NPB FINAL RESEARCH GRANT REPORT FORMAT

Project Title and NPB project identification number: Assessing the feasibility of the mRNA vaccine technology for use against ASF, NPB #21-126
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Industry Summary:

African swine fever virus (ASFV) is the causative agent of a fatal disease in swine. The virus has spread to many countries and regions, causing significant economic losses, and posing a substantial risk to global food security. There is an urgent need for a safe and effective vaccine to control this virus. Multiple live-attenuated ASFV (LAV) vaccine candidates have been developed which induce solid protection against virulent parental strains. However, one inherent risk associated with the use of LAVs is that the vaccine strains might revert to virulence. Furthermore, ASFV is designated as a select agent, leading to stringent regulations on virus handling and study. Consequently, live-attenuated ASFV vaccines must be produced at designated facilities.

On the other hand, subunit vaccines provide a safer alternative. Moreover, this type of vaccine can be produced in standard production facilities as they do not require the handling of live ASFV strains. Typically, subunit vaccines contain only one or a few viral antigens; thus, allowing the use of serological assays for differentiating vaccinated from naturally infected animals. However, the major challenges to the development of an effective subunit vaccine against ASFV include the gap in knowledge regarding viral protective antigens and the lack of efficient platforms for the delivery of vaccine immunogens in pigs.

Recently, mRNA vaccine technology has emerged as a promising platform for the development of subunit vaccines. Therefore, we explored the feasibility of applying mRNA vaccine technology to develop a subunit vaccine against ASFV. mRNA transcripts are extremely sensitive to degradation and do not penetrate cells effectively. Thus, the mRNA transcripts must be packaged into nanoparticles to enhance their stability and facilitate their cellular entry. Among the several types of nanoparticles, lipid nanoparticles (LNPs) have proven the most effective technology for delivering mRNA transcripts. Typically, LNPs are formed by four types of lipids: a cationic lipid, a phospholipid, cholesterol, and a polyethylene glycol (PEG)-conjugated lipid. The types and molar ratios of lipids used to formulate LNPs influence the uptake and endosomal releases of the encapsulated nucleic acid cargo and consequently the potency of the mRNA vaccines.

In this pilot project, we chose to work with four well-characterized ASFV genes, namely p32, p54, C-type lectin, and CD2v. We successfully generate mRNA transcripts, some of which show high levels of protein expression when transfected into HEK-293T cells by using a commercial transfection reagent. We then use the green fluorescent protein (GFP) mRNA as a model to develop several lipid nanoparticle formulations. However, none of these formulations demonstrated effective transfection of the GFP mRNA transcripts into HEK-293 cells cultured *in vitro*. Subsequently, when administered to mice, only one LNP formulation showed a modest induction of antibody responses. We believe that the inefficient transfection efficiency and low immunogenicity observed with the LNP-mRNA vaccines may be attributed to the specific lipid types and formulation methods employed. Therefore, further efforts are required to develop an effective LNP formulation for encapsulating and delivering mRNA to pigs.

Key Findings:

- Four well-characterized ASFV genes, namely p32, p54, C-type lectin, and CD2v were selected to explore the feasibility of the mRNA vaccine approach.
- mRNA transcripts for these genes were successfully generated using an in vitro transcription kit.
- High levels of protein expression were observed in HEK-293T cells when p32 and p54 mRNA transcripts were transfected using a commercial transfection reagent.
- Three lipid nanoparticle formulations were developed for encapsulating the mRNA transcripts.
- None of these LNP formulations were effective in transfecting the mRNA transcripts into HEK-293T cells.
- One of the LNP formulations induced a modest antibody response when administered to mice.
- Further efforts are required to develop an effective LNP formulation for encapsulating and delivering mRNA to pigs.

