of the components carried over to the final preparation of the vaccine do not cause any harm to human health; however, in some individuals, they may induce allergic reactions.

Vaccines protect the immunized individual through various mechanisms (Fig.1.2) involving both B and T cells and the protection occurs mainly through the production of antibodies. The vaccine induces immunological memory central to protection against future infections. When the body encounters the pathogen for the second time, the memory of immune response attained by vaccination to that particular pathogen mounts an immune response faster and more robustly.

1.3.1. Protein vaccines

Most vaccines against infectious diseases consist of inactivated or live attenuated pathogens. The attenuated and inactivated vaccines are considered conventional vaccines, and vaccination using these have successfully decreased the incidence and burden of several infectious diseases over the last several decades.

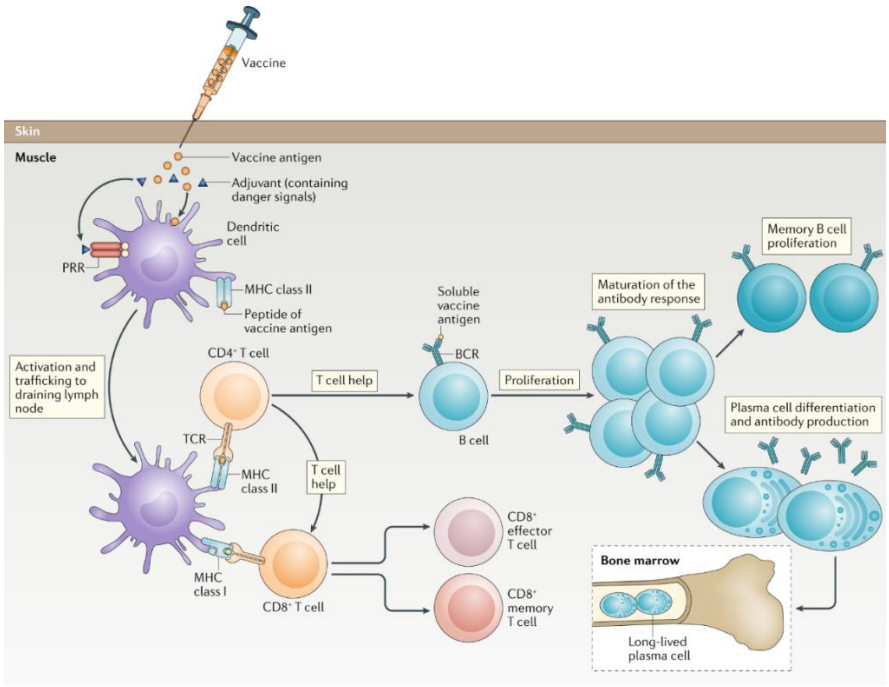


Fig. 1.2. Generation of immune response to the vaccine. Modified and adopted from the reference (16). The immune response is followed by administering the protein (antigen) vaccine to muscle cells. The PRRs on the APCs recognize adjuvant as a danger signal and initiate the immune response.

Attenuated vaccines, for example, viruses generally take years to develop because a lot of time is required to isolate these viruses and subsequently adapt them in-vitro to decrease their virulence. Cultivating the pathogens in specialized biosafety facilities may also be challenging and adapting them to grow in such conditions. Purification and testing of these attenuated vaccines may also require complex methods. In addition, attenuated vaccines always have the risk of reversion and cause disease in humans. As mentioned above, inactivated vaccines, on the other hand, need to be combined with adjuvants to increase their immunogenicity as the danger signals are often lost during their preparation. Because of these reasons development of traditional vaccines is a complex, expensive, slow, and laborious process requiring substantial investment (17). It is estimated that the

development of a conventional vaccine candidate is estimated to cost up to one billion dollars before it can be mass-produced before entering the market. It is estimated that the average time for developing a conventional vaccine from the pre-clinical phase to marketing requires appx. Ten years and only 6% of them reach the market. The lengthy time and the multiple and complex steps involved in manufacturing a conventional vaccine necessitate the development of new strategies to accelerate the variety of vaccine platforms. Therefore, traditional methods of manufacturing attenuated and inactivated vaccines are ineffective in protecting the world population in contrast to both established and evolving pathogens. In this regard, viral vector and DNA or RNA-based vaccine platforms have been created to overcome vaccine challenges during the past few decades.

Attenuated or inactive whole microorganisms can induce a strong immune response since they have both T and B cell epitopes presented in a way that mimics the pathogen. Due to the weak immunogenicity of protein/peptide vaccines, they may need to perform many immunizations to reach comparable efficacy to the whole virus vaccines. Nevertheless, various approaches, such as presenting epitopes in various formats, have been utilized to increase the effectiveness of subunit vaccines (e.g., nanoparticles or virus-like particles) or formulation with adjuvants. Peptides presented on MHCs should meet the sequence of amino acid requirements; however, it is important to note every epitope is immunogenic (18). Currently, Trimer (adjuvants; Matrix M), SCB-2019 trimer (adjuvants; Alum+CpG 1018 or AS03) , Covax-19 (adjuvant; AdvaxCpG55.2), and SARS-CoV-2-RBDN1C1 (adjuvants;Alum+CpG), recombinant vaccines are under clinical trials for COVID-19 (19, 20).

1.3.2. DNA and RNA vaccine

Nucleic acid vaccines: One of the vaccine platform already proven to be safe and effective against infectious diseases are nucleic acid vaccines. They induce immune responses which target only the selected antigen in the Pathogen. There are 2 types of nucleic acid vaccine: RNA vaccine (as messenger RNA or mRNA) and DNA vaccine (plasmids). DNA vaccines include a gene that encodes the desired part of a pathogen into a bacterial plasmid. DNA vaccines can be delivered through intramuscular, intradermal, mucosal, and transdermal routes. They contain plasmid DNA carrying the gene representing the gene of the antigen encapsulated with lipid nanoparticles (LNPs). Once the DNA vaccines are administered, the DNA is transferred to the nucleus of the cells (for example, professional APCs), where it is transcribed; the mRNA produced is transported to the cytoplasm to produce the foreign antigen (21). Upon expressing the foreign antigen, the professional APCs can degrade them to peptides and then relocate to lymphoid structures (lymph nodes) for presenting the antigen to resident cytotoxic T cells and T_h CD4+ cells to mount an adaptive immune response. Notably, the professional APCs may also acquire and process the foreign antigen shed by other transfected cells at the injection site, eventually presenting the peptides to the helper T cells.

The DNA vaccines is to deliver the load into the nucleus of the cell, DNA transcription occurs and later translated into protein (antigen) in the cytoplasm. One limitation of DNA vaccines is the comparatively low immune activation profile, which hampers the required clinical effect. However, there are still safety concerns for DNA vaccines. For example, the risk of generating anti-DNA antibodies will lead to autoimmune disease conditions. Prior studies indicated that the (HBV) vaccine produced anti-DNA

antibodies and activated autoimmune disorders (22, 23). Moreover, the major risk of DNA vaccines is the possibility of incorporation into the host genome. This potentially causes the mutations, which will be seen as malfunctioning or inactivating gene expression (e.g.tumor suppressor gene) (24).

RNA vaccines are proven successful and could be explored for various infectious diseases (25). RNA vaccines contain mRNA encapsulated LNPs. When injected into the body, several cell types, including the APCs, take up these mRNAs, which undergo translation and produce proteins in the cytoplasm. These proteins are then breakdown into peptides and presented by MHCs to T cells. In addition to regular routes of antigen presentation, macrophages can present the antigens generated through the lysosomal/endosomal pathway via the MHC I molecules to the cytotoxic T cells (26).

1.4. Vaccine adjuvants

The adjuvant is part of a vaccine that enhances a more robust immune response.

In conjugate, recombinant, toxoid vaccines, and inactivated, adjuvants are employed significantly for improved and long-lasting immune responses. Traditionally aluminum-based salts are used as adjuvants for commercially used vaccines. More adjuvants are available but constrained to a few vaccines, e.g., CpG ODN, MF59, AS01, AS03, MF59, and AS04 are FDA-approved vaccines for human consumption. So far, FDA-approved adjuvants for commercial use are o/w emulsions (AS03 and MF59, aluminum salts, AS01) (27).

The adjuvant molecular mechanisms by which they work still need to be explored to understand better. However, the knowledge of the stimulation of the innate responses

through PRRs is refining the effectiveness of adjuvants. The research studies also pointed out that sensor activation (metabolic, nutrient) and tissue damage can regulate the innate immune system to stimulate cellular or humoral immunity. Some of the adjuvants are applied AS01 (malaria vaccine Mosquirix), AS03 (Pandemrix and Arepanrix), AS04 (human papilloma vaccine Cervarix), and CpG-1018 (hepatitis B vaccine Heplisav-B) (28). These adjuvants are essential TLR receptor activators, usually present on APCs. In the late 1990s, it was found that stimulation of TLRs, which sense PAMPs in microbes, are the source for activation of APCs, which in turn promotes antigen-specific (T and B cell) reactions (29-32).

There are membrane-bound TLRs (TLR 1–TLR 13) that have been reported. So far, TLR is known to identify the danger signals located on the cell membrane or the endosomes. Lately, it has been reported cytosolic recognition systems such as RLRs and NLRs. The RLRs are receptors that detect viral RNAs and control anti-viral systems by IFN production (33). On the other hand, NLRs comprise several types, such as NOD1, NOD2, and LRR (leucine-rich repeat) (33).

1.4.1. Toll-like receptors (TLRs)

Additional benefits of utilizing adjuvants are dose sparing, increasing vaccine efficacy in older persons, and increasing the vaccine capability to work against variable pathogens by eliciting a broader immune response. TLR agonists are urged to be used as an adjuvant because of their ability to coordinate with adaptive immune response. TLRs are cell membrane receptors primarily present on APCs. They are categorized into the cell membrane (TLR 1,2,4,5 and 6) and intra-cellular TLRs (TLR3, 7 -9), present on endosomal membranes (Fig.1.3.) (34). Bacterial lipopolysaccharide (LPS) has been shown as a ligand

for TLR4 receptors and initiates the inflammatory signal (35). In one study, it was demonstrated that TLR4 knockout mice did not respond to LPS in an in-vivo study (36). The same trend was observed for (CpG) DNA; an agonist for TLR9 failed to stimulate in the knockout model (37). The inherent downstream signaling pathways of mammalian TLRs have been demonstrated in the picture (Fig.1.3). TLRs recruit the downstream activating proteins after binding with the agonist. It included the TIR-containing adaptor proteins molecule MyD88, which activated the MAP kinases and NF- kB signaling to initiate articulation of TNF- α (38). The TLRs also can trigger IFN responses, for example, IRF-3 and IRF-7, which perform an vital role in connecting innate and antigen-specific immune activation by activating co-stimulatory fragments on immune cells (39, 40).

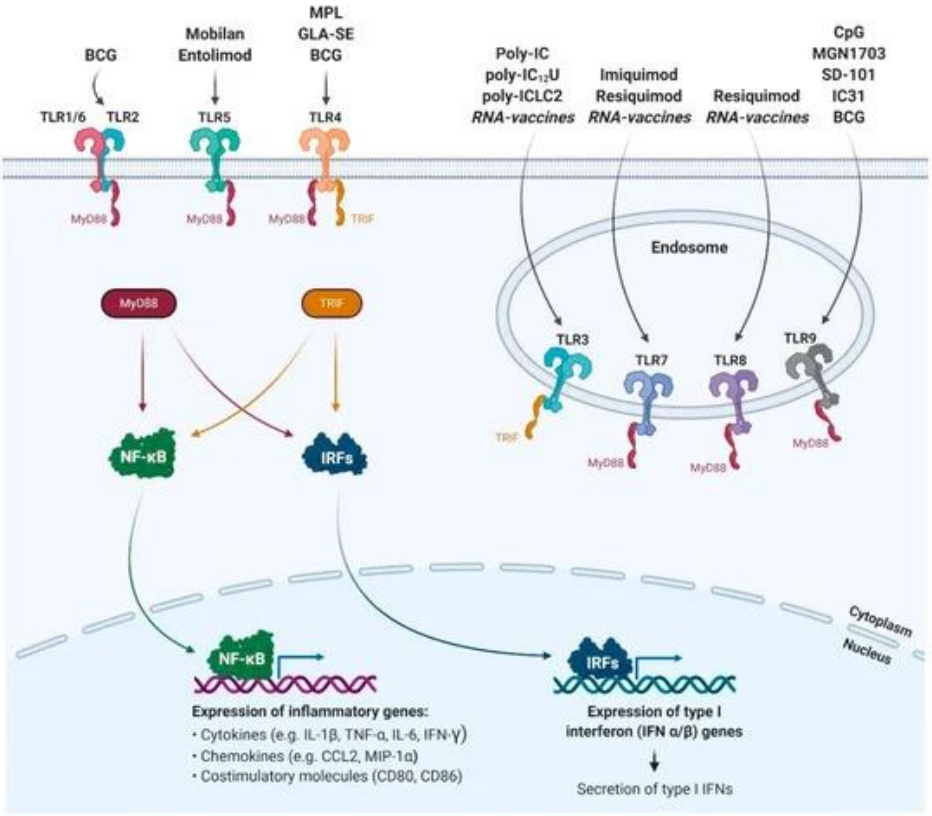


***Fig. 1.3. TLR-activating adjuvants and the induced signaling pathway*.** Modified and adopted from the reference (34). TLRs on the cell surface or intracellular targets for the immunopotentiators (adjuvants). With the binding of ligand to the leucine-rich repeat, TLRs recruit the proteins for activating the signaling pathway.

The inflammatory response trigger dendritic cells to induce local and systemic inflammatory responses and produce pro-inflammatory cytokines, including TNF, and Interleukin-6. The cytokines TNF initiate the stimulation of local endothelium to increase vasodilation and raise the penetrability of the blood vessel, permitting serum proteins and leukocytes to be recruited at the site of infection (41). Further, microbial spreading could be prevented by coagulation factor III by initiating a coagulation cascade. Furthermore, the stimulation of hepatocytes by IL-1β, along with IL-6, generates acute phase proteins with collectins. These proteins help in phagocytosis by immune cells (antimicrobial response) (41).

TLRs can also directly generate antimicrobial proteins by stimulating macrophages. In mouse macrophages, nitric-oxide synthase (iNOS) is produced and has a vital role in antimicrobial defense (42). Based on the current state of vaccines, weak immunogenic subunit vaccines than whole virus vaccines are being utilized. To overcome this, addition of adjuvant boosts immune response.

TLR3 agonists

The TLR3 recognizes the dsRNA templets and promotes NF-kB production. The dsRNA polyinosinic-polycytidylic acid (poly-IC) works by acting on TLR3 and RLRs, activating innate immune response and stimulating the adaptive immune activation (34).

TLR4 agonists

Monophosphoryl Lipid (MPL)

The discovery of MPL as a TLR4 adjuvant accelerated the search for more adjuvants. MPL was the first TLR agonist to receive licensed commercial use in vaccine preparation as an adjuvant. The AS01 adjuvant, mixed with saponin and liposomes, is used in the herpes vaccine (43). In addition, MPL is adsorbed on aluminum salts within the AS04 adjuvant, used in the human papillomavirus vaccine Cervarix (44-47). MPL as an agonist, activate TLR4, and APCs produce the NF-kB to release prominent cytokines, such as IL-6 and TNF-α (proinflammatory). These produced proinflammatory markers enhance the immune response by macrophage maturation, decrease the regulatory T-cell and suppress the tolerance (48).

Inulin Acetate (InAc)

Our laboratory previously discovered a TLR4 agonist from a plant fiber inulin. Inulin is modified to InAc to make it more active. (Fig. 1.4) (49). The TLR4 agonist activity studies were established in multiple immune cells: microglia, dendritic, and peripheral blood mononuclear cells (50-52). We observed that incubation of InAc with the above immune cells could release pro-inflammatory cytokines. However, the InAc failed to activate

immune cells in the presence of TLR4 antagonist or immune cells absent in TLR4 or deficient in adapter proteins involved in TLR signaling (Mal/MyD88). The antigen encapsulated water-insoluble particles of InAc mimicked the pathogen properties. They offered improved antigen delivery compared to soluble antigen or antigen delivered through poly(lactic-co-glycolic acid) (PLGA) particles (49). We named this "pathogen-mimicking vaccine delivery system (PMVDS)" (50). The distinctiveness of PMVDS is high antigen delivery to the APCs as an efficient vaccine delivery system, and concurrently as an adjuvant, it activates the TLR4 on APCs to release the cytokines/chemokines. A mouse study was conducted, and the PMVDS administered mice results suggested robust stimulation of both humoral (>32 times vs. alum) and cellular immune responses to encapsulated antigen (Ovalbumin). The InAc particles as a delivery system stimulated strong cell-mediated immunity to provide protection in around 40% of the vaccinated mice against tumor (B16-ova-Melanoma) progression (50, 51).

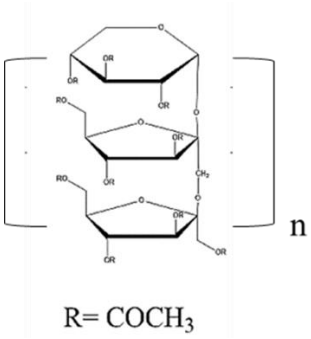


Fig. 1.4. Structure of Inulin Acetate. Modified and adopted from the reference (49). The soluble form of inulin was modified to InAc by acetylating the hydroxyl groups. Inulin acetate is water-insoluble, and its backbone structure (poly-fructose with beta (2-->1) linkages in linear chains.

Glucopyranosyl Lipid A in a Stable Emulsion (GLA-SE)

Based on the established MPL adjuvant properties, a second-generation of stable emulsion with glucopyranosyl lipid A activated the TLR4 s. Evaluated the GLA-SE activity in enhancing the H5N1 flu vaccine in clinical trial as an adjuvant. (53). In some other studies, investigated GLA-SE for its adjuvant (combination with four proteins) activity with ID93 tuberculosis (TB) vaccine (54). Additionally, when the GLA-SE was combined with the flu vaccine, Fluzone observed improved T cell and antibody levels and boosted the specificity in evaluation with respect to Fluzone (55).

TLR5 agonists

Entolimod is a rprotein and a derivative of flagellin for pharmacological activity. The TLR5 binds to bacterial flagellin, activates the NF-kB signaling and initiates the innate immune response. In mouse studies, anti-tumor effects were started through the CD183-dependent immune cell activation (56).

TLR7 agonists

Resiquimod, a dual agonist for TLR7/8, is a typical imidazoquinoline molecule (57). It failed to elicit the local immune response while tested as an immunopotentiator in the influenza vaccine against infectious diseases. However, due to its solubility, resiquimod diffused quickly from the injection site to the nearby tissues and throughout the body.

In conclusion, the adjuvants combined with the subunit vaccines could potentially stimulate the immune system. The immunopotentiator effect of adjuvants could be explored in systemic or mucosal vaccines.

1.5. Mucosal immunity: Oral and intranasal vaccination

The worldwide problem of morbidity and mortality related to infectious diseases, especially mucosal viruses, is extreme. The severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) pandemic reminded the world of the constant threat of mucosal infectious disease and the danger caused by many mucosal viral infections for which not many vaccines exist. Due to the lack of effective vaccines, there is a need for vaccines for respirational pathogens (58).

Respiratory pathogens persist as a leading cause of global deaths. The fourth leading cause of death worldwide is respiratory tract infections and infecting around 2.4 million deaths per year. Few of the diseases were streptococcus pneumonia and influenza virus, remarkably infecting the younger and older population (59). Currently, there is no commercially available vaccine for respiratory syncytial (RSV) infection, most common in younger people under 10 years (60, 61). Even though few commercial vaccines target mucosal pathogens like bordetella pertussis, streptococcus P, and influenza virus, there is still a need to enhance the vaccine effectiveness for protection, especially at the site of infection. Studies show that the advanced mucosal vaccine strategy offers promising treatments for these infections. For instance, for influenza vaccines, live attenuated doses are given intranasally, and lately, these vaccines have become a central part of influenza vaccination approaches, especially to children (62, 63); vaccines for B. pertussis are administered intranasally and successfully enrolled for phase II (64, 65). The rise of SARS-CoV-2 has strongly shown how fatal respiratory pathogens can be, with around two hundred eighty million people infected and 5,400,000 deaths attributed to this pathogen

(66, 67) and predicting that the pandemic will continuously impede the world economy, especially third world countries (68, 69).

Even though there are multiple effective vaccines for SARS-CoV-2, there require developing a vaccine; to meet the challenges posed by mass production and distribution.

To overcome the vaccination attempts in third-world countries, developing orally delivered vaccines (SARS-CoV-2 or influenza) will improve convenience and compliance (70). Although it is challenging to design the universal mucosal vaccines with conserved antigens, it is a feasible option for preventing the upcoming pathogen infections (71).

Several microorganisms invade the mucosal routes (mouth and the upper respiratory tract) and infect and lead to an alarming disease condition in the lower respiratory tract. The upper respiratory tract's secretion of bactericidal enzymes and mucociliary transport helps eradicate pathogens before reaching the lower respiratory tract. Mucosal immunity comprises protection mechanisms comparable to the gastrointestinal and respiratory tract (72).

Secretory IgA (sIgA), a dimer, is the primary secretory antibody secreted in mucous fluids. sIgA lacks the complement activation or bacteriolytic effects (as IgG) because it cannot destroy the pathogen. Alternatively, the neutralizing and agglutinating activity of sIgA prevents the binding of pathogens to the cells. The pathogen bind by sIgA will be cleared through mucociliary transport from the mucosal routes. sIgA is one of the contributing factors in maintaining the upper respiratory tract homeostasis (immunological and microbiological).

Some pathogens reside in the upper respiratory tract, including Streptococcus P. and Haemophilus influenza. Increased colonization of these pathogens in healthy children

leads to acute otitis media (AOM) & Otitis media with effusion (OME). Therefore, to prevent these conditions, there is a need to develop mucosal vaccines that protect the nasopharynx.

These vaccines elicited the only systemic immune response and were administered through the subcutaneous route. To overcome this problem, administering through mucosal routes (intranasal or oral) elicits better immune responses comprising mucosal level immunity and systemic immune responses. One of the successful vaccines approved for commercial use for flu and secondary AOM is FluMist. This vaccine contains live attenuated influenza and is intranasally administered (73).

1.6. Lessons from licensed mucosal vaccines

In the past years in vaccine research, there has been a wide range of formulation types, from the traditional whole-cell killed/attenuated vaccines to vector-based, nucleic acid (DNA or RNA) or adjuvant subunit vaccines (74, 75). These changes will affect the disadvantages of whole viral vaccines by introducing delivery systems and adjuvants.

However, the success rate of the mucosal vaccine is different than other routes in terms of its efficacy. Of the nine mucosal vaccines (only live-attenuated or whole-cell inactivated formulations) approved for human consumption, one is intranasal, and eight are orals (Fig.1.5).

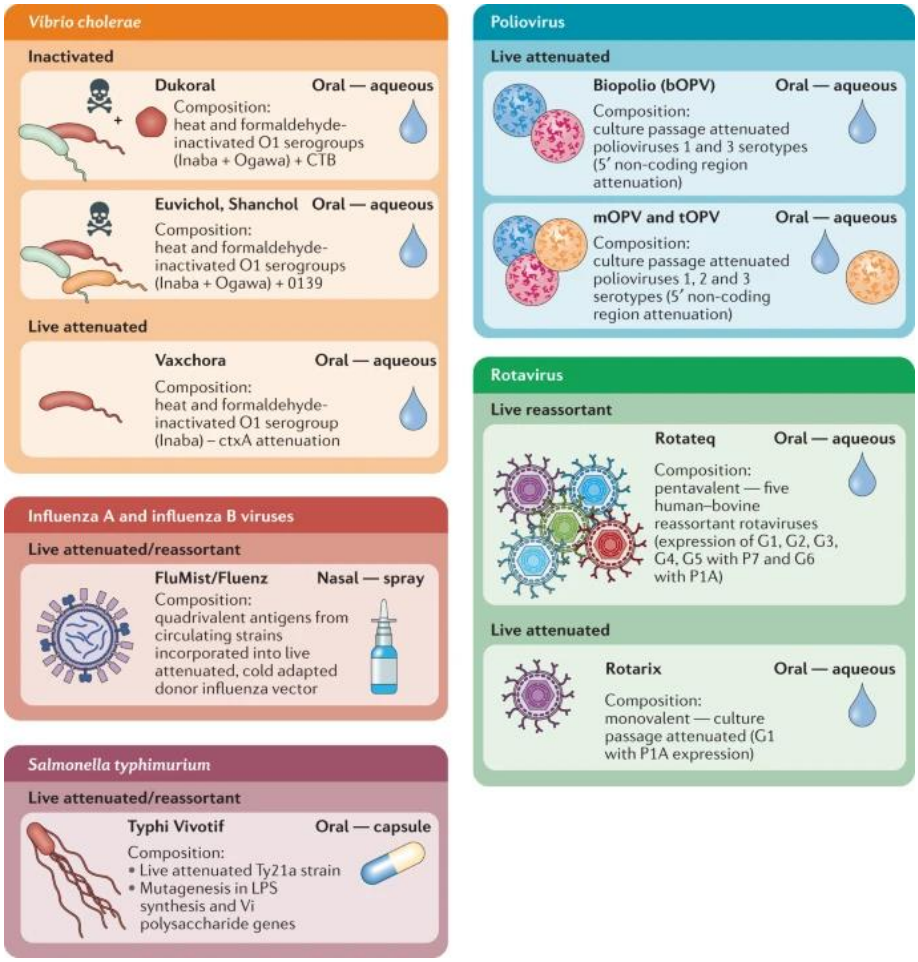


Fig. 1.5. Successful platforms for mucosal vaccine design. Modified and adopted from the reference (58). There are eight oral vaccines licensed against poliovirus, cholera, rotavirus, and salmonella. So far, the whole virus vaccines (live attenuated and killed vaccines) have demonstrated the effectiveness of mucosal vaccines.

The cholera toxin B subunit (CTB) was the first subunit antigen licensed as a mucosal vaccine in 2004 as an added ingredient to the Dukoral (inactivated whole-cell Vibrio cholerae vaccine). CTD is highly immunogenic and attaches part of cholera toxin; (76-78) and binds with high affinity to ganglioside receptor (GM1) on epithelial cells.

Moreover, extrapolation of vaccine effectiveness from rodents to human beings can be difficult because of variation in physiological conditions, including GI tract residence

times, pH of the GI tract, and intestinal surface area. The vaccine ingredients need to be considered based on the candidate's capability of eliciting a mucosal response. When the antigens are in the particulate form, whether as attenuated, whole bacterial cells, synthetic particulate formulations, or virus-like particles, they are more potent in immunogenicity than purified proteins. Further, the particulate nature enhances the uptake and targeting APC site when mucosal is delivered. The nature of antigen uptake (Fig. 1.6) is a vital factor when considering a vaccine candidate. The property of the antigen will determine the type of immune activation, such as particulate, living, or soluble nature.

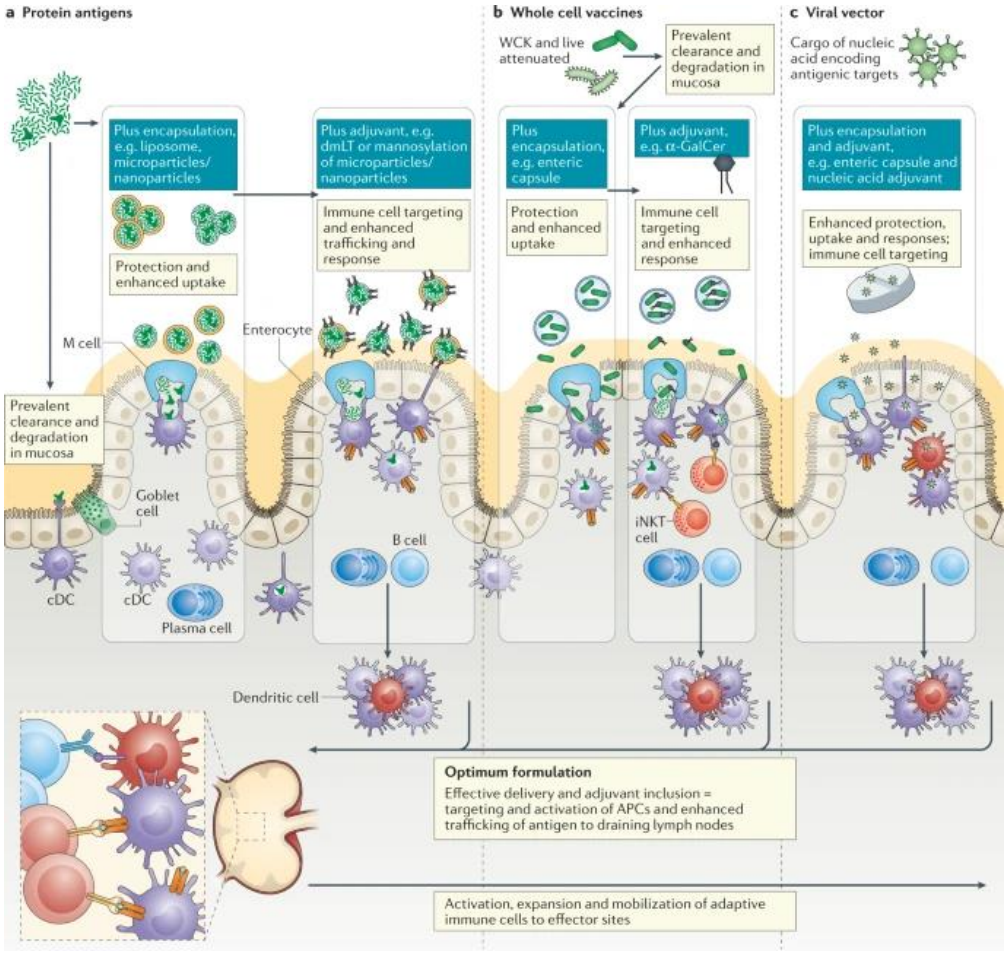


Fig. 1.6. The nature of antigen uptake depends on the vaccine system. Modified and adopted from the reference (58). Disposition of antigen uptake depends on the vaccine components.

The immune homeostasis in mucosa due to tolerance plays a critical role in vaccine effectiveness (79). The oral route is constantly exposed to different antigens procured from the food and pathogens. This leads to oral tolerance and maintains the immune homeostasis of the intestine (80). The gut tolerogenic can be caused due to acclimatization to inflammatory conditions triggered by microorganisms (81) for innate immune response. Combined with adjuvants in the vaccine will overcome the tolerance and induce innate and adaptive immunity immune responses. However, more research needs to explore a potential mucosal adjuvant. Few adjuvants are under investigation (Table.1.1) (82, 83).

Table. 1.1. Adjuvants and their targets

Composition	Target	Reference
Muramyl dipeptide (MDP)	TLR2	(84)
Monophosphoryl lipid A (MPLA)	TLR4	(85)
Flagellin	TLR5	(86)
Cholera toxin	GM1	(81)
Quillaja saponins	DCs	(87)
Dimethyldioctadecylammonium (DDA)	DCs uptake	(88)

In conclusion, there is a need for safe and effective adjuvants to boost mucosal immunity when administered with the antigen against respiratory viruses, including influenza viral infection.

1.7. Influenza

Influenza (flu) is highly infectious and enters by respiratory tract and leads to sickness. The influenza viruses contain a negative-sense RNA (ssRNA) genome and can infect animals and humans. The difficulties of influenza can cause significant morbidity and mortality. As per the recent information from the Center for Disease Control and Prevention (CDC), it was estimated that there were 41 million illnesses, among them 140,000 – 710,000 hospitalizations and 12,000 – 52,000 deaths yearly between 2010 and 2020 (89, 90).

1.7.1. Structure and types of Influenza

So far, they have discovered 4 types of influenza viruses (A, B, C, and D) based on their differences in antigen present on nucleoprotein and matrix protein. The influenza genome is separated into different subtypes 8 types (A, B) or 7 types (C, D) influenza strains. Out of all these strains, the major respiratory infections reported are caused by Type A strain and lead to illness or death. There is a possibility of a new influenza epidemic or pandemics. It is also reported that the influenza B strain can cause infection in humans. In seasonal flu infections, influenza B lineages, B/Victoria and B/Yamagata, are used in vaccine preparation due to their circulation annually (91). Influenza C viruses generally trigger moderate symptoms. Influenza D infects small farm animals, including sheep, swine, and cattle. There is limited data on how it infects humans (92-95). Influenza viruses

have a filamentous or spherical shape. The influenza virus has surface lipid membrane glycosylated proteins hemagglutinin (HA) and neuraminidase (NA) (Fig.1.7). The protein unit number may vary between the different types of influenza based on the antigenicity (96, 97).

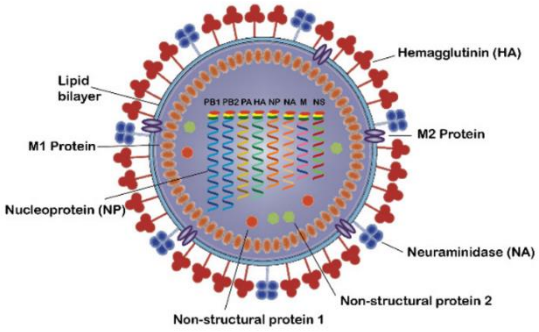


Fig. 1.7. Structure of influenza virus. Modified and adopted from the reference **(98).** Schematic representation of Influenza A virus, illustrating the location of different antigen sites including surface glycoproteins, nonstructural, and nucleoproteins.

The influenza virus contains the lipid envelope membrane proteins and non-structural protein 2 (NS 2), RNA segments layered with nucleoprotein (NP) in the RNA complex. Influenza viruses are subdivided based on the surface HA and NA glycoproteins; it has reported eighteen subtypes of HA and eleven subtypes of NA (98). The 3 proteins (HA, NA, and M2) are present on the M1 lipid envelope. The M2 ion channel protein is present in small numbers, around ten per hundred molecules of HA. On the other hand, viruses have polymerase acid protein (PA) and RNA-dependent RNA polymerases (PB1 and are PB2) (99).

1.7.2. Influenza vaccine

Vaccination signifies an effective and economically affordable way to restrict epidemics caused by various viral infections like influenza and protects human health. Annual influenza vaccine efficacy varies due to antigenic drift or shift. Influenza viruses undergo genetic mutations and evade the immune system, and vaccine strains must be revised every year. The effectiveness of commercial vaccines

The protective effects of the currently licensed vaccines differ annually (Fig. 1.8) and depend on the antigenic similarity between the vaccine strains and viruses. The vaccine efficacy can also be varied by the host immune system. For instance, elderly and young individuals are more vulnerable to influenza infection (100-102).