Keywords: ASFV, mRNA vaccine, lipid nanoparticles, DNA vaccine

Scientific Abstract

African swine fever virus (ASFV) is spreading to many countries and regions, causing significant losses to swine producers. Currently, there are no commercial vaccines or treatments available for ASF. Although multiple live-attenuated virus (LAV) vaccines have been generated and demonstrated efficacy in inducing protection against parental virulent ASV strains, there is a significant concern about the use of LAV vaccines, especially the risk of reversion to virulence. The primary objective of this project was to assess the feasibility of utilizing the mRNA vaccine technology to develop a subunit vaccine against ASFV. Four well-characterized ASFV genes, namely p32, p54, C-type lectin, and CD2v were selected and mRNA transcripts of these selected ASFV genes were successfully generated. When transfected into HEK-293T cells using a commercial transfection reagent, two of these mRNA transcripts (p32 and p54) exhibited high levels of protein expression. Three different lipid nanoparticle formulations were developed to encapsulate the mRNA transcripts. However, none of these LNP formulations were effective in transfecting the mRNA transcripts into HEK-293T cells. Subsequently, when administered to mice, only one LNP formulation showed a modest induction of antibody responses. We believe that the inefficient transfection efficiency and low immunogenicity observed with the LNP-mRNA formulations in this study may be attributed to the specific lipid types and formulation methods employed. Therefore, further efforts are required to develop an effective LNP formulation for encapsulating and delivering mRNA to pigs.

Introduction

ASFV infects all members of the family *Suidae* including domestic pigs, wild boars (*Sus scrofa ferus*), warthogs (*Phacochoerus aethiopicus*), and bushpigs (*Potamochoerus porcus*) (1). In addition, the virus can infect soft ticks of the *Ornithodoros* species. ASFV infection does not cause any serious clinical signs in warthogs, bush pigs, and soft ticks, allowing them to serve as reservoirs for the virus. The sylvatic cycle between soft ticks and warthogs contributes significantly to the endemic cycle of ASFV in the Africa (2).

ASFV infection in domestic pigs can result in a broad spectrum of clinical manifestations, depending on the virulence of the infecting viral strains(3). In the case of highly virulent ASFV strains, the infected pigs typically exhibit peracute signs, progressing rapidly to fatal outcomes within 6-13 days post-infection, with mortality rates approaching 100% (4). On the other hand, moderately virulent ASFV strains often induce sub-acute or chronic infections, characterized by various clinical signs including weight loss, intermittent fever, respiratory signs, chronic skin ulcers, and arthritis. In these cases, mortality rates can range between 30% and 70% (5).

Under field conditions, there is a small portion of pigs that can survive the infection with virulent ASFV strains (6). Notably, these surviving pigs are resistant to reinfection with closely related virulent ASFV strains (7-9). Likewise, pigs immunized with candidate ASF live attenuated vaccines (LAVs) are protected against lethal infection with the parental virulent ASFV strains (10). These findings highlight the ability of ASFV to induce protective immunity in pigs, however, this immunity tends to be only effective against homologous ASFV strains (11). The specific immune correlates of

protection are still not well understood, but both humoral and cell-mediated immune responses are believed to play crucial roles in providing complete protection against ASFV.

ASFV is a large double-stranded DNA virus with a genome size ranging between 170 and 193 kbp and typically contains between 150 and 170 open reading frames (ORFs) (12). More than 100 viral proteins have been identified in ASFV-infected cells (13). The ASFV virion contains at least 68 virus-encoded proteins, with half of them having no known functions (14). While the specific viral proteins responsible for inducing protective immunity are not yet fully elucidated, certain proteins such as p32, p54, C-type lectin, and CD2v have been demonstrated to be able to induce neutralizing antibodies and provide partial levels of protection against lethal ASFV challenge (15).

Considerable efforts have been dedicated to the development of subunit vaccines against ASFV but the outcomes have shown considerable variability (15). While the subunit vaccine candidates elicit specific immune responses against the incorporated viral antigens, most experimental vaccines tested thus far have failed to confer protection against lethal challenges with virulent ASFV strains. The main hurdles to the development of an effective subunit vaccine against ASFV include the limited understanding of the viral proteins that can induce protective immunity and the lack of an efficient platform for the delivery of vaccine immunogens in pigs.

Recently, mRNA vaccine technology has emerged as a highly promising platform for subunit vaccine development. mRNA vaccines offer several advantages, including their safety, potent immunogenicity, and the ability for rapid development and production (16). However, significant challenges associated with mRNA are its inherent instability and limited cellular penetration. Thus, the mRNA molecules are often packaged into nanoparticles to enhance stability and facilitate cellular entry. Among the several types of nanoparticles, the lipid nanoparticle (LNP) has emerged as the most effective technology for delivering mRNA vaccines. The lipid nanoparticles are typically formed by four types of lipids: a cationic lipid, a phospholipid, cholesterol, and a polyethylene glycol (PEG)-conjugated lipid (17). The types and molar ratios of lipids used to formulate LNPs influence the uptake and endosomal releases of the encapsulated nucleic acid cargo (18).

In this study, we aim to explore the potential of mRNA vaccine technology for the development of a subunit vaccine against ASFV. For this pilot project, we focused on assessing the immunogenicity of mRNA vaccines containing four well-characterized ASFV antigens: p32, p54, C-type lectin, and CD2v. Each of these antigens plays a distinct role in the viral lifecycle. Phosphoprotein p32, also known as p30, is an early-expressed protein that primarily localizes in the cytoplasm of infected cells (19). The transmembrane protein p54 is crucial for ASFV morphogenesis (20). Both p32 and p54 play important roles in viral entry into susceptible cells. Particularly, p54 is involved in the viral attachment while p32 is involved in the viral internalization (21). Both p32 and p54 are highly immunogenic and antibodies specific to these two proteins are capable of neutralizing virus infection (19). C-type lectin and CD2v are two viral glycoproteins that mediate hemadsorption to viral infected cells (22, 23). Pigs infected with ASFV developed antibodies that inhibit hemadsorption to viral-infected cells. Thus, antibodies specific to C-lectin and CD2v might be important for immune protection against ASFV.

Objectives

Objective 1: Generation and *in vitro* characterization of ASFV mRNA lipid-nanoparticle vaccine candidates.

Objective 2: Evaluation of the immunogenicity of the mRNA-LNP vaccine candidates in pigs.

Materials & Methods

Cells and lipids

HEK-293T (ATCC CRL-3216) cells were used for the evaluation of transfection efficiency. The lipids used in this study included DLin-MC3-DMA (MC3) (Nanosoft Polymer, Winston-Salem, NC), 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) (Cayman Chemical, Ann Arbor, MI), Cholesterol (Sigma Aldrich), distearoylphosphatidylcholine (DSPC), and 1,2-dimyristoyl-rac-glycero-3-methoxypolyethylene glycol-2000 (DMG-PEG2000) (Avanti Polar Lipids, Birmingham, AL). The lipids were separately dissolved in absolute ethanol.

Generation of DNA plasmids and mRNA transcripts

Genes encoding four ASFV antigens: p32, p54, C-type lectin, and CD2v were designed based on the genome sequence of the ASFV strain Georgia 07 (GenBank accession number FR682468). The genes were codon optimized for optimal expression in swine cells (*Sus scrofa*) and a flag-epitope sequence (DYKDDDDK) was fused in-frame to the 3' end of the gene to facilitate protein detection. The genes were chemically synthesized using a commercial DNA synthesis service (GenScript, Piscataway, NJ). To produce mRNA transcripts, the genes were cloned into an expression vector between the optimal 5' and 3' untranslated regions (UTRs) (Figure 1). The bacterial phage T7 promoter sequence was incorporated to the 5'end of the gene, immediately upstream of the 5' UTR to facilitate in vitro transcription to produce mRNA transcripts. To develop DNA vaccines, the genes were cloned into the pCI plasmid (Promega), immediately downstream of the human cytomegalovirus promoter. In both cases, large-scale DNA plasmids were amplified in *Escherichia coli* DH5α and purified by using a plasmid giga prep kit (Zymo Research, Costa Mesa, CA). DNA sequencing was performed to confirm the plasmid sequence's authenticity.

To generate mRNA transcript, the plasmid containing either ASFV genes or the green fluorescent protein (GFP) gene was linearized by restriction enzyme digestion. Subsequently, the linearized DNA was used as the template for an *in vitro* transcription reaction to produce 5'-capped and 3'- polyadenylated mRNA transcripts utilizing the Hiscribe™ T7 mRNA kit with CleanCap Reagent AG (Promega).

Lipid nanoparticle formulation

Lipid nanoparticles encapsulating either mRNA or DNA plasmids were prepared following a method described previously with some modifications (24). Briefly, the lipids were individually dissolved in ethanol and mixed at specific molar ratios to form lipid mixtures. The mRNA transcripts or DNA plasmids were diluted in citrate or sodium acetate buffer at an acidic pH. The lipid (organic phase) and DNA solution (aqueous phase) were mixed by using a mixer-4 chip and the NanoGenerator™ Flex-M Nanoparticle Synthesis System (Precigenome, San Jose, CA). The resulting products were dialyzed against 100 mM Tris-Cl buffer at pH 7.4 using the Slide-A-Lyzer™ G2 dialysis cassettes with the molecular weight cut-off of 10 kDa (Thermo Fisher Scientific, Carlsbad, CA). After dialysis, LNPs were passed through 0.45 μm PES filters and the encapsulated mRNA or DNA plasmid concentration was adjusted to 100 μg/mL.