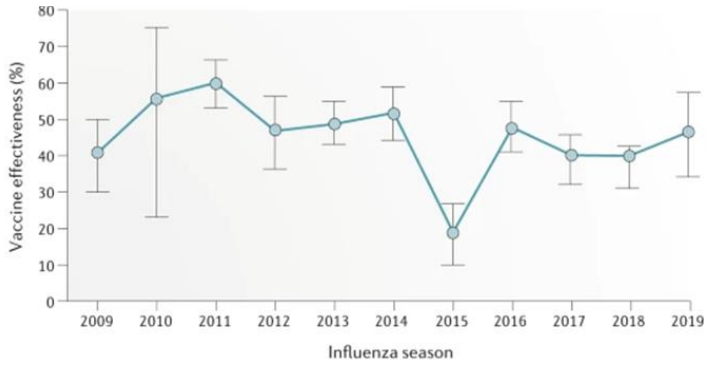


Fig. 1.8. Seasonal influenza vaccine effectiveness from 2009 to 2019. Modified and adopted from the reference (103). The efficacy of the annual flu vaccines depends on the antigen match among the spreading strains and vaccine strain.

There are threats from the virus in multiple ways; it may be due to the risk of re-evolving earlier endemic viruses and may be due to novel viruses emphasizing the need for robust and cross-protective influenza vaccines. The interest in designing a new formulation that effectively generates the neutralizing antibodies and provides cross protection could be achieved by targeting the conserved regions of the virus (104-106).

Research studies are in progress to develop vaccine formulation and move towards a universal vaccine for influenza (Table. 1.2). The goal of designing the vaccine is to elicit a cross-protection against different strains of influenza or the potential to work on mutated novel strains and stimulate the long-term response.

Currently, there are multiple platforms or universal influenza vaccines in clinical trials. New platforms have been developed using recombinant proteins, VLPs, or nucleic acid-based delivery (107). Moreover, along with the conserved epitopes as HA, extracellular domain of M2 and the NA, are also being studied. The conserved regions (M1 and NP) of influenza virus like M1 and NP, are also have under investigation for the stimulation of cross protection by T cells (73).

1.8. Conclusion

In this dissertation, we are designing the nanovaccine particulate system for influenza vaccines in swine to evaluate the vaccine efficacy in eliciting humoral immunity; and the oral vaccine delivery system for influenza and investigating its mucosal immune response against the vaccine antigen in mice.

Table. 1.2. Currently licensed vaccines in the United States and Europe. Modified and adopted from the following reference (103).

Vaccine technology/platform	Vaccine type	Vaccine name (manufacturer)	Target/ MOA	Adjuvant used
Inactivated virus	Split virus	Afluria (Seqirus)	HAI	None
		Fluarix (GSK)	HAI	None
		FluLavel (GSK)	HAI	None
		Fluzone, Fluzone HD (Sanofi Pasteur)	HAI	None
		Fluvirin (CLS Limited)	HAI	None
	Subunit	Flucelvax (Novartis)	HAI	None
Live-attenuated	Live, cold-adapted	FluMist (AstraZeneca)	HAI	None
Recombinant protein	Non-purified HA	FluBlok (Sanofi Pasteur)	HAI	None
Inactivated virus	Split virus	Influvac, Imuvac (Abbot)	HAI	None
		Fluarix, Alpharix, Influsplit (GSK)	HAI	None
		3Fluart (Omninvest)	HAI	None
		Afluria, Enzira (Pfizer/CSL)	HAI	None
		Vaxigrip, Vaxigrip Tetra (Sanofi Pasteur)	HAI	None
	Subunit	Agrippal (Seqirus)	HAI	None

Chapter II

Multifunctional Pathogen-Mimicking Vaccine Delivery System for Influenza Vaccine

2.1. Introduction

Viral diseases such as porcine reproductive and respiratory syndrome (PRRS), porcine circovirus type 2 (PCV2), porcine epidemic diarrhea (PED), and influenza pose a severe challenge to the productivity of today's swine farms (108, 109). Influenza viruses are single-stranded RNA enveloped viruses of the family Orthomyxoviridae. Serological and virological studies showed a 23- 28% prevalence of swine influenza in the swine population of the Midwest and north-central united states. Influenza infection in pigs causes weight loss, fever, cough, anorexia, and nasal discharge. Further influenza infections in pigs increase the risk of transmission to humans. Often the best route for managing viral diseases is prevention through efficient vaccines. However, not all viral antigens are immunogenic enough to stimulate a potent immune response. Therefore, immune-stimulatory agents called adjuvants have become an integral part of vaccines (29, 110, 111).

Adjuvants are molecule carriers dispensed with vaccines to enhance the immune response. The adjuvants in the biological system will be recognized by the immune system as a foreign antigens. The early responders in innate immunity to infection are APCs such as dendritic cells (DCs), Langerhans cells, and macrophages will identify pathogens or microbes through PRRs. PRRs are expressed both on the cell membranes and inside the cells (112-114). The transmembrane receptors C-type lectin receptors (CLR), TLRs, and NLRs are well-studied PRRs. They recognize PAMPs or MAMPs and DAMPs that initiate the signaling to release cytokines and chemokines and alter the surface receptors that modulate humoral and cellular immune responses. The activation of the TLR signaling pathway is vital against influenza viral infection. Significantly among various TLRs,

targeted activation via TLR4 ligands triggers instant defensive responses such as inflammation and sets up antigen-specific immune responses which include humoral, and cell mediated responses. Very few vaccine adjuvants are available which can stimulate both humoral and cellular type immune responses (E.g., monophosphoryl lipid (MPL), AS04 (it is a combination of aluminum hydroxide and monophosphoryl lipid A (MPLA)). Nevertheless, these advanced adjuvants are too expensive for mass vaccinations for farm animals (5, 33, 115-119).

To overcome the economic burden for farmers due to adjuvant cost, our laboratory previously discovered inulin acetate (InAc), a plant-based polymer that acts as a novel TLR4 agonist. Inulin acetate is an acetylated product of plant-derived polysaccharide Inulin. The TLR4 agonist activity was established in multiple immune, microglial, and peripheral blood mononuclear from human and swine origin. Antigen encapsulated in micro- or nanoparticles prepared with InAc improved antigen delivery to dendritic cells (49, 50).

The nanoparticle (NPs) based vaccine delivery platforms have successfully elicited a robust immune response against the encapsulated antigen. The first step for any successful vaccine is that the antigen must be internalized and processed by APCs. Being particulate, the NPs containing the antigen are efficiently phagocytosed by the APCs. Previously, our laboratory designed a novel nanoparticulate-based pathogen-mimicking vaccine delivery system (PMVDS) using a TLR4 agonist InAc as a polymeric matrix to target APCs such as dendritic cells (50). This dual mechanism of efficiently delivering antigen to APCs and activating them through TLR4 produced robust humoral and cell-mediated immune responses against the encapsulated antigen (ovalbumin) in mice (52).

In adjuvant research, the success of an adjuvant in one animal species cannot be extended to another species until proven. In this study, we are investigating the InAc as a TLR4 adjuvant in pigs for the first time by designing an InAc-based nanoparticulate influenza vaccine using surface protein hemagglutinin (HA) and extracellular peptide of matrix protein (M2e) from Influenza-A/California/04/2009 (H1N1) as model antigens. Testing the efficacy of an influenza vaccine in the swine model is also more clinically relevant because of the immunological similarities between pigs and humans in terms of structure and function. The humoral response was assessed as the antibody responses against the HA, which has been established to be protective against influenza virus infection (120). Similarly, a highly conserved ectodomain of M2e peptide has been selected due to its conserved sequence among multiple variants of influenza, which could potentially provide cross-protection between the various variants (121, 122). The study establishes the application of a TLR4 based nanovaccine delivery system in delivering viral vaccines in pigs.

2.2. Material and methods

2.2.1. Materials

Inulin (cat# 198971) and polyvinyl alcohol (cat# 151937) were purchased from MP Biomedicals, Solon, Ohio. Poly (D, L-lactide-co-glycolide) (Resomer® RG 503, cat#739952), albumin from chicken egg white, lyophilized powder (cat# A5503), and albumin from bovine serum (cat# A3059) were obtained from Sigma-Aldrich, St. Louis, MO. The M2e peptide (MSLLTEVETPTRNEWECRCSDSSD) was synthesized at GenScript USA Inc., Piscataway, NJ. Fluorescein isothiocyanate isomer I (cat# sc-

206015A) was purchased from Santa Cruz Biotechnology, Inc. Dallas, TX. Hemagglutinin (HA) protein from Influenza A Virus, A/California/07/2009 (H1N1) pdm09, Recombinant from Baculovirus (NR-51668), Anti-HA monoclonal antibodies (mAbs) [Anti-Influenza Virus H1 Hemagglutinin (HA), A/California/04/2009 (H1N1) pdm09, Clone 5C12 (produced in vitro; cat#NR-42019], were obtained from BEI resources, ATCC, Manassas, VA. AddaVax™ (cat#vac-adx-10) was purchased from Invivogen, San Diego, CA. Immulon™ 4 HBX (cat#3855) clear plates were purchased from Thermo Scientific, Waltham, MA, USA. Goat anti-porcine IgG-HRP conjugated secondary antibody (Cat# 6050-05) was purchased from Southern Biotech (Birmingham, Alabama, USA), Bethyl Laboratories, Montgomery, TX. BD detergent solution concentrate (cat# 660585), BD sheath additive (cat# 660584), and BD FACS clean (cat# 340345) were purchased from BD Biosciences, San Jose, California. Magnesium Chloride (cat# BP214) and other solvents and reagents were purchased from Fisher Scientific, Waltham, MA, USA.

2.2.2. Cell lines and animals

Pig alveolar macrophages (3D4/31) were obtained from ATCC (cat# CRL-2844™). Cells were cultured in DMEM-high glucose medium (Hyclone Laboratories, Logan, Utah) and supplemented with antibiotics (gentamycin and penicillin/streptomycin) and 10% fetal bovine serum (FBS). The piglets (3-weeks old, either gender) were obtained from Mid-west research swine (Yorkshire, cross-bred), Glencoe, MN, and housed according to the approved South Dakota State University, IACUC protocol.

2.2.3. Synthesis of fluorescein isothiocyanate labeled ovalbumin (FITC-Ova)

Twenty milligrams of fluorescein isothiocyanate (FITC) and 100 mg of Ovalbumin were incubated in 10 mL of 220 mM sodium carbonate buffer (pH 9.5) for 8 hrs. The mixture was dialyzed (SnakeSkin Tubing; 10 K.Da. cutoff, cat# 88245, Thermo fisher) against the deionized water. The final solution was lyophilized and stored in the dark at 4 °C (123).

2.2.4. Synthesis of InAc polymer and preparation of InAc nanoparticles (InAc-NPs)

InAc was synthesized and characterized using FT-IR, and InAc nanoparticles were prepared as described previously (49). In brief, antigen (0.5 mg of HA, 3 mg of M2e, or 20 mg of FITC-Ova) dissolved in 200µl of 10 mM phosphate buffer (pH 7.4) was mixed with 50 µL of 2% Pluronic-F68 solution as a surfactant. The oil phase was prepared by dissolving 100 mg of InAc in 5 mL of dichloromethane (DCM). The aqueous solution was added dropwise to the InAc solution by vertexing to form a primary emulsion (w/o). Later, the primary emulsion was added to water containing 0.5% w/v of polyvinyl alcohol as a stabilizer (45 mL) with continuous stirring for 14 hrs. The precipitated particles were collected via centrifugation (20,000g), lyophilized with 20 mg of mannitol as a cryoprotectant, and stored at 4^{O}C until further use. Nanoparticles were also prepared with PLGA as a polymer and FITC-Ova as an antigen, as described above. HA and M2e containing InAc-NPs were labelled as InAc-HA-NPs and InAc-M2e-NPs, respectively.

2.2.5. Size, zeta potential, and morphology

The size and charge (ζ potential) nanoparticles were measured as described previously (49, 50)

2.2.6. Scanning electron microscope (SEM)

The morphology and size of InAc-NPs were examined using scanning electron microscopy as described previously in (49, 50).

2.2.7. Determination of antigen loading

A weighed amount of antigen-loaded NPs was added to the acetone to dissolve the polymer. The precipitated antigen was pelleted by centrifugation and dissolved in a 1% sodium dodecyl sulfate (SDS) solution. The concentrations of extracted HA and M2e were determined by the micro-BCA method (Micro BCA™ Protein Assay Kit; cat# 23235) and in-house developed RP-HPLC method as described in section 2.2.8. The results are reported as microgram of antigen per milligram of InAc-NPs.

2.2.8. Development of reverse phase HPLC method for the quantification of M2e peptide

HPLC analysis was conducted using the Waters HPLC system (Milford, MA, USA) with a W2998 PDA detector. A gradient method with the conditions described (Table.2.1) were used to elute the peptide in the reverse phase using Syncronis C18 column (150×4.6 mm, 5 µm column) (Thermofisher, USA). The mobile phase-A contains 0.125% TFA in water,

and the mobile phase-B contains 0.1% TFA in acetonitrile with a constant flow rate of 0.7 mL/min. The sample injection volume was 20µl, and the eluted peptide was detected at 220nm wavelength. From the chromatograms obtained from a known amount of the M2e peptide (n=3) a standard curve was prepared which was used to determine the unknown amounts in the sample.

Table.2.1. Gradient conditions for elution of M2e peptide

Time (minutes)	Flowrate	Mobile phase A	Mobile phase B
0.0	0.7	95.0%	5.0%
7.0	0.7	25.0%	75.0%
13.0	0.7	95.0%	5.0%

2.2.9. Determination of endotoxin Levels

The presence of the endotoxins may interfere with the vaccine efficacy and its immune assays. Therefore, the endotoxin levels in the formulation were assessed by the commercially available ToxinSensor^{TM} Chromogenic LAL Endotoxin Assay Kit [GenScript (Piscataway, NJ, USA)], and it is within the limits specified by the United States Pharmacopeia (USP).

2.2.10. In-vitro antigen release

The InAc-M2e-NPs (1mg/mL) were dispersed in 0.1M phosphate-buffered saline (PBS) at pH 7.4 and incubated at 37^{o}C at 100 rpm using an orbital shaker. The samples were collected and centrifuged at 20,000g for 15 minutes at pre-concluded time intervals. The

supernatants were filtered (0.2 μm), and the amount of M2e released was determined by RP-HPLC as described in section 2.2.8.

2.2.11 Storage stability of HA antigen

The antigen (HA) was stored as a solution from the commercial source or in InAc-NPs for twelve months at 4^{o}C. After storage, the antigen was extracted, as discussed in section 2.2.7. A freshly obtained recombinant HA solution (<1 month) was used to compare the stability with the stored samples. Seven micrograms of protein samples were loaded onto SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 10 %). After separating the proteins, the gels were stained with Coomassie Brilliant Blue (R-250) (0.25%) as described by the manufacturer. The images of the stained gel were collected using Bio-RAD molecular imager, ChemiDoc. XRS+ with Image lab software.

2.2.12. Antigen delivery by InAc-NPs to porcine macrophages

The uptake of InAc-NPs containing FITC-Ova by porcine macrophages was evaluated by flow cytometry. The (3D4/31) porcine alveolar macrophages cells (1×10^{6}/well) were seeded in a 24-well plate. The groups include no treatment (only media), InAc-FITC-Ova-NPs, or PLGA-FITC-Ova-NPs for 1hr at 37^{o}C. The amount of antigen added is normalized to 25 μg/well using the fluorescence of FITC-Ova. After incubation, cells were washed and trypsinized. Subsequently, the percent of cells containing FITC-Ova and the relative amount of FITC-Ova per cell were analyzed using flow cytometry.

2.2.13. Immunization in piglets

The 3-weeks old littermate's piglets with mixed gender and (n = 5 per group) were immunized subcutaneously (s.c) with 25 µg of each antigen in 500 µl of vaccine formulation using a 21G needle. The formulations were prepared as shown below (Table 2.2).

Table. 2.2. Formulation dilutions for pig immunization

Adjuvant	Saline q.s.	Blank NPs (0 µg/mg)	HA-NPs (2.42 µg/mg)	M2e-NPs (2.071 µg/mg)	HA Solution (0.641 µg/µL)	M2e Solution (2.5 µg/mL)	Addavax
Blank	3.0 mL	150 mg	0	0	0	0	0
Saline	3.0 mL	0	0	0	234 µL	60 µL	0
InAc-NPs	3.0 mL	15 mg	62 mg	73 mg	0	0	0
Addavax	3.0 mL	0	0	0	234 µL	60 µL	1.5 mL

The antigens were delivered with 3 different adjuvants/delivery systems: a) in saline solution, b) encapsulated in InAc-NPs and suspended in saline, and c) in 50 % v/v AddaVax^{TM} (squalene-based oil-in-water nano-emulsion) in saline. The blank particles containing no antigen were used to eliminate the background immune response due to the adjuvant. Primary immunization (day 1) was followed by one booster dose after 2 weeks. The blood was collected 14 days post-immunization, and the serum was separated and stored at -20^{o}C until further use**.**

2.2.14. Determination of serum antibody titers

The antigen-specific antibody levels in the immunized pig serum were determined by the indirect enzyme-linked immunosorbent assay (ELISA). The ELISA plates were coated

with HA (1μg/well) protein or M2e peptide (~2μg/well) in sodium carbonate buffer, pH 9.2. Plates were sealed with parafilm to prevent evaporation and incubated overnight at 4^{o}C. The plates were washed five times with a wash buffer (0.05 % Tween 20) and blocked with a blocking solution containing 5% nonfat dry milk in PBS. Different dilutions of serum samples containing the primary antibody were added to the wells and then incubated for 2 hrs at room temperature. The plates were washed with wash buffer and incubated with the anti-IgG pig secondary antibody for 1 hr before washing thrice with the wash buffer. The plates were exposed to an HRP substrate (1-step Ultra EMB-ELIS) for 7 minutes. Finally, the stop solution was added, and measured the absorbance at 450 nm wavelength.