LNP sizes were measured by following the nanoparticle tracking analysis (NTA) method by using the Flow NanoAnalyzer (NanoFCM, Tokyo, Japan). The polydispersity index (PDI) and zeta potential (mV) were determined using the Malvern Zetasizer® (Nano ZS, Malvern Instrument, Worcestershire, UK). mRNA or DNA plasmid encapsulation efficiency (EE%) was quantified using Quant-iT™ Ribogreen Assay Kit or Quant-iT™ PicoGreen™ dsDNA assay kit (Thermo Fisher Scientific) as described previously (24).

Transfection efficiency in vitro

To assess the transfection efficiency *in vitro*, 500 ng of LNP-mRNA or LNP-DNA plasmids was directly added to one well of the 24-well plate containing HEK-293T cells. An equal amount of naked-DNA plasmid was added to another well to serve as a negative control. For positive controls, the TransIT®-mRNA kit (Mirus Bio) was used to transfect mRNA transcripts while polyethyleneimine (PEI) was used to transfect DNA plasmids. Protein expression was assessed using indirect immunofluorescent assay (IFA) and western blotting.

Evaluation of the immunogenicity of the GFP-LNP preparations in mice

Twenty 6-week-old female BALB/c mice were obtained from Charles River Laboratory and were randomly divided into 5 groups. Each group received a different intramuscular injection. Three groups were administered 10 μg of each GFP-LNP vaccine formulation, one group received 10 μg of naked mRNA, and the final group was injected with PBS, serving as the negative control. Blood samples were collected at various time intervals following vaccination to assess the anti-GFP antibody responses using an indirect ELISA assay.

Evaluation of the immunogenicity of the p54 LNP-DNA plasmid preparation in pigs

Two pig experiments were conducted to assess the immunogenicity of the lipid nanoparticle encapsulating the DNA plasmid encoding the ASFV p54 gene.

In the first experiment, nine 4-week-old pigs seronegative for swine influenza virus and PRRSV were randomly assigned to three treatment groups. Groups 1 and 2 were administered intramuscularly with 100 μg and 500 μg of the p54-LNP vaccine preparation, respectively, while group 3 was injected with PBS to serve as a negative control (Figure 7A).

In the second experiment, twelve 4-week-old pigs, also seronegative for swine influenza virus and PRRSV, were randomly assigned into three treatment groups. Groups 4 and 5 were administered intramuscularly with 100 μg and 500 μg of the naked p54 DNA plasmid, respectively, while group 6 was injected with PBS to severe as the negative control (Figure 7B).

In both experiments, blood samples were collected at various time intervals post-vaccination, and anti-p54 antibodies were measured using the luciferase immunoprecipitation system (LIPS) assay as described previously (25). The assay involved incubating the test serum samples with luciferase-tagged ASFV p54 protein and protein-A Sepharose beads in a 96-well filter plate. If the test samples contained antibodies (IgG) specific to the luciferase-tagged p54, the antigen-antibody complexes would be captured by the protein-A Sepharose beads and retained in the well. The retained luciferase-tagged antigen would then react with a luciferase substrate, generating a quantifiable bioluminescence signal proportional to the amount of antigen-specific antibody in the test serum samples. This allowed for the quantitative assessment of antigen-specific antibody levels in the samples.

Results

Objective 1. Generation and *in vitro* characterization of mRNA lipid-nanoparticle vaccine candidates.

1a. Design of a plasmid backbone to clone the target genes for in vitro transcription to produce mRNA transcripts.

For nucleic acid-based vaccines (such as mRNA and DNA vaccines), the expression levels of the vaccine antigens critically affect the magnitudes of immune responses. It is known that the expression levels of the mRNA transcripts are affected by the 5’ and 3’ untranslated regions (UTRs) flanking both sides of the coding sequence. Hence, we conducted a thorough analysis of existing literature to identify the most effective 5' and 3' UTR sequences. We then chemically synthesized the 5’ and 3’ UTRs and cloned them into a bacterial plasmid to generate an expression vector depicted in Figure 1. Subsequently, the four ASFV target antigens (p32, p54, C-type lectin, and CD2v) were separately inserted into the expression plasmid between the 5’ and 3’ UTR sequences. The flag-tag sequence was fused in-frame to the C-terminus of each antigen to facilitate the detection of protein expression. The resulting plasmids were subjected to DNA-sequencing to verify the sequence authenticity.

1b. Production of mRNA transcripts and evaluation of protein expression.

mRNA transcripts of each of the four ASFV genes (p32, p54, C-type lectin and CD2v) were produced by using an *in vitro* transcription kit. The resulting mRNAs were then transfected into HEK-293T cells using a commercially available transfectant commonly used for mRNA transfection. Two different methods were used to detect protein expression: indirect immunofluorescence assay (IFA) and western blotting.

Through western blotting analysis, specific protein bands corresponding to the expected molecular weight were only observed in cells transfected with p32 and p54 mRNA. Conversely, no protein bands were detected in cells transfected with C-type lectin and CD2v mRNA (Figure 2A).

By utilizing IFA, we could observe specific fluorescence signals from cells transfected with all four ASFV mRNA transcripts. The highest number of positive cells and signal intensity were detected in cells transfected with p32 mRNA, followed by p54 mRNA (Figure 2B). Cells transfected with C-type lectin and CD2v mRNA exhibited a lower frequency of positive cells. These results demonstrated the expression of all four ASFV mRNA transcripts in the transfected cells. However, the expression intensity of C-type lectin and CD2v was low and could only be detected using the IFA method, not by western blotting (Figure 2A and 2B).

1c. Generation and in vitro characterization of lipid nanoparticles encapsulating a model mRNA transcript

The detection of ASFV protein expression requires assays such as IFA or Western blot, which are time-consuming. Therefore, we utilized the green fluorescent protein (GFP) transcript as a model antigen to optimize the procedures to produce lipid-nanoparticles (LNPs) as it allows us to assess protein expression in a more efficient and timely manner.

We used three different formulations to prepare the LNPs encapsulating the GFP mRNA transcripts, herein referred to as GFP-F1, GFP-F2, and GFP-F3. The physical characteristics of the resulting LNPs are presented in Figure 3. The average diameters of GFP-F1, GFP-F2, and GFP-F3 were approximately 100 nm (Figure 3A), slightly larger than the commonly reported size of LNP-mRNA in the literature (18, 24). The polydispersity index (PDI) is an indicator of particle size heterogeneity. LNP preparations with a PDI closer to zero exhibit more homogeneity in particle size, while those closer to one indicate greater polydispersity. In our study, the PDIs of GFP-F1, GFP-F2, and GFP-F3 ranged between 0.2 and 0.3 (Figure 3B), suggesting a slight heterogeneity in particle sizes for these three LNP preparations. The mean zeta potentials of GFP-F1, GFP-F2, and GFP-F3 were +28 mV, +30 mV, and +5 mV, respectively (Figure 3C). Additionally, the mean encapsulation efficiencies (EE%) of GFP-F1, GFP-F2, and GFP-F3 were above 85% (Figure 3D), indicating successful encapsulation of most mRNA transcripts within the LNPs.

To evaluate the transfection efficiency, 0.5 μg GFP-F1, GFP-F2, and GFP-F3 were directly added directly into the medium of HEK-293T cells in a 24-well plate. Naked mRNA transcripts were used as a negative control while mRNA transcripts complexed with TransIT®-mRNA, a commercial transfectant reagent commonly used for mRNA transfection, were used as a positive control. At various time points post-transfection, the expression GFP was directly observed using a fluorescence microscope. As anticipated, cells transfected with naked DNA plasmid did not exhibit any fluorescent-positive cells. In contrast, approximately 60% of cells transfected with TransIT®-mRNA demonstrated fluorescence (Figure 4). These results confirm the functionality of the GFP mRNA transcripts utilized in this study. However, there were only a few fluorescent-positive cells observed in the wells treated with GFP-F1, GFP-F2, and GFP-F3. Thus, the three LNP formulations that were used to encapsulate GFP mRNA in this project had limited potency in facilitating the transfection of HEK-293T cells.