2.2.15. Binding kinetics of anti-HA antibody from the immunized pig serum

The kinetic analysis was performed on Nicoya Open surface Plasmon Resonance (SPR) using a carboxyl sensor following standard procedures. The instrument was primed with a running buffer (PBS + 1% Tween 20 (PBST)) using a blank sensor chip without any surface chemistry. The instrument separates the inserted sensor chip into two separate channels. Channel-1 was coated with a control protein BSA and channel-2 with the target protein HA for this experiment. The molecule coated on the sensor is called a ligand, and the molecules that bind to the ligand are called analyte. Before coating with the ligand protein, the sensor chip was surface conditioned by injecting 10mM HCL (pH 2) on both channels at 150 μL/min. Subsequently, the carboxyl groups on the sensor were activated by injecting a 1:1 ratio of EDC/NHS (10 mM) on both channels at 20μL/min. The optimized concentration of recombinant HA protein (50 μg/mL) dissolved in 10 mM acetate buffer,

pH 5.6, was injected to only channel-2 at 20µL/min. The activated surface on channel-1 and any other open sites on channel-2 were blocked with two injections of 1% BSA followed by one injection of Nicoya blocking solution. After the ligand immobilization, the running buffer was switched to filtered 10% naïve pig serum in 1%PBST, and the instrument was auto conditioned. The kinetics of antigen-antibody interactions were established at first using a commercial anti-HA monoclonal antibody (anti-HA mAb, analyte). The anti-HA mAb (100 µL) was injected over both channels at 20µL/min. The binding response was measured as response units (RU). After each passage of the analyte (antibody), the surface of the sensor was regenerated using magnesium chloride (4M) to remove non-covalent interactions between the immobilized HA and the injected antibody. Subsequently, the sensor was stabilized with the running buffer before injecting the next analyte solution. The binding data were processed and evaluated by Trace drawer software (ON, Canada). The Association (ka), dissociation (kd) rate constants, and equilibrium dissociation constant (K_D) were obtained by fitting a 1:1 ligand to the analyte binding model. The experiment was repeated with different analyte concentrations (monoclonal anti-HA antibody) and diluted serum (1 in 10) from the immunized and unimmunized piglets. To confirm the consistency of the degree of availability of HA ligand molecules on the surface, ant-HA mAbs at 10 µg/mL concentration was run intermittently. Each set of experiments was completed on a single sensor.

2.2.16. Statistical analysis

The results were expressed as mean ± standard deviation (SD). The variance between groups was compared using one-way ANOVA followed by Bonferroni's posthoc multiple

comparison tests. The data was analyzed in Instant graph pad prism software (CA). $P<0.05$ was considered a statistically significant difference unless specified under figure legends.

2.3. Results & Discussion

Influenza viruses often cause acute respiratory infections in pigs. Influenza infections not only produce significant economic losses for the global pig industry but also increases the risk of zoonotic transmission to humans (124, 125). The transmission of infection from swine to humans occurs due to the formation of novel influenza subtypes through genetic assortment by mixing various flu viruses (Avian, porcine, human, e.t.c.) that could potentially infect pigs. (126) Development of vaccines made a significant contribution to reducing viral infectious disease and preventing death from infectious pathogens. While designing the vaccine, one should consider the vaccine's efficacy and health, economic, and social benefits (127). To overcome the problem and protect the pigs from severe influenza infections, in this study, we have designed and tested a subunit influenza vaccine using InAc-based polymeric nanoparticles as a vaccine adjuvant and delivery system. InAc is an acetylated form of polysaccharide inulin established as a TLR4 agonist .

Previously, our laboratory has shown that the nanovaccine system utilizing the InAc as a polymer made an efficient delivery of antigen to APCs and was able to robustly stimulate both humoral (> 32 times vs. alum) and cell-mediated immune response in mice . Based on these published reports, we utilized the InAc polymer to make a nanovaccine particulate system and encapsulated the HA and M2e as antigens.

2.3.1 Physicochemical characterization of the InAc polymer and vaccine formulations

Inulin acetate was synthesized and characterized as described earlier (49, 50). The acetylation was confirmed by the absence of hydroxyl groups in the polymer determined by using FTIR (Fig. 2.1) and peak shift in ^{1}H-NMR spectroscopy as described earlier (49).

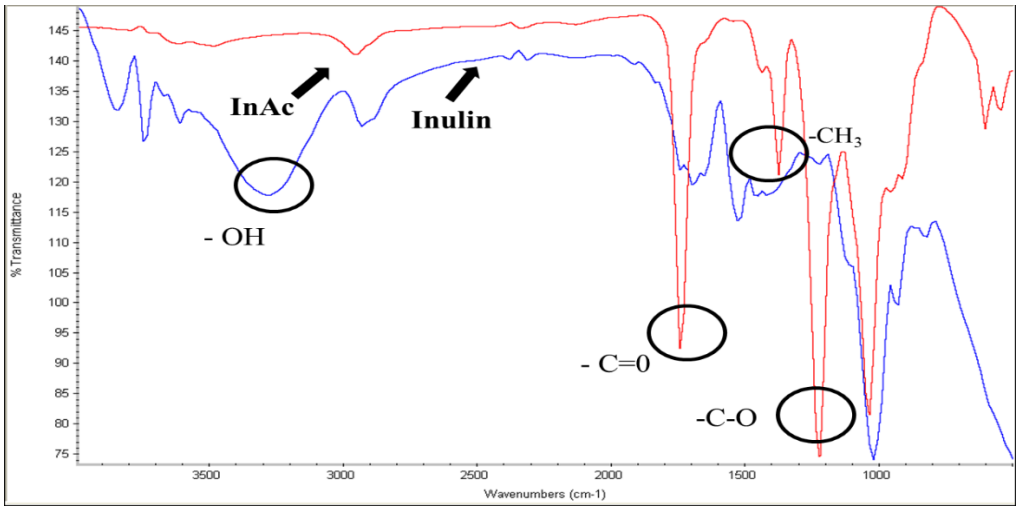


Fig. 2.1. FTIR spectrum of inulin and inulin acetate. Indicating the disappearance of the -OH peak of inulin (~ 3326 cm− 1) and the appearance of peaks for – C=O (~ 1743 cm− 1), -C-O (~ 1224 cm− 1), and for -CH3 groups (~ 1369 cm− 1) implying the acetylation of inulin.

The M2e or HA-loaded nanoparticles were prepared using InAc as a polymer. The size of InAc-HA-NPs and InAc-M2e-NPs were around 361 ± 0.52 and 327 ± 0.30 nm in average diameter, respectively, and with a slightly neutral charge as determined by DLS (Fig. 2.2 A, B, and Table. 2.3). Additionally, the shape (spherical) and size were further confirmed by SEM (Fig.2.2 C).

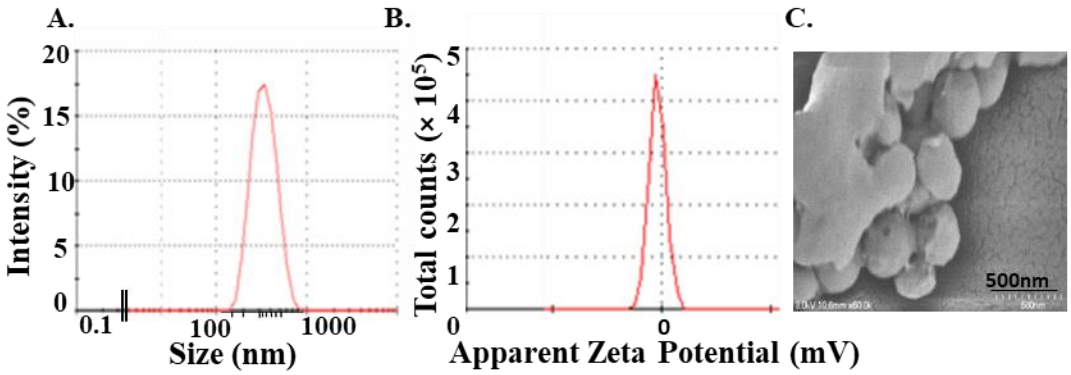


Fig. 2.2. Characterization of Inulin-acetate nanoparticles (InAc-HA-NPs). **A)** The mean particle size distribution was measured using DLS and represented as intensity (percent). **B)** Zeta potential shows an illustration of surface charge. **C)** The morphology of InAc-NPs as shown by scanning electron microscopy (SEM).

Table. 2.3. Physicochemical properties of InAc-NPs.

Parameter	Blank InAc-NPs	Blank PLGA-NPs	InAc-HA-NPs	InAc-M2e-NPs
Size (nm)	314 ± 0.62	297± 0.22	361 ± 0.52	327 ± 0.30
Zeta-potential (mv)	-0.8 ± 0.12	-1.7 ± 0.67	-1.2 ± 0.35	-0.6 ± 0.44
Loading (μg/mg)	N/A	N/A	2.42 ± 0.2	2.071 ± 0.28

A measured amount of InAc-NPs loaded with antigens were dissolved in acetone. The precipitated antigens (HA and M2e) were pelleted by centrifugation and dissolved in 1% sodium dodecyl sulfate (SDS) solution as described in section 2.2.7. The extracted HA & M2e amount was determined by RP-HPLC and micro-bicinchoninic acid (BCA) protein assay (as per the manufacturer's instructions), respectively.

The antigens selected are water-soluble, and the InAc is water-insoluble. As shown previously, preparing NPs by using the double emulsion technique (w/o/w) provided higher antigen encapsulation. The amount of HA and M2e antigens present in the NPs was estimated by micro-BCA and HPLC methods, respectively. To determine the concentration of M2e peptide, a new HPLC method was established (Fig. 2.3). The details of the optimized method are described in section 2.2.8. Using the above methods, it was estimated that 2.42 ± 0.2 and 2.071 ± 0.28 µg of HA and M2e antigens were present per milligram of InAc-HA-NPs and InAc-M2e-NPs, respectively (Table.2.3). The use of nanoparticles to deliver vaccine antigen(s) has several advantages: a) sustains the release of antigen, b) improves antigen stability, c) targets the delivery to APCs, d) and being insoluble, enhances the immunogenicity. The InAc-based nanovaccine delivery system is evaluated for the above advantages in this study.

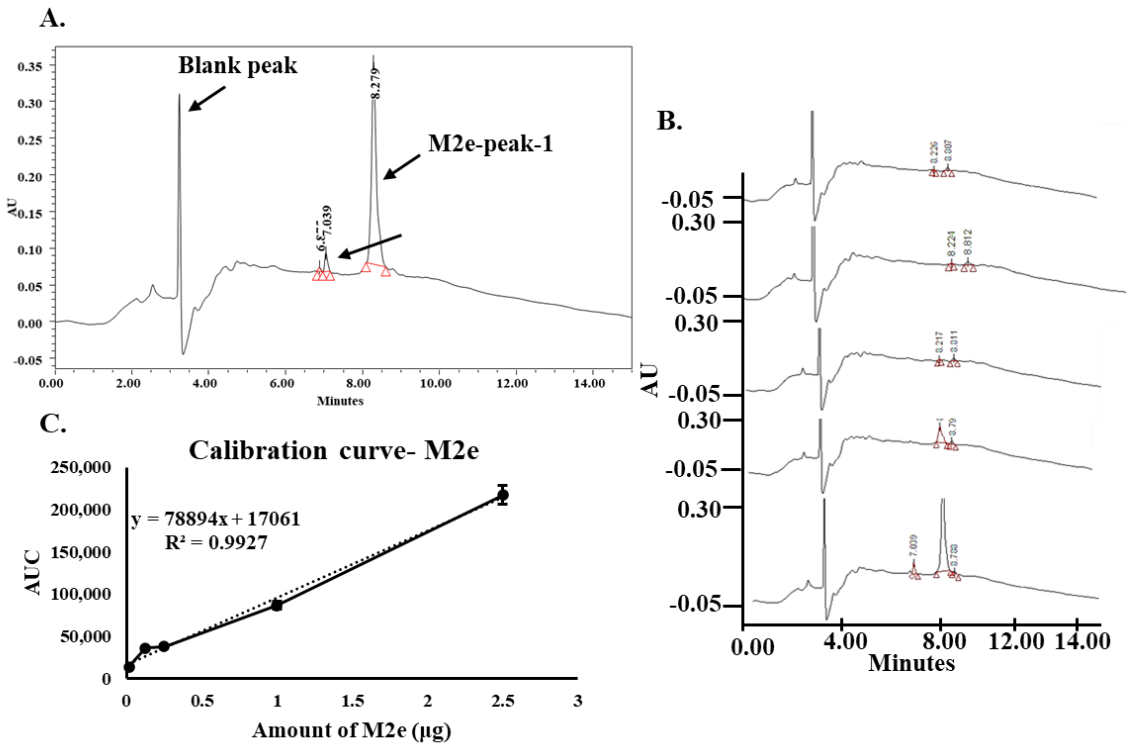


Fig. 2.3. Development of HPLC method for M2e peptide. **A).** Using the gradient program (described in the methods section table 2.1), the peptide peak was eluted at 8.2 minutes (Retention Time). **B).** The chromatograms were obtained at various amounts of The M2e peptide was dissolved in phosphate buffer. **C).** The calibration curve was plotted from the area under the curve of the peptide peak vs. the amount of the peptide.

2.3.2. Antigen release from InAc-NPs

It is known that hydrophilic macromolecules such as HA and M2e when encapsulated, release from hydrophobic particles in a sustained pattern (51, 128). To model the antigen release from InAc-NPs, M2e-encapsulated NPs were selected. As shown in Fig.2.4, there was a burst release of around 20% of the antigen within 30 minutes of incubation in PB, which may be due to a quick release of surface adsorbed peptide. However, most of the antigen (~80 %) was released within 5 days of incubation. Although peptide release from InAc-NPs was sustained over 3-5 days, it is much faster than the reported release of another antigen (Ovalbumin) from InAc-NPs (~21 days) (50). The differences in the release kinetics may be due to the smaller size of the M2e peptide (2.7 kDa.) compared to ovalbumin protein (45 kDa.).

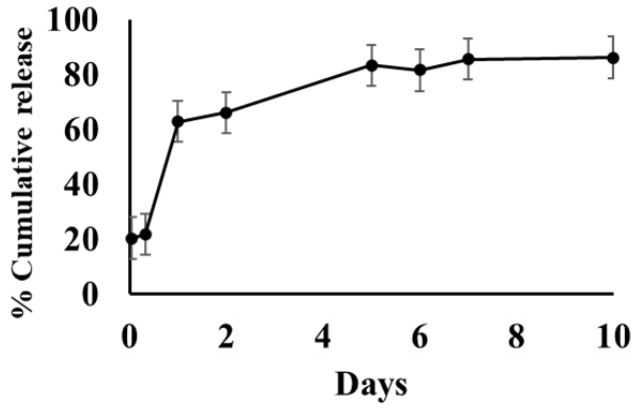


Fig. 2.4. In-vitro antigen (M2e) release from InAc-NPs. InAc-NPs (1 mg/mL) were dispersed in 0.1M PB, pH 7.4 at ~100 RPM. M2e concentration in the soluble fraction was measured at different time points using HPLC (n=3) as described in section 2.2.8.

2.3.3. InAc-NPs improve the stability of antigen

In subunit vaccines, the antigens are either peptides or proteins prone, which are to proteolysis. Instability of vaccines/antigens is a major challenge during vaccine development, vaccine transportation, or storage in third-world countries. The vaccine formulation must preserve the stability of the antigen. The swine HA protein used in this study is highly unstable and therefore, must be stored at <-80°C to preserve its stability (data not shown). One of the significant advantages of InAc-NPs based vaccines is that they can be stored as a lyophilized powder. Therefore, we have evaluated the ability of InAc-NPs to protect the HA protein by storing the HA protein at 4°C either as a solution (50 mM Tris-pH 8, 500 mM NaCl, and 10% glycerol) versus encapsulated in InAc-NPs as a lyophilized powder. After 12 months of storage, the stability of HA was analyzed using SDS-PAGE (Fig.2.5). The HA protein in the solution degraded completely, whereas encapsulation inside InAc-NPs protected the protein. However, there is a slight degradation in the encapsulated HA due to harsh encapsulation or extraction procedures used.

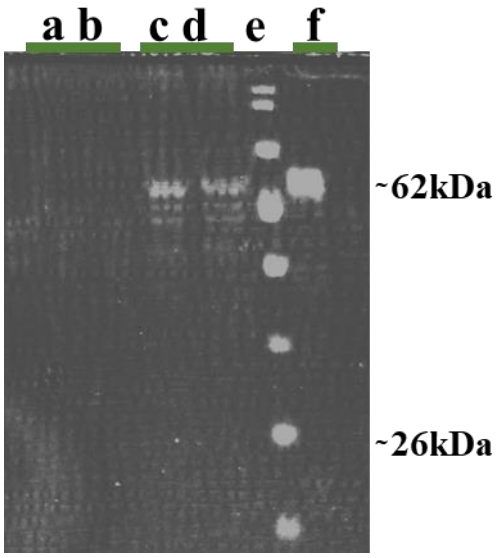


Fig. 2.5. The ability of InAc-NPs to protect the antigen (HA) from degradation during storage. The HA protein was stored at 4°C as a solution or encapsulated inside InAc-HA-NPs. After 12 months, the protein was extracted from NPs as described in methods (section 2.2.7) and analyzed by SDS-PAGE followed by Coomassie blue staining. Lane 'a' and 'b', HA solution (50 mM Tris, pH 8 with 500 mM NaCl and 10% glycerol), Lane 'c' and 'd', InAc-HA-NPs, Lane 'e', molecular weight marker, Lane 'f', fresh HA protein solution (50 mM Tris, pH 8 with 500 mM NaCl and 10% glycerol).