Objective 2. Evaluation of the immunogenicity of the mRNA-LNP vaccine candidates in pigs

2a. Evaluation of the immunogenicity of the lipid nanoparticles encapsulating a model mRNA transcript in mice

Although the three LNP formulations utilized for encapsulating GFP mRNA transcripts fail to mediate transfection HEK-293T cells, we still wanted to assess their potential to induce an immune response in mice. Five groups of mice were included in this study, each group contains 4 mice (Figure 5A). Indirect ELISA was used to measure GFP-specific antibody responses. As expected, the PBS control group and the Naked mRNA group did not exhibit any detectable levels of anti-GFP antibodies at any sampling dates (Figure 5B). Similarly, mice immunized with the GFP-F2 or GFP-F3 did not show any presence of anti-GFP antibodies. Conversely. mice immunized with GFP-F had low levels of anti-GFP antibodies at day 28 post-vaccination. Notably, there was no significant increase in antibody titers in this group of mice following the booster shots administered at day 28 and day 50 post-vaccination. Together, the results demonstrate that the three LNP formulations employed for encapsulating the model mRNA transcripts (GFP) in this study were not effective in inducing a robust immune response in mice.

Given the lack of a robust immune response in mice, an immunogenicity study in pigs was not conducted.

3. Additional work extended beyond the scope outlined in the original proposal

3a. Generation and *in vitro* characterization of the lipid nanoparticles encapsulating the DNA plasmid encoding the ASFV p54 gene.

While conducting this project, we simultaneously pursued a distinct line of research focused on the development of lipid nanoparticles as a delivery system for DNA plasmids in pigs. It is important to note that this research was funded by different funding sources and did not involve the National Pork Board (NPB) in any capacity.

We employed the hemagglutinin (HA) antigen of the H3N2 influenza A virus of swine (IAV-S) as the model vaccine immunogen to evaluate the immunogenicity and protective efficacy of our lipid nanoparticle-based DNA vaccine (LNP-

DNA) in pigs. The use of IAV-S and its HA antigen is particularly advantageous in studying vaccine effectiveness in pigs, as the viral HA antigen alone has been shown to confer complete protection (26) and the hemagglutinin inhibition antibody titers are a reliable immune correlate to predict vaccine-induce protection. Additionally, we have developed a swine model to assess the vaccine's protective efficacy. Our study demonstrated that a single-dose intramuscular administration of the LNP encapsulating the DNA plasmid encoding the HA antigen, referred to as LNP-H3 DNA, resulted in high HI antibody titers within 7-14 days after vaccination. Notably, pigs vaccinated with the LNP-H3 DNA vaccine displayed complete protection against challenge infection with the homologous H3N2 strains. These findings indicate the potential of LNP-DNA as an effective approach for developing vaccines for swine.

Building on the success of the LNP-DNA vaccine utilizing the influenza virus HA gene in pigs, we sought to explore the feasibility of employing the same LNP-DNA vaccine approach for delivering African Swine Fever Virus (ASFV) antigens. To investigate this, we utilized the LNP formulation initially employed for encapsulating the HA gene and adapted it to encapsulate the ASFV p54 gene.

The physical characteristics of the p54-LNP are presented in Figure 6. The average diameter of the p54-LNP was about 65 nm (Figure 6A), significantly smaller than the LNP-mRNA (GFP-F1, GFP-F2, and GFP-F3) described above (Figure 3A). The polydispersity index (PDI) of the p54-LNP was around 0.1, indicating a monodispersed particle distribution (Figure 6A). The mean zeta potential of the p54-LNP was +12 mV (Figure 6B) and the mean encapsulation efficiency of p54-LNP was around 90% (Figure 6C).

To evaluate the transfection efficiency, 0.5 μg of the p54-LNP was directly added into the medium of HEK-293T cells in a 24-well plate. The p54 DNA plasmid complexed with polyethyleneimine (PEI-DNA), a transfectant commonly used for DNA plasmid transfection, was used as a positive control. At 48 hr post-transfection, the cells were fixed, and an indirect immunofluorescence assay was performed to detect p54-expressing cells. As expected, approximately 90% of cells transfected with p54-PEI were stained positive for p54 by an IFA (Figure 6D). Similarly, many p54-positive cells were observed in the well transfected with p54-LNP. Western blotting was utilized to further evaluate the protein expression. Notably, the intensity of the p54 band on the western blot membrane was comparable between cells transfected with p54-LNP and those transfected with p54-PEI (Figure 6E). Collectively, these results demonstrate the efficiency of the lipid nanoparticle formulation employed in this study for delivering the p54 DNA plasmid to HEK-293T cells.

3b. Assessment of the Immunogenicity of lipid nanoparticles encapsulating ASFV p54 gene DNA plasmid in pigs.