2.3.4. InAc-NPs promoted internalization of antigen by swine macrophages

For an efficient vaccine response, the antigen needs to be processed and presented by antigen-presenting cells (APCs) such as dendritic cells or macrophages. APCs are more efficient in internalizing an insoluble antigen than a soluble one (129, 130). The nanosized InAc-NPs mimic the size and shape of viruses or pathogens (Fig. 2.2). Further, the TLR4 agonistic activity of the InAc particles functions as PAMPs to enhance the recognition and interaction by antigen-presenting cells. Nanoparticles encapsulated with a fluorescent

antigen (FITC-labeled ovalbumin) were prepared using InAc or PLGA as a polymeric matrix. The uptake of these particles by porcine alveolar macrophages was quantified by flow cytometry. In general, porcine alveolar macrophages were less efficient in phagocytosis than murine macrophages. After incubation for 1 hr with NPs, around 31 and 49 % of porcine macrophages tested positive for a detectable antigen for PLGA-NPs and InAc-NPs, respectively (Table 2.4). However, previous studies have shown that 61% and 98 % of murine macrophages were positive for the same antigen when incubated with PLGA-NPs and InAc-NPs, respectively (51). In all these experiments, PLGA-NPs was used for comparison as they have similar size, shape, and lipophilicity as InAc-NPs. However, InAc could activate TLR4 receptors on macrophages, whereas the PLGA is immunologically inert and failed to activate macrophages (52). As shown here, InAc-NPs were taken up by porcine macrophages 1.2 folds higher than PLGA-NPs (Fig. 2.6). The increased uptake of the InAc-NPs by macrophages is likely to be mediated by the recognition of the InAc by TLR4. These events may eventually contribute to potent adaptive immune responses.

The size, shape, and rigidity of the particles also play a role in the internalization by macrophages (131) (132). Further, nanoparticles are also elicited a stronger cytokine response relative to microparticles (133). The particles with the size of 20- 100 nm range will quickly enter the lymphatic system, while the particles ranging from 200-500 nm (InAc-NPs in this study) have to be taken up by immune cells (Fig. 2.6), which gets mobilized to the lymph nodes (134). Taken together, InAc-NPs provided additional advantages as a nanovaccine delivery system by sustaining the release of antigen, protecting it from degradation, and efficiently delivering it to macrophages.

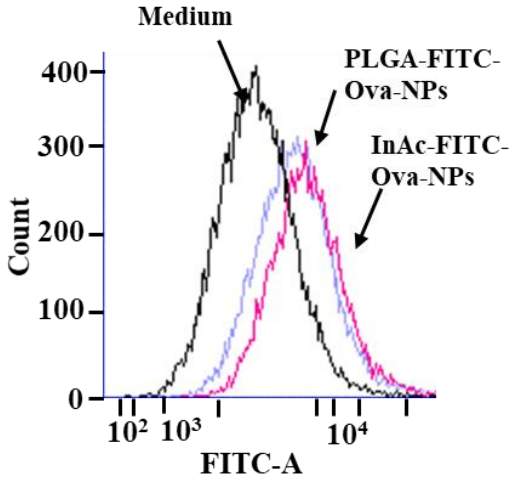


Fig. 2.6. Antigen delivery to porcine alveolar macrophages. The InAc-FITC-Ova-NPs or PLGA-FITC-Ova-NPs (25µg equivalent to FITC-Ova) were added to 100,000 cells. After 1hr incubation, the cells were analyzed by flow cytometry for the presence of green fluorescence. The data were represented as mean ± standard deviation (n=3).

Table. 2.4. Quantification of antigen delivery to pig alveolar macrophages

S.No.	Treatment groups	Mean fluorescence intensity (MFI)	% Green cells
1.	Medium	8547.80 ± 32.5	13.24 ± 0.60
2.	PLGA-FITC-Ova-NPs	11453.85 ± 704.06	31.61 ± 3.07
3.	InAc-FITC-Ova-NPs	13958.40 ± 253.80	49.02 ± 3.32*

The data from Fig. 2.6. was quantified and reported. The MFI of FITC-Ova in the green channel was fluorescence units (counts). Data represent the mean ± standard deviation (n = 3). * $p < 0.05$ InAc-FITC-Ova-NPs vs. PLGA-FITC-Ova-NPs using one way-ANOVA followed by Bonferroni's multiple comparison test.

2.3.5. Antigen delivery by InAc-NPs generated strong antibody titers.

After establishing the efficacy of InAc-NPs using in-vitro, the ability of InAc-NPs to induce the humoral responses in pigs was evaluated by immunizing 3-week-old piglets through a subcutaneous route (2 doses of 25 μg of antigens HA and M2e). The antigen-specific total IgG levels in serum collected 14 days after booster immunization was determined by indirect ELISA. Antigens dissolved in saline, or along with a commercial adjuvant Addavax were used as controls for comparison.

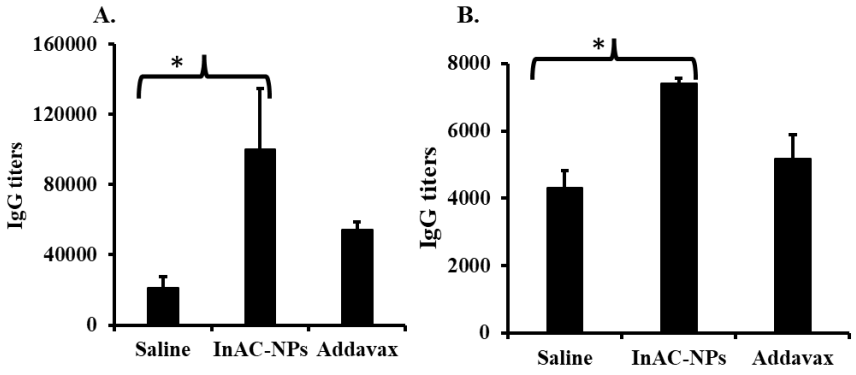


Fig. 2.7. Titers of HA (panel A) or M2e (panel B)-specific IgG antibodies in the serum. Pigs (n = 5 per group) were immunized subcutaneously with HA and M2e (25 μg each/dose; 2 doses) in saline or encapsulated in InAc-NPs or along with Addavax, and the antibody titers in the srum collected 14-days post final immunization were determined by using indirect ELISA. The titer is defined as a minimal experimental serum dilution at which the absorbance is more than average absorbance plus two standard deviations from the blank serum (no antigen). * $p < 0.05$ InAc-NPs vs. saline as an adjuvant using one way-ANOVA followed by Bonferroni's multiple comparison test.

InAc-NPs displayed significantly higher (*$p < 0.05$) anti-HA (4.7-fold) and anti-M2e (1.7-fold) antibody titers than saline as an antigen delivery vehicle (Fig. 2.7). InAc-NPs were able to generate similar antigen-specific serum antibody titers to a commercial vaccine adjuvant (Addavax) (Fig. 2.7). Similar results were observed with InAc-NPs in mice studies (50, 51). In mice, InAc particles have shown superior serum IgG antibody response against the antigen compared to Alum (32 times) as an adjuvant (50). InAc-microparticles formed a depot at the injection site despite being non-toxic. However, InAc-NPs (200-300 nm) were cleared from the injection site within 30 hrs of injection (51), which is beneficial in meat food-producing animals.

Serum antibodies can function in multiple ways to provide protection or quick recovery from an infection. Antibodies can neutralize the virus and prevent its entry into a host cell or through antibody-dependent cellular cytotoxicity (ADCC) or phagocytosis (ADCP); antibodies promote the clearance of the virus. In this study, InAc-NPs were able to generate high titers of anti-HA or anti-M2e antibodies. HA protein is easily accessible to antibody binding and thus leads to virus neutralization, unlike the M2e peptide. Despite the failure to neutralize the virus (135-137), generating strong anti-M2e antibodies seems to be effective against influenza infection, as evident by the protection shown with passive transfer of M2e anti-serum or anti-M2e monoclonal antibodies (135, 138-140) or poor protection of M2e vaccination in B cell-deficient mice despite generating strong CD4 T cell response against M2e (141). Therefore, in this study, the presence of high levels of serum antibodies against both HA and M2e peptides is expected to provide protection against influenza (142).

2.3.6. Antigen-specific serum antibodies generated by InAc-NPs have high avidity.

For a vaccine to be successful in preventing or reducing the severity of a disease, in addition to generating high levels of antibodies (Fig. 2.7), it is critical that the generated antibodies must be of high quality (affinity and avidity) with respect to binding to the targeted antigen. Avidity and affinity will determine the binding strength. Avidity is for determining the total binding strength, and affinity is for determining the binding strength at a single binding site. The Open-SPR was used to investigate and compare relative binding affinities and kinetic parameters of antibodies present in immunized swine serum (with different adjuvants) to an immobilized recombinant HA protein (H1 Hemagglutinin (HA) Protein from Influenza A Virus, A/California/07/2009 (H1N1) pdm09, recombinant from Baculovirus, NR-51668). SPR measurements are based on the mass and indicate the proportional concentration of analyte bound to the ligand at a given concentration and a defined flow rate to determine the binding interaction parameters (143). SPR can analyze the association and dissociation phases of interaction and detect even weak binding events (144, 145). The sensorgram indicates the different binding events, such as association and dissociation phases of binding, which are indicated by rate constants, k_a and k_d, respectively (Fig. 2.8 B). These parameters are used to determine the equilibrium dissociation constant (k_D) for the binding of a ligand (immobilized) to the analyte (in flow) using the Tracedrawer software. The approximate amount of ligand to coat the chip surface was calculated using the following formula: $\text{Response}_{max} = \text{Response}_{Ligand} \times \text{Mass}_{analyte} \times \text{Valency}_{Ligand})/\text{Mass}_{Ligand}$.

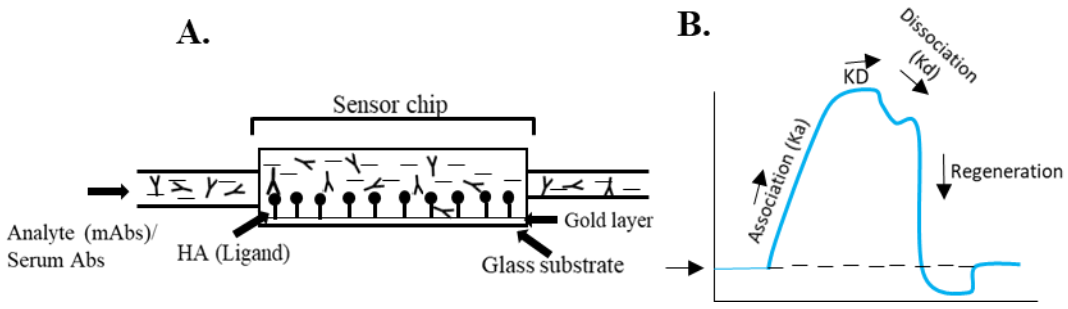
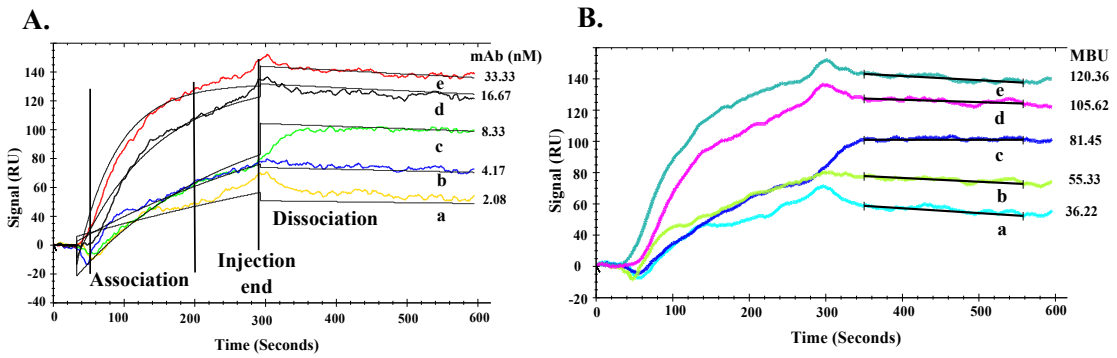


Fig. 2.8. Schematic representation of Surface Plasmon Resonance (SPR) **A)** procedure for injection of samples, ligand immobilization, and analyte binding **B)** demonstrating the association (k_a), dissociation (k_d), and equilibrium rate constants (K_D) in SPR sensorgram.

Before we analyze the polyclonal serum from the immunized pigs, we have standardized the binding of anti-HA antibodies to an immobilized HA antigen using a commercial anti-HA monoclonal antibody (NR-42019, BEI Resources). Various concentrations of the mAb (2.08 nM to 33.33 nM) were injected over an immobilized HA protein on an SPR sensor. The association and the dissociation phases of binding were recorded (Fig. 2.9 A). The values of association rate constant (k_a), dissociation rate constant (k_d), equilibrium dissociation binding constant (k_D), and the maximum binding units were calculated using Tracedrawer software. The antigen-specific antibody concentration was determined using the binding response measured during early mass transport limited binding phase (146). The Binding rate (slope) represents the rates dependent on the antibody concentration but not binding kinetics. This is distinctly observed in the binding response curve from titration of known concentration of anti-HA (NR-42019) monoclonal antibodies in Fig. 2.9 A.



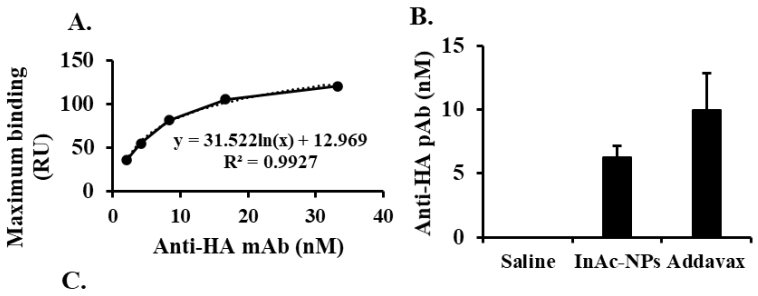
C.

k_a ($M^{-1}s^{-1}$)	k_d (s^{-1})	K_D (M)
5.50E+05	2.87E-04	5.21E-10

Fig. 2.9. Determination of association, dissociation rate, and maximum binding of anti-HA mAb to recombinant HA protein. **A).** An SPR sensogram of binding of Anti-HA mAb (2.08 nM to 33.33 nM, (a-e)) to immobilized HA protein. The binding is recorded as Response units (RU). **B).** A graph showing maximum binding units (MBU) for different concentrations of anti-HA mAbs (a-e), and **C).** Binding rate constants determined using the trace drawer software.

A logarithmic relationship was observed between maximum binding units (MBU) vs. concentration of anti-HA mAbs (Fig. 2.10A) (R^2=0.9927). The calibration curve of known anti-HA monoclonal antibodies was used to calculate the relative concentration (equivalent nM) of HA-specific antibodies in polyclonal serum collected from the immunized pig, as mentioned previously (145). The serum from the immunized pigs from each group was pooled, filtered, and diluted to 1:10 before injecting over the immobilized HA protein on a sensor chip. The specific binding of anti-HA antibodies from the serum to the HA protein on the sensor was recorded after baseline was stabilized with 10 % serum from non-immunized pigs (naïve pigs). From the MBU values obtained from the sensogram of polyclonal serum antibodies, the relative anti-HA antibody concentration in

the serum was calculated (Fig. 2.10 B) using the standard curve prepared using anti-HA mAbs (R^2=0.993; Fig. 2.10 A) (145). Serum from the pigs immunized with InAc-NPs as an adjuvant showed 6.27 times more anti-HA antibodies compared to saline as an adjuvant (Fig. 2.10 B). Similar to the data shown using ELISA (Fig. 2.7), SPR data also indicates high antigen-specific antibody titers for both InAc-NPs and Addavax as adjuvants. The strength of antibody-antigen binding is indicated by the K_D value. Most antibody-antigen interactions have K_D values in the range of 1E-6 to 1E-10M. The affinity and rate kinetics also indicate that InAc-NPs generated a high-quality antibody in the serum that binds to the target antigen at a nanomolar affinity (3.49E-09 M). The strength of binding of serum antibodies generated by InAc-NPs as an adjuvant is comparable to anti-HA mAbs and serum antibodies generated by a commercial adjuvant Addavax. In contrast, when adjuvant is not used during vaccination (saline), the concentration of serum anti-HA antibodies, as well as their affinity towards the antigen, is very low (Fig. 10C). For establishing the avidity of serum antibodies, HA protein was selected instead of M2e because of high serum titers observed against HA antigen (Fig. 2.7). Although InAc has been shown to be superior in activating mice immune cells compared to pig immune system, the avidity of mouse antibodies has yet to be investigated.



C.

S.No.	Saline (mean± SD)	InAc-NPs (mean± SD)	Addavax (mean± SD)
ka (1/(M*s)	4.22E+06 ± 5.46E+06	1.15E+06 ± 1.38E+06	6.12E+05 ± 3.24E+05
kd (1/s)	1.38E-02 ± 9.68E-03	6.81E-04 ± 3.44E-04	6.48E-04 ± 2.39E-04
KD (M)	2.34E-07 ± 3.97E-07	3.49E-09 ± 4.65E-09	1.26E-09 ± 6.16E-10

Fig. 2.10. Quantification of serum antibodies concentration and affinity. **A).** Calibration curve of antibody binding was derived from titrating the commercially available anti-HA mAbs by considering maximum binding (RU) as a parameter, **B).** The HA-specific antibodies concentration from unknown serum samples from the experimental group was calculated from the calibration curve with possible total binding combinations (avidity) (n=3, experimental triplicate), **C).** The curve-fitting analysis 1:1 dissociation model (Langmuir model) was applied and calculated the association rate constants (k_a), dissociation rate constants (k_d), and equilibrium rate dissociation constant (K_D) from the immunized serum.

Influenza viruses significantly affect the health of humans, livestock, and several wild species. Controlling the infections in swine is very important for not only preventing the economic loss to the swine industry across the globe but also preventing zoonotic transmission to humans, which was observed during the 2009 “Swine Flu” outbreak. Swine influenza, like in humans, cause high morbidity in pigs along with respiratory illness that

is characterized by coughing, sneezing, fever, and loss of appetite (147). In addition to the weight loss, influenza infection is also connected to higher abortion rates and smaller litter size in breeding sows (148). Vaccination is the only major medical intervention for influenza that can be translated to the large population. Current commercial swine vaccines are focused on inactivated whole viruses. Just like in humans, swine influenza vaccines also face challenges due to the presence of high number of genetic variants and difficulties in predicting the upcoming variant(s). Although, the final direction for all Influenza vaccine researchers is to design a universal influenza vaccine, it is far from a reality in the near future (149). Recently, M2 protein and nucleoprotein (NP) was being investigated as a vaccine antigens for broader protection than frequently mutating surface proteins such as HA and Neuraminidases (149). However, these proteins are weakly immunogenic and require a strong vaccine adjuvant. As shown in this study, the use of a selective TLR4 agonist such as InAc as an adjuvant and delivery system may be possible to provide broader protection against influenza.

2.4. Conclusion

In the present study, we investigated the ability of InAc-NPs as a vaccine adjuvant in pigs using influenza antigens. InAc-NPs have produced high serum antibody titers that have a high affinity to the targeted antigen. Further, InAc particles protected the degradation of HA antigen for at least 12 months when stored at 4^{o}C. This study is a proof-of-concept study, for the first time shows the ability of InAc as an adjuvant and a vaccine delivery system in pigs. 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 pigs and perhaps even for humans.

CHAPTER III

A Stable Oral-Vaccine Delivery System and Adjuvant for Influenza

3.1. Introduction

Most viruses responsible for infectious diseases will enter by mucosal surfaces (150). The mucosal areas of gastrointestinal, genitourinary tracts, respiratory, and the ear cavity include a large surface area (approximately 400m^2) of mucus membranes lining (151). Due to their physiological nature, these cavities are prone to the external environment and susceptible to opportunistic infections. About 70% of pathogens are known to enter the host by mucosal surfaces (152). The mucosal surfaces comprise massive immunological surveillance. This constant observation enables the innate and adaptive mucosal immune system to defuse possible threats from the external environment (153). The most efficient strategy to protect these mucosal surfaces from viral infections is vaccines. Vaccines reduce the global burden of infectious diseases, such as preventing diseases like pertussis polio and eliminating smallpox (154, 155). Most vaccines are administered via parental routes, which induce a systemic immune response. Systemic immunity, in general, is not potent enough to restrict the entry of pathogens via mucosal surfaces (156). The mucosal vaccination, such as oral vaccination, could provide mucosal immunity, indicated by the generation of secretory antibodies (sIgA) and systemic immunity, and acts as the first line of defense during the entrance of the virus through mucosal surfaces.

However, oral vaccines must encounter a harsh environment composed of gastric fluids after administration. Vaccines face the challenge of surviving in the GI tract under highly acidic pH conditions and intestinal enzymes, including proteases. Subsequently, vaccines need to reach the GALT by overcoming the physical and chemical barriers that influence their internalization. Moreover, oral vaccines have to overcome the tolerogenic responses present in the intestinal tract, which suppresses vaccine efficacy (154).

Therefore, oral immunization is relatively not successful. There are two approaches showing promise to overcome these obstacles: entrapping of antigens in the protective delivery system from degradation (nanovaccine), which promote uptake and or target of vaccines to particular regions or cells in the GIT.

Limited mucosal vaccines have been approved, especially oral vaccines, for human consumption, including the polio vaccine for rotavirus and cholera (153). So far, these approved vaccines are whole pathogens, either inactivated or live-attenuated. Therefore, they are prone to the risk of virulence, and extensive quality control measures are to be considered during production. There is a need for safe and effective vaccines to overcome this re-emerging of virulence from traditional mucosal vaccines. One of the effective strategies is to explore the subunit vaccines. The rationale for subunit vaccines is that the vaccine comprises only viral components that elicit a protective immune response. However, these subunit vaccines are poorly immunogenic compared to traditional whole virus vaccines, which may be due to conventional whole virus-containing vaccines having immunostimulatory components known as pathogen-associated molecular patterns (PAMPS).

The lack of PAMPS in subunit vaccines demanded the need for vaccine adjuvant to augment the vaccine response. The adjuvants are typically classified as immunopotentiators or delivery systems, while some have both properties (151). Some of the adjuvants are PAMPS or modified forms of PAMPS from known microbes. Inulin acetate is a plant-based polysaccharide discovered in our laboratory previously known for its activation of immune cells via TLR4 (50, 52, 157).

Besides as a potent vaccine adjuvant, nanoparticles prepared with InAc as a polymer will have unique properties that could qualify them as an oral vaccine delivery system. Inulin acetate is water-insoluble, and its backbone structure (poly-fructose with beta (2-->1) linkages in linear chains) will not be degraded by known gastric enzymes or conditions. Therefore, it is expected not to release its encapsulated antigens within the GI transit time. Further, being a particulate in nature and prepared with a TLR4 agonist, InAc-NPs are expected to be taken up by APC in the GI tract. In this study, for the first time, Inulin Acetate (InAc) is proposed as a nanoparticle system to deliver the encapsulated antigen orally in mice with a peptide from influenza-A nucleoprotein (Inf-A) as a model antigen and evaluated its ability to stimulate a mucosal immune response.

3.2. Materials and methods

3.2.1. Materials

Inulin (cat# 198971) and polyvinyl alcohol (cat# 151937) were purchased from MP Biomedicals, Solon, Ohio. Poly (D, L-lactide-co-glycolide) (Resomer® RG 503, cat#739952), albumin from chicken egg white, lyophilized powder (cat# A5503), and albumin from bovine serum (cat# A3059) was obtained from Sigma-Aldrich, St. Louis, MO. The Monophosphoryl lipid A (MPLA) was purchased from InvivoGen, San Diego, CA, USA. DAF-FM diacetate (3',6'-bis(acetyloxy)-4-amino-2',7'-difluoro-5-(methylamino)-spiro[isobenzofuran-1(3H),9'-[9H] xanthen]-3-one, cat# 18767), was purchased from Cayman Chemical, Ann Arbor, Michigan. Fluorescein isothiocyanate isomer I (cat# sc-206015A) was purchased from Santa Cruz Biotechnology, Inc. Dallas, TX. BD detergent solution concentrate (cat# 660585), BD sheath additive (cat# 660584),

and BD FACS clean (cat# 340345) were purchased from BD Biosciences, San Jose, California. All the other chemicals were purchased from Fisher Scientific, Waltham, MA, USA.

3.2.2. Cell lines and animals

Mouse macrophage cells derived from primary bone marrow cells were sponsored by BEI Resources, ATCC, Manassas, VA, USA (NR-9456 & NR-9458). Macrophages were cultured in DMEM-high glucose medium (Hyclone Laboratories, Logan, Utah) and supplemented with antibiotics (gentamycin and penicillin/streptomycin) and 10% fetal bovine serum (FBS). Balb/c mice were purchased from Taconic Biosciences, IN, USA.

3.2.3. Reverse phase HPLC method for the quantification of Inf-A peptide

HPLC analysis was conducted using the Waters HPLC system (Milford, MA, USA) with a W2998 PDA detector. A gradient method with the conditions described (Table.3.1) was used to elute the peptide in the reverse phase using Eclipse XDB-C18 4.6×150mm. Mobile phase-A contains 0.05% TFA in water, and mobile phase B has 0.01% TFA in acetonitrile with a constant flow rate of 0.7 mL/min; the sample injection volume was 20µl, and the eluted peptide was detected at 220 nm wavelength. A standard curve was prepared from the chromatograms obtained from a known amount of the Inf-A peptide (n=3), which was used to determine the unknown amounts in the sample.

Table. 3.1. Gradient conditions for elution of Inf-A peptide

Time (minutes)	Flow rate	Mobile phase-A	Mobile phase-B
0.0	0.7	80.0%	20.0%
5.0	0.7	25.0%	75.0%
10.0	0.7	80.0%	20.0%

3.2.4. Nano vaccine formulation

Antigen (Inf-A) loading into InAc-NPs was achieved by double (w/o/w) double emulsion solvent evaporation technique as described in Chapter II (section 2.2.4) with minor modifications. Inf-A antigen (5.0mg) was dissolved in the aqueous phase and added dropwise to the organic phase containing 100mg of InAc during the primary emulsion.

3.2.5. Scanning electron microscope (SEM)

The morphology and size of InAc-NPs were examined using scanning electron microscopy as described previously in (49, 50).

3.2.6. Quantifying the antigen loading of InAc-Inf-A-NPs

A weighed amount of antigen-loaded NPs was added to the acetone (0.5 mL) to dissolve the polymer. The precipitated Inf-A peptide (antigen) was pelleted by centrifugation, and the collected pellet was dissolved in 10 mM PB, pH 7.4. The concentrations of extracted Inf-A peptide in solution were determined by HPLC (section 3.2.3). The results are reported as microgram of antigen per milligram of InAc-NPs.

3.2.7. Stability of InAc-NPs in gastric fluids: diffusion of the cargo

The InAc-NPs loaded with Fluorescein sodium dye (3 µg equivalent of dye) were dispersed in three media; deionized water, simulated gastric fluid (SGF), and simulated intestinal fluid (SIF). The dispersed samples were incubated at 100 rpm; 37^{o}C, and samples were collected at pre-determined intervals. The samples were centrifuged at 20,000g, and the supernatant solution was analyzed using a fluorimeter at excitation of 460nm and emission of 515nm.

3.2.8. ***Stability of InAc-NPs in gastric fluids: surface erosion of the particles***

The weighed amount (3mg/5mL) of blank InAc-NPs was dispersed in simulated gastric fluid and intestinal fluid incubated at 37^{o}C at 100 rpm using an orbital shaker. At pr-concluded time intervals, the sample was collected and centrifuged at 20,000g, and the pellet was resuspended in 10mM PB, pH 7.4, and measured the size of particles using DLS.

3.2.9. ***Stability of antigen in encapsulated InAc-NPs***

The InAc-Ova-NPs or Ovalbumin protein (Ova) were dispersed in three media: a) SGF and b) SIF, and c) 10 mM PB, pH 7.4. At pre-determined intervals, samples were collected, and the Ova was extracted as described in section 2.2.7. Four micrograms (µg) of equivalent protein samples were loaded onto 10% SDS-polyacrylamide gel electrophoresis (SDS-PAGE, 10 %). After separating the proteins, the gels were stained with Coomassie Brilliant Blue (R-250) (0.25%) as described by the manufacturer.

3.2.10. Release of Inf-A peptide from InAc-NPs

The Inf-A- NPs (1mg/mL) were dispersed in 0.1M phosphate-buffered saline (PBS) at pH 7.4 and incubated at 37^{o}C at 100 rpm using an orbital shaker. The samples were collected and centrifuged at 20,000g for 20 minutes at pre-determined time intervals. The samples supernatants were filtered (0.2 μm), and the amount of Inf-A released was determined by RP-HPLC as described in section 3.2.3.

3.2.11. Internalization of InAc-NPs by murine macrophages

The uptake of InAc-NPs containing FITC-Ova by mouse macrophages was evaluated by flow cytometry. The labeling of Ovalbumin with FITC is conducted as described under section 2.2.3. The murine macrophage cells (1×10^{6}/well) were seeded in a 24-well plate and incubated with the following groups: no treatment (only media), InAc-FITC-Ova-NPs, or PLGA-FITC-Ova-NPs for 1hr at 37^{o}C. The amount of antigen added is normalized to 25 μg/well using the fluorescence of FITC. After incubation, cells were washed three times and trypsinized to make a single-cell suspension. Subsequently, the percent of cells containing FITC-Ova and the relative number of FITC-Ova per cell were analyzed using flow cytometry.

3.2.12. TLR4 activation assay

TLR4 specific activation vt InAc-NPs was investigated using bone marrow-derived mouse wild type (WT) and TLR4 knockout ($TLR4^{-/-}$) mice. The cells were treated for 48 hrs without antigen, blank PLGA-NPs or InAc-NPs (250 μg/mL), and MPLA (2 μg/mL) was

used to compare the results. The supernatant was collected and centrifuged at 20,000g and determined the Interleukin-6 (IL-6) levels (52).

3.2.13. Intracellular nitric oxide estimation

Mouse macrophages were seeded in black cell culture plates and incubated with the treatments mentioned in section 3.2.12. After 12 hrs, the cells were washed with 1X PBS, and the cells were incubated for 2 hrs with 10 µM DAF-FM diacetate in an FBS-free medium. Later, the cells were washed 4 times with 1X PBS, and the intracellular fluorescence was measured at an excitation and emission at 485/520 nm (52).

3.2.14. Mice immunization

The Balb/c mice (10-weeks old and mixed-gender) were purchased from Taconic Biosciences, IN. Upon arrival, mice were divided into three groups with equal males and females for acclimatization in laboratory conditions for one week. After one week, mice were fasted overnight and performed the oral gavage with 50µl saline (group 1), 100 µg Influenza A peptide (Influenza A NP (366-374) Strain A/PR/8/35 peptide, GeneScript, NJ) (group 2), or InAc-Inf-A-NPs containing 100 µg Influenza A peptide (group 3). A booster dose for oral vaccination was given after one week while blood was drawn at 0 days, one week, two weeks, and four weeks of initial vaccination from the tail vein. Mice were sacrificed at four weeks of immunization; intestine, lung, and spleen were collected and snap freezed in liquid nitrogen to measure tissue-specific IgA titer. All animal procedures were conducted per the approved IACUC protocol at Arkansas Tech University, AR.

3.2.15. Measuring Inf-A specific IgG and IgA concentration in serum

Blood collected at 0 days, one week, two weeks, and four weeks of vaccination was used to separate serum. Serum was analyzed for Inf-A specific (IgG) concentration using comparative ELISA/ Influenza A Virus NP Antibody Inhibition ELISA (Virusys Corporation, MD) as per manufacture protocol. Obtained Nucleoprotein Reduction Index (NPRI) for each sample was converted to fold change in influenza-A specific IgG concentration compared to control (e.g., Mice fed with saline). With slight modification, influenza-specific serum IgA was measured with IgA Mouse Uncoated ELISA Kit (Invitrogen, MA). Briefly, the ELISA plate was coated with Influenza A peptide (Influenza A NP (366-374) Strain A/PR/8/35 peptide, GeneScript, NJ). Influenza A peptide was dissolved in 1x coating buffer to achieve a final concentration of 0.4 mg Influenza A peptide/mL. A 100µl of this solution was added to all the wells, and plates were incubated at 4 °C overnight. After incubation plate was washed and blocked as per recommended protocol. IgA standard and serum samples were diluted in assay buffer and added to respective wells while assay buffer was added to wells to determine background. After incubation for 2 hours, plates were washed four times, and detection antibodies were added to each well. After 1-hr of incubation, plates were washed four times, and a 100 µl substrate was added to each well. The reaction was stopped by adding a 100 µl stopping solution, and plates were analyzed for optical density (OD) at 450 nm. OD values for standards were used to estimate each sample's Influenza A specific antibodies.

3.2.16. Measuring total and Inf-A-specific IgA concentration in the small intestine (ileum), lungs, and spleen

To measure the Inf-A specific IgA concentration in mucosal tissue, the distal part of the ileum (2 inches segment from the ileocecal junction) and all lobes of the lungs were collected at four weeks of vaccination. Tissue samples were snap-freezed in liquid nitrogen and stored at -80 °C until analyzed. During analysis, frozen tissue was weighed and homogenized in phosphate-buffered saline (1XPBS, pH 7.4) to achieve a 200mg/mL final concentration. Tissue homogenate was centrifuged at 8000 rpm for 3 minutes to collect supernatant containing IgA. Protein concentration in each tissue supernatant was measured using the Pierce BCA protein assay kit (Thermo Fisher Scientific, MA) and normalized as 100mg/mL. Influenza-specific serum IgA in homogenized tissue supernatant was measured using IgA Mouse Uncoated ELISA Kit (Invitrogen, MA) with slight modification and converted to IgA/gram of tissue.

3.2.17. Hemagglutination inhibition assay (HI assay) to measure influenza-A specific antibodies in the ileum and lungs

Influenza A Virus, A/Puerto Rico/8-9NMC1/1934 (H1N1) (kindly provided by BEI Resources, NR-29023) was used to measure the Inf-A specific neutralizing antibodies in the tissue using HA-HI assay as a method described earlier (158). Influenza A virus was diluted in PBS to obtain its final concentration as 4 HA units using 1% chicken red blood cells (Innovative Research Inc, Novi, MI). A 25 µl supernatant of tissue homogenate was serially diluted two folds in a round bottom 96 well plate with 1X PBS, while 1X PBS alone was used as a negative control. An equal amount of influenza virus (25 µl: 4 HA unit) was added to each well and incubated for 30 minutes for virus neutralization. After 30 minutes, 1% chicken red blood cells were added to all wells and incubated for another

30 minutes. The highest tissue homogenate dilution with “bottom” formation was used to estimate the sample's HI unit for Influenza A virus-specific antibody.