Two immunization experiments were conducted in 4-week-old pigs to evaluate the immunogenicity of the p54-LNP vaccine. In the first experiment, pigs in groups 1 and 2 were administered intramuscularly with 100 μg and 500 μg of the p54-LNP, respectively, while group 3 received a PBS injection to serve as a negative control (Figure 7A). The luciferase immunoprecipitation system (LIPS) assay was used to measure anti-p54 antibody development (25). Anti-p54 antibodies were not detected in the PBS-control group or in pigs immunized with 100 μg of the p54-LNP. However, anti-p54 antibodies were detected in two out of three pigs vaccinated with 500 μg of p54-LNP starting from day 19 post-vaccination (Figure 7C).

In the second experiment, groups 3 and 4 were intramuscularly injected with 100 μg and 500 μg of the naked p54 plasmid, respectively, while group 6 received a PBS injection to serve as a negative control. Once again, the LIPS assay was employed to measure anti-p54 antibody development. The results indicated that anti-p54 antibodies were not detected in pigs injected with PBS or with the naked p54 DNA plasmid at any time point post-vaccination Figure 7D).

Discussion

mRNA vaccine technology is an attractive platform for subunit vaccine development. However, mRNA molecules are extremely sensitive to degradation. Additionally, naked mRNA molecules do not enter the animal cells effectively, leading to limited protein expression. LNPs have proven effective nanocarriers that can be utilized for the delivery of various nucleic acids, including siRNA, microRNA, mRNA, and DNA (27). LNPs function as a capsule that protects the encapsulated nucleic acids from degradation and facilitate attachment and internalization of the nucleic acid cargo into target cells. LNPs typically consist of four types of lipids: a cationic lipid, a phospholipid, cholesterol, and a polyethylene glycol (PEG)-conjugated lipid (17). The types and molar ratios of lipids used to formulate LNPs influence the uptake and

endosomal releases of the encapsulated nucleic acid cargo, and consequently the expression levels of the encapsulated genes.

Using the GFP mRNA transcript as a model antigen, we generated three different LNP formulations for the encapsulation of GFP mRNA molecules. However, none of these formulations demonstrated effective transfection of GFP mRNA *in vitro* using HEK-293T cells. Subsequently, when evaluated in mice, only LNP formulation 1 (GFP-F1) showed a modest induction of antibody responses. These results suggest that the inefficient transfection efficiency and low immunogenicity observed with the LNP-mRNA vaccines may be attributed to the specific lipid types and formulation methods employed.

During the course of this project, we also pursue a distinct line of research aiming toward developing lipid nanoparticles as a delivery system for DNA plasmids in pigs. We were able to optimize several LNP formulations for the effective encapsulation of the HA gene of swine influenza viruses. Notably, these LNP-DNA formulations demonstrated high transfection efficiency in HEK-293T cells. Importantly, when administered to pigs, the LNP-DNA vaccines elicited robust immune responses and provided complete protection against challenge infection with the corresponding influenza virus strain. These findings highlight the promising potential of LNP-DNA as an effective platform for swine vaccine development.

By employing the LNP formulation optimized for delivering the influenza virus HA gene, we successfully encapsulated the DNA plasmid encoding the ASFV p54. Similar to the LNP-DNA based on the influenza HA gene, the LNP-DNA based on ASFV p54 also exhibited high transfection efficiency in HEK-293T cells. Moreover, when administer to pigs, the p54-LNP vaccine p54-LNP induced a substantial antibody response, detectable from day 19 post-vaccination. These results demonstrate the potential of LNP-DNA as a reliable delivery platform for administering vaccine-candidate genes to pigs.

Various viruses such as SARS-CoV2, influenza virus, and porcine circoviruses, pestiviruses use a single viral protein for attachment to cellular receptors to facilitate virus entry into target cells. Consequently, subunit vaccines containing a single viral attachment protein are sufficient for inducing complete protection. However, in the case of ASFV, the viral proteins responsible for receptor binding and the cellular receptors involved in ASFV entry remain unknown. Consequently, the specific protective antigens of ASFV are yet to be identified. It is highly likely that subunit ASFV vaccines require a combination of multiple viral antigens to be effective (15). In this project, we have demonstrated the efficacy of LNP-DNA in eliciting antibody responses against the p54 antigen. Our future studies will focus on identifying potential protective antigens of ASFV and optimizing LNP formulations for the efficient encapsulation of multiple ASFV genes.

Figures