3.2.18. Statistical analysis

The results were expressed as mean ± standard deviation (SD). The variance between groups was compared using Student’s t-test or one-way ANOVA as required, followed by Bonferroni's post-hoc multiple comparison tests. The data was analyzed in Instant graph pad prism software (CA). $P<0.05$ was considered a statistically significant difference unless specified under figure legends.

3.3. Results and Discussion

Influenza vaccines provide the protection by producing neutralizing antibodies against the virus surface glycoproteins hemagglutinin (HA) and neuraminidase (NA). Annually, mutations in influenza viruses lead to the antigenic drift and shift especial in HA and NA genes. Thus, developing cross-reactive influenza vaccines against influenza viruses is essential. Several peptides from nucleon protein (NP) or a transmembrane protein M2 have been tested for providing protection against broader strains of influenza (159). In this study, Inf-A peptide (a.a. 366–374) from Influenza strain A/PR/8/35 (ASNENMETM)) was selected as an antigen due to its ability to elicit CD8+ T cell response to influenza virus and the generation of anti-viral cytokines (IFN-γ and TNF-α) (160, 161). Peptide antigens are weakly immunogenic, including Inf-A peptide, which

require vaccine adjuvants in the formulation (121). The current study used an Inf-A peptide as a model antigen and InAc-NPs as a vaccine adjuvant and delivery system.

3.3.1. Physicochemical characterization of the InAc polymer and vaccine formulation

The size of InAc-Inf-A-NPs was around 515 ±0.86 nm in average diameter and with a slight negative charge of -0.9 ± 0.21 mV as determined by dynamic light scattering (DLS) (Figure 3.1A, B). Additionally, the shape and size were confirmed by SEM (Fig. 3.1 C). The amount of antigen (Inf-A) present in InAc-NPs was analyzed using an RP-HPLC described in section 3.2.3. Approximately, 15.43±0.7 µg/mg of Inf-A peptide was loaded in InAc-Inf-A-NPs.

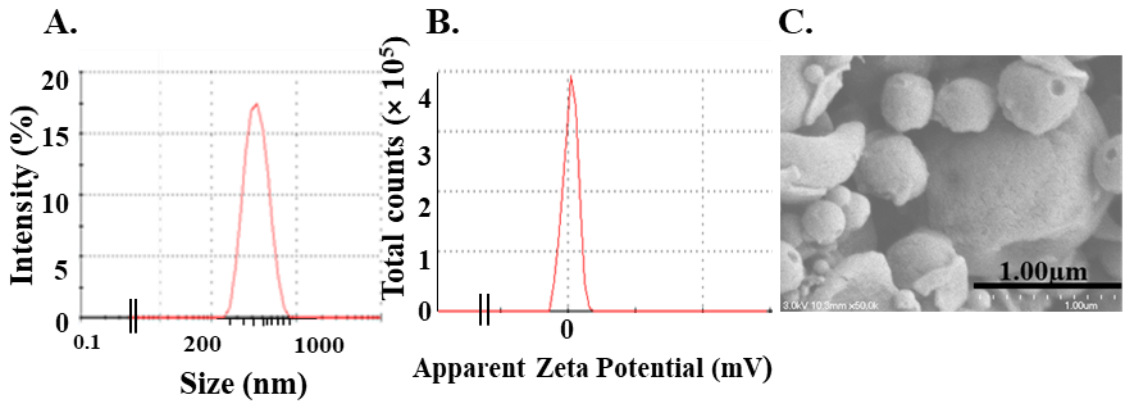


Fig. 3.1. Characterization of InAc-Inf-A-NPs. **A).** The mean particle size distribution was measured using DLS and represented as intensity (percent). **B).** Zeta potential shows an illustration of surface charge, which is close to a neutral charge (-0.9±0.2 mV). **C).** The morphology of InAc-NPs as shown by scanning electron microscopy (SEM). The InAc polymer is a hydrophobic polysaccharide-based TLR4 agonist and activates Toll-like receptors on APCs to release cytokines which were previously studied.

3.3.2. InAc-NPs protect the antigen in gastric conditions

Orally delivered nano vaccines will encounter a harsh environment, increased or triggered enzymatic activity, a shift in acidic pH, or reductive or oxidative conditions (162). The most vital task of nanoparticles in the oral vaccine delivery system is to protect protein/peptide molecules from the GIT enzymes and deliver encapsulated protein to the desired cells. For a antigen to be protected the particulate delivery system should not release the antigen in the gastric fluids. The encapsulated material from the hydrophobic polymeric particles is released by two different mechanisms; a) it is released due to surface erosion of the polymeric matrix through the cleavage of chemical bonds at the exterior, and b) by diffusion of the physically entrapped drug (163).

The InAc polymer is hydrophobic, suggesting surface erosion controls the drug release (164). The stability analysis of blank InAc-NPs in simulated gastric and intestinal fluid (n=3) was performed based on the nanoparticles' size and measured by DLS. If there is a surface erosion, you expect a decrease in the size of the particles with respect to time of incubation. The sizes and PDIs of both InAc and PLGA-NPs were not significantly

altered (Fig. 3.2) as we incubated them in SIG or SIF for up to 12 hrs., which suggests that the particles are stable in SGF and SIF.

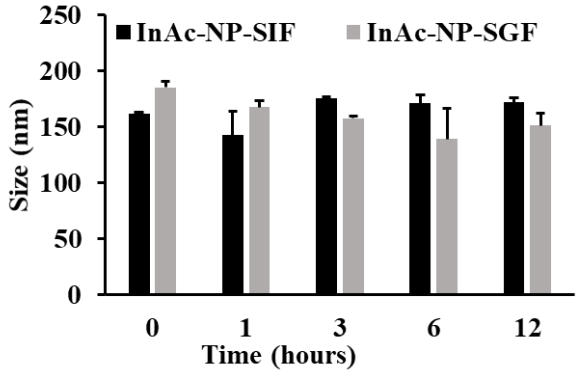


Fig. 3.2. Stability of InAc-NPs against erosion. The InAc and PLGA blank particles were dispersed in a) SGF and b) SIF at ~100 rpm, 37 °C. Dispersed samples were collected and measured for particle size at pre-determined time intervals using DLS. Data represent mean ± standard deviation (n=3).

To check whether InAc-NPs prevent the pre-mature release of the antigen in the gastric environment, the stability of InAc NPs in the gastric fluids and the release of the encapsulated cargo through diffusion are studied. The InAc-NPs loaded with Fluorescein sodium released negligible amounts of the encapsulated cargo (Fluorescence sodium) in Di water (0.8%), SIF (0.1%), and SGF (0.04%), respectively, by 24 hrs (Fig.3.3). This data suggests that the InAc-NPs are stable, and there is no diffusion of the cargo in the time studied.

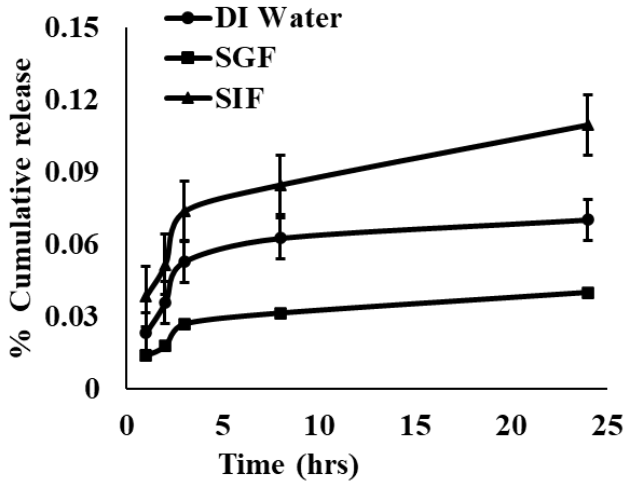


Fig. 3.3. Stability of InAc-NPs in preventing premature release of the cargo. Release of Fluorescein sodium from InAc-NPs. InAc-NPs (3 mg/mL) were dispersed in a) DI Water, b) Simulated Gastric Fluid (SGF), or c) Simulated Intestinal Fluid (SIF) at ~100 rpm, 37°C. Fluorescein concentration in the supernatant solution at different time points was measured using a fluorimeter at excitation and emission wavelengths of 460 nm and 515nm, respectively. Data represent mean ± standard deviation (n=3).

3.3.3. Inf-A release kinetics by InAc-NPs

Peptide and polymer interaction study is critical to formulating a nanovaccine system in a sustained release delivery system so that the desired antigen release can be achieved (165). The cumulative amount of Inf-A released from the InAc-NPS was determined in 10mM PB, pH 7.4. The Inf-A peptide concentration was estimated using RP-HPLC, as mentioned in section 3.2.3.

We found a burst effect within the first 60 minutes for all systems below 20% of the loaded peptide. This can be associated with the surface adsorbed peptide having immediate contact with the buffer and dissolved into the solution. Over the 12 days of

duration, we observed the cumulative release of peptides of around 80% in the solution (Fig.3.4). This is advantageous for the nanovaccine delivery system to release the antigen constantly.

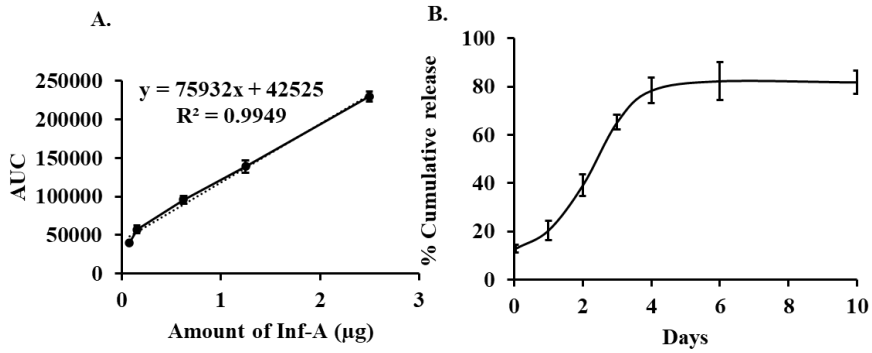


Fig. 3.4. In-vitro antigen (Inf-A) release from InAc-NPs. Calibration curve for Inf-A peptide (panel A) and in-vitro antigen (Inf-A) release kinetics from InAc-NPs (Panel B); The calibration curve (panel A) was plotted from the area under the curve of the peptide peak vs. the amount of the peptide. For panel B, InAc-NPs (1 mg/mL) were dispersed in 0.1M PB, pH 7.4 at ~100 RPM. Inf-A concentration in the soluble fraction was measured at different time points by using HPLC (n=3) as described in section 3.2.3.

3.3.4. Internalization of InAc-NPs by murine macrophages

The nanosized InAc particles mimic the size and shape of viruses or pathogens. Further, the TLR4 agonistic activity of the InAc particles functions as PAMPs to enhance the recognition and interaction by APCs such as macrophages and dendritic cells.

Nanoparticles encapsulated with a fluorescent antigen (FITC labeled Ovalbumin) were prepared using InAc or PLGA as a polymeric matrix. The uptake of these particles by murine macrophages was quantified by flow cytometry (Fig. 3.5). The data suggest that

the percentage of uptake in the cell is higher for InAc-FITC-Ova-NPs vs. PLGA-FITC-Ova-NPs.

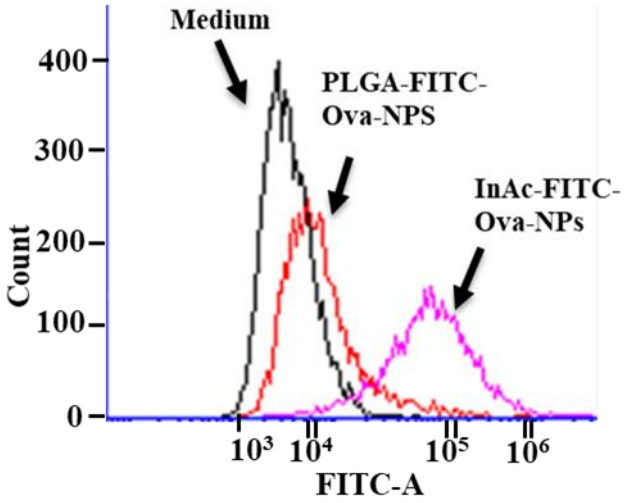


Fig. 3.5. InAc-NPs uptake by murine macrophages. The InAc-FITC-Ova-NPs or PLGA-FITC-Ova-NPs (25µg equivalent to FITC-Ova) were added to 100,000 cells. After 1hr incubation, the cells were analyzed by flowcytometry for the presence of green fluorescence. The experiments were performed in triplicate, and data were represented as mean ± standard deviation.

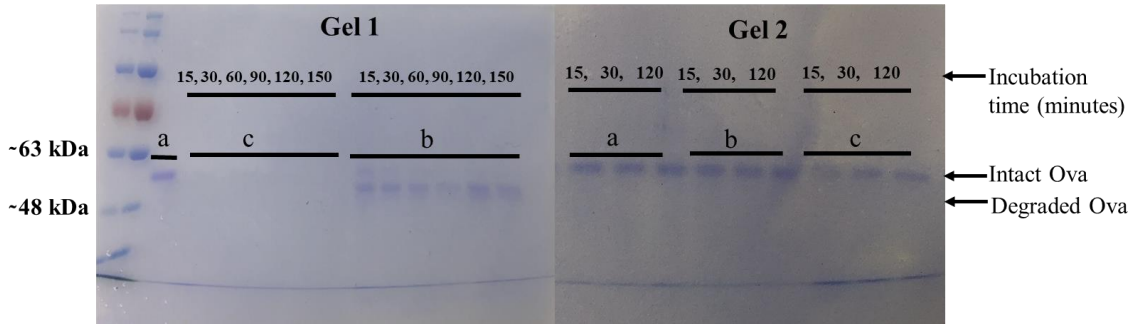
Table. 3.2. Quantification of antigen delivery to mouse macrophages

S.No.	Treatment groups	Mean fluorescence intensity (counts)	% Green cells
1.	Media	5678.48 ± 346.15	10.92 ± 3.80
2.	PLGA-FITC-Ova-NPs	21828.27 ± 2018.09	84.19 ± 4.20
3.	InAc-FITC-Ova-NPs	13958.40 ± 253.80	99.80 ± 0.05*

The data from Fig. 3.5. was quantified and reported. Data represent the mean ± standard deviation (n = 3). * $p < 0.05$ InAc-FITC-Ova-NPs vs. PLGA-FITC-Ova-NPs using one way-ANOVA followed by Bonferroni's multiple comparison test.

3.3.5. Stability of antigen (Ova) InAc-NPs in SGF and SIF

The InAc-NPs were loaded with the Ovalbumin (OVA) as a model protein antigen to evaluate the impact of the formulation on protecting the antigen integrity from the harsh environment. After the InAc-NPs were pre-treated for 30 minutes with 10 mM, PB, pH 7.4, the particles were incubated in SGF and SIF for 30 minutes and 2 hrs. The Ova was extracted from the treated In-Ac-Ova-NPs as described in section 3.2.6. After the protein extraction from InAc-NPs, the concentration was calculated using micro-BCA as per manufacturer instructions. The SDS-PAGE was performed on the protein samples to evaluate any change in molecular weight of the protein or degradation. Compared with the pure OVA protein band, the data suggested OVA was intact during antigen loading to InAc-NPs and upon exposure to SGF and SIF. The SDS-PAGE showed no additional bands, indicating the absence of the aggregates or fragments besides the bands observed in untreated OVA (166). However, there is a decrease in the band intensity for the NPs treated with SIF; this could be due to the pancreatin enzyme present in the intestinal fluid (Fig.3.6).



F***ig. 3.6. Protection of antigen (Ova) by InAc-NPs in SGF and SIF.*** The InAc-Ova-NPs or pure Ova protein was incubated in10 mM PB, pH 7.4 (a), SGF (b), and SIF (c). At different time points, as shown in the figure, the antigen (Ova) was isolated and separated by SDS-PAGE and detected by Coomassie blue. Gel 1 represents Ova protein solution, and gel 2 represents Ova protein encapsulated in InAc-NPs.

3.3.6. TLR4 selective activation by InAc-NPs

An in vitro assay was conducted to investigate the activation of mouse macrophages by InAc-NPs. Upon activation, macrophages releases lipids, cytokines, and nitric oxide. We studied the proinflammatory cytokine (IL-6) production in the supernatant and analyzed for nitric oxide production (Fig. 3.7A). The InAc-NPs were able to activate the mouse macrophages and produced significant levels of IL-6 compared to the PLGA-NPs. The PLGA was chosen because of its inert activity in the stimulating immune cells. In contrast, InAc-NPs failed to stimulate the TLR4 knockout macrophages (Fig. 3.7B). This indicated the specific activation is TLR4 dependent. MPLA was used in the experiment as a positive control for TLR4 activation. From our laboratory, we showed the immune activation properties of InAc on different types of APCs through TLR4 (49). For the first time, we investigated the stimulatory effect of InAc-NPs on in mouse macrophages.

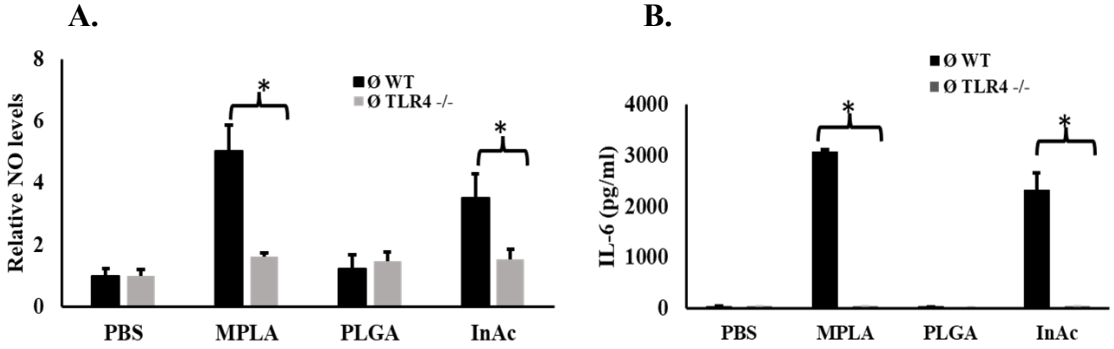


Fig. 3.7. Activation of macrophages by InAc-NPs. The activation effect of InAc-NPs on TLR4 was examined using mouse macrophage cell lines (WT and TLR4 -/-). The cells were incubated with InAc-NPs or PLGA-NPs (no antigen) for 48 hrs. MPLA (known TLR4 agonist) was used as a positive control. The stimulation of macrophages was evaluated by assessing the levels of panel **A).** nitric oxide and panel **B).** interleukin-6 (IL-6) in the culture supernatants. The significant difference between groups was measured by paired t-tests compared to InAc-NPs or MPLA treatments of Ø WT cells versus MPLA or InAc-NPs treatment of Ø TLR4 (−/−) cells at a 95% level of significance. (*$p<0.05$).

3.3.7. Antigen delivery by InAc-NPs generated strong secretory (sIgA) antibody titers

Extensive vaccine research is underway to develop needle-free platforms; a few delivery systems include live recombinant bacterial and viral vectors. The vectors used for delivery are inert and not capable of generating immune responses (167). Live bacterial or viral vectors induce sIgA via various mechanisms depending on the PAMPs they stimulate and the type of the cargo (for example, cytokines) they deliver together with the antigen. The non-living delivery systems such as nano- and microparticles, nanogels, or liposomes do not possess the possible safety concerns usually observed in the whole live pathogen. Many non-living carriers induce SIgA after mucosal immunization either by oral, vaginal, or nasal routes (168, 169). Secretory IgA (sIgA) plays a crucial role in host defense against respiratory pathogens by inducing mucosal immunity (170).

After immunization of mice groups with saline, Inf-A peptide in saline solution, and InAc-Inf-A-NPs, the mice were sacrificed after four weeks, and the tissues were

collected. Saline, Inf-A peptide in saline solution, and InAc-Inf-A-NPs elicited antigen-specific IgA antibody responses in homogenized organs (Fig.3.8 A). In Lung homogenate, compared to the titer elicited by Inf-A peptide alone, the titers were ~2 fold higher for the InAc-Inf-A-NPs formulation; in the intestine (Ileum) homogenate, compared to the titer elicited by Inf-A peptide alone, the titers were ~2 fold higher for the InAc-Inf-A-NPs formulation. However, the control mice immunized orally with saline had background titers similar to the peptide solution; this could be because the mice before the experiment were exposed to any other respiratory pathogens. In turn, it generated the secretory antibodies.

On the other hand, we conducted the sandwich ELISA to evaluate the total IgA in the Intestine (Ileum), Lung, and spleen homogenates (Fig. 3.8 B). In Lung homogenate, compared to the titer elicited by Inf-A peptide alone, the titers were ~1.3 fold higher for the InAc-Inf-A-NPs formulation ($P < 0.05$), In the intestine (Ileum), homogenate, compared to the titer elicited by Inf-A peptide alone, the titers were ~1.6 fold higher for the InAc-Inf-A-NPs formulation ($P < 0.05$). The same background was observed in the mice immunized with mice alone as in antigen-specific IgA. However, there is no difference between the treatments in the spleen.

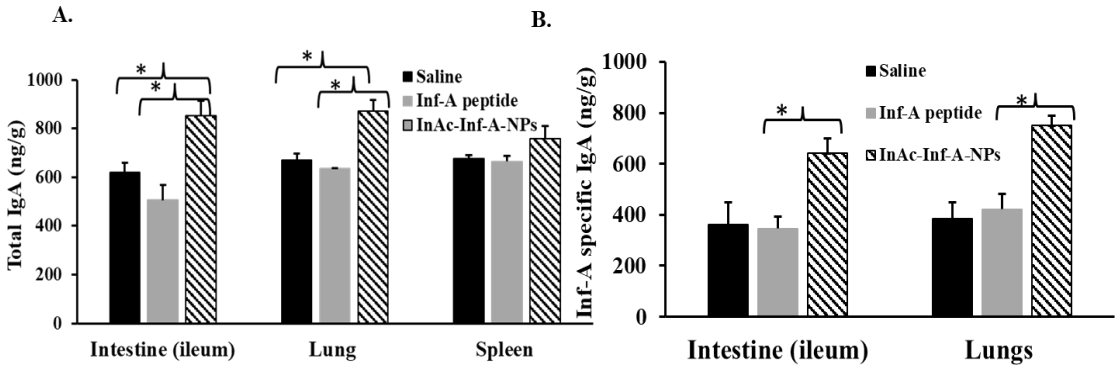


Fig. 3.8. Total IgA (panel A) & Inf-A specific IgA (panel B) antibody response in the tissues following oral vaccination. Balb/c mice were orally administered with saline, peptide alone in saline, or InAc-Inf-A-NPs for immunization. After four weeks, the mice were sacrificed, and the tissues were collected. The tissue samples were homogenized in protease inhibitor and normalized for equal protein concentration followed by measuring the concentration of anti-Inf-A IgA and total IgA by sandwich ELISA. * $p < 0.05$ InAc-Inf-A-NPs vs. saline or peptide using one way-ANOVA followed by Bonferroni's multiple comparison test.

3.3.8. Antigen delivery by InAc-NPs generated strong antigen-specific IgG and IgA antibody titers

B cell responses play a key role in adaptive immunity to a viral infection (171). sIgA antibodies mediate viral neutralization and are involved in distinctive roles in immunity from the entry of pathogen at distal sites (172). Following the vaccination by Saline, Inf-A peptide in saline solution, and InAc-Inf-A-NPs, the serum was collected at 1 and 4 weeks of immunization for Inf-A (antigen) specific IgG (Fig. 3.9 A). The Serum was analyzed for influenza-A specific (IgG) concentration using comparative ELISA/ Influenza A Virus NP Antibody Inhibition ELISA. The titers from the saline-treated mice have the same background after two doses of vaccine admonition. At the same time, the serum titers were collected after one week of primary vaccination. We observed antigen-specific IgG response in peptide alone and InAc-Inf-A-NPs, but there were not statistically significant. However, in the serum collected after in 4th week, compared to the titer elicited by Inf-A peptide alone, the titers were ~1.5 fold higher for the InAc-Inf-A-NPs formulation; thus, the

data indicates with the administration of two doses of InAc-Inf-A-NPs, it is possible to have antigen-specific antibodies production. The sandwich ELISA was conducted to evaluate the Inf-A specific (antigen) sIgA in the serum following the immunization after 2nd dose (Fig. 3.9 B). In serum, compared to the titer elicited by Inf-A peptide alone, the titers were 9 fold higher for the InAc-Inf-A-NPs formulation. The background was observed in the mice immunized with saline alone, and this could be due to mice exposed to other respiratory pathogens, which elicited the IgG antibodies.

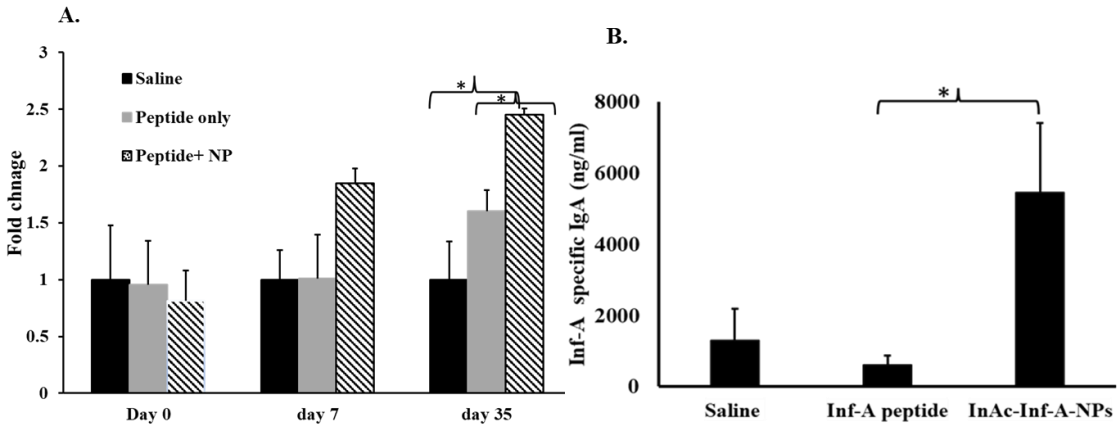


Fig. 3.9. Inf-A specific IgG (panel A) & IgA (panel B) antibody response in the serum following oral vaccination. Balb/c mice were orally administered with saline, peptide alone in saline, or InAc-Inf-A-NPs for immunization. Two doses were given on weeks 1 and 3. The Inf-A specific IgG was quantified in serum collected at 1 and 4 weeks of immunization (panel A). The antigen-specific IgA titers in the serum were determined on week 4 (panel B) measured by sandwich ELISA. * $p < 0.05$ InAc-Inf-A-NPs vs. peptide using one way-ANOVA followed by Bonferroni's multiple comparison test.

3.3.9. Hemagglutination inhibition (HI)

Influenza viral surface protein HA will bind to sialic acid receptors. The virus has other property of binding to the erythrocytes, causing a lattice formation. The lattice formation is called hemagglutination. The RBCs have the unique property of binding to the virus and forming a layer on the round wells; on the other hand, button formation will be observed if the virus is not bound to the RBCs.

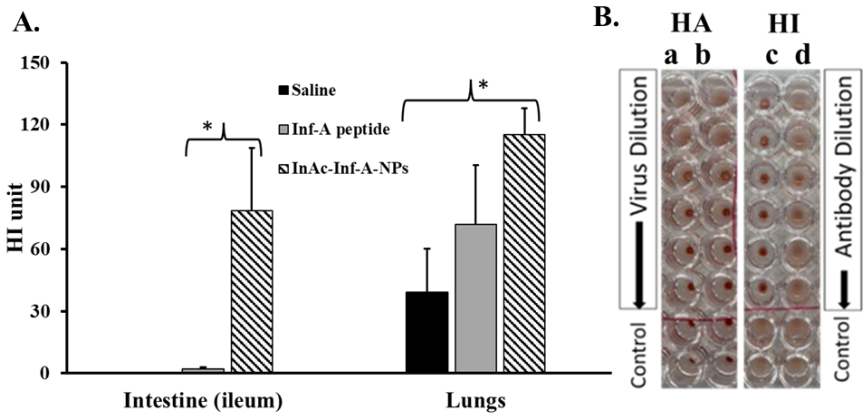


Fig. 3.10. Hemagglutination inhibition (HI) response following oral vaccination.

Balb/c mice were orally administered with saline, peptide alone in saline, or InAc-Inf-A-NPs for immunization. **A).** Following four weeks, the mice were sacrificed, and tissues were collected. The tissue samples were homogenized in protease inhibitor and normalized for equal protein concentration. * $p < 0.05$ InAc-Inf-A-NPs vs. saline or peptide using one way-ANOVA followed by Bonferroni's multiple comparison test. **B).** Hemagglutination inhibition 96-well plate for better understanding of the design of the experiment. Hemagglutination (HA), column ‘a, b’; assay showing the 2 HA units of diluted virus (Influenza A virus, A/puertorico/8-9NMC1/1934(H1N1). Column ‘d’ shows the Hemagglutination inhibition (HI) unit for control as zero HI units, while ‘c’ shows more than 64 HI units in tissue lysate.

The fundamental basis for the hemagglutination inhibition assay is that antibodies produced against the influenza virus will inhibit the virus from binding to the RBCs. The maximum dilution of serum that inhibits hemagglutination is called the HI titer of the serum (173) . After immunization of mice groups with saline, Inf-A peptide in saline solution, and InAc-Inf-A-NPs, the mice were sacrificed after four weeks, and the tissues were collected. The HI units of homogenized organs lysate of the intestine (Ileum) and lungs for groups saline, Inf-A peptide in saline solution, and InAc-Inf-A-NPs were reported in (Fig. 3.10. A). In Lung homogenate, compared to the HI unit of saline alone, the HI units were ~3 fold higher for the InAc-Inf-A-NPs formulation. However, peptide alone in the saline group has HI units; this could be due to the lungs being exposed to other antigens. The generated antibodies could be interfering with the data. The antibodies in intestinal (Ileum) lysate homogenate, compared to the titer elicited by Inf-A peptide alone, the titers were ~39 fold higher for the InAc-Inf-A-NPs formulation. This data suggests the InAc-Inf-A-NPs nanovaccine was able to induce the mucosal antibodies, and they were able to bind the influenza virus. The picture of the experiment for both hemagglutination and hemagglutination inhibition (HI) (Fig. 3.10 B) was reported for better understanding.

3.4. Conclusion

In the present study, we have investigated for the first time the ability of InAc-NPs encapsulated with Inf-A antigen as an oral delivery system to elicit a mucosal and systemic immune response as a proof-of-concept study to develop a vaccine against the influenza virus. We observed that the antigen encapsulated in the InAc nanoparticles were robustly

taken up by the mouse macrophages, demonstrating the ability of APCs to respond to the presence of antigens delivered by InAc polymer as the particulate system showed a 99.80% green cells for InAc-FITC-Ova-NPs compared to uptake by PLGA polymer, which is 84% green cells. In addition, we demonstrate that InAc-NPs are stable in the gastric environment and protect the model antigen Ovalbumin. Furthermore, after immunization of mice, the presence of sIgA in the serum and mucosal tissue lysates suggests that the InAc-Inf-A-NPs were able to elicit the mucosal immunity, and the antibodies produced demonstrated functional activity by inhibiting the hemagglutination.

SUMMARY AND FUTURE STUDIES

Due to the evolving respiratory pathogens (viral and bacterial), growing antibiotic resistance, and rising occurrences of inflammatory mucosal diseases, there is an urgent medical need to discover a safe vaccine technology that activates both systemic and mucosal protection. However, there are limitations in the availability of vaccine technologies or vaccine adjuvants that address the above need, which has hampered the development of successful preventive vaccines against challenging mucosal pathogens such as HIV, influenzas, and HPV SARS-CoV-2 virus.

Our laboratory previously reported a "Pathogen Mimicking Vaccine Delivery System" (PMVDS) prepared with inulin acetate, a TLR4 agonist, as a polymer to generate strong humoral and cell-mediated immunity. The main goal of this dissertation is to investigate the potential of InAc-based nanoparticles (InAc-NPs) as a delivery system for an oral route to generate mucosal immunity and evaluate its potential as an adjuvant and delivery system for influenza vaccine in pigs.

As in Chapter II, subcutaneous delivery of InAc-NPs encapsulated with influenza antigens HA and M2e generated robust systemic antibody titers (IgG) in pigs, which was comparable to the commercial vaccine adjuvant Addavax. Various in-vitro mechanistic investigations have shown that InAc-NPs improve the stability of the antigen during storage and efficiently deliver it to APCs such as macrophages. Previously, our laboratory has identified the signaling pathways of InAc activating macrophages to initiate an immune response cascade. This is the first study that showed the efficacy of InAc as a subcutaneous vaccine in pigs.

This preliminary study signifies the ability of InAc-NPs as an adjuvant and a delivery system to generate robust antibody titers in pigs. More studies are underway in our laboratory to test the vaccine in protecting the pigs from challenges with homologous and heterologous strains of porcine influenza. A universal vaccine that covers multiple strains of influenza is the long-term goal of this project. Further, the technology will be extended to other viral diseases in pigs, such as porcine epidemic diarrhea virus (PEDV), porcine reproductive and respiratory syndrome (PRRS), and porcine epidemic diarrhea (PED) pose a serious challenge to the productivity of today's swine farms.

Further, in chapter III, using InAc-NPs as a delivery system, we have developed an oral vaccine with Inf-A as a model antigen and tested it in mice. InAc-Nps activated mouse macrophages to release inflammatory cytokines necessary for vaccine efficiency. InAc-Inf-A-NPs generated high titers of antigen-specific sIgA (secretory and mucosal) and IgG antibodies in serum and mucosal organs such as the intestine and lungs. The generation of mucosal secretory antibodies by the InAc-NPs-based vaccine delivery system is significant in designing oral vaccines against other mucosal pathogens. A significant discovery of this aim is to identify an oral delivery system that protects encapsulated materials such as antigens from degradation by gastric juices. In future studies, it will be tested to deliver microbes to the colon or antigens to generate mucosal immunity. In addition, preliminary studies were performed to explore InAc-NPs as a delivery system for other TLR agonists and polymeric adjuvants, which could have implications for oral vaccine delivery or subcutaneous delivery for influenza infections.

In conclusion, the dissertation advanced InAc-based platform technology for mucosal vaccines for animal applications vat this stage and will be advanced for human application.

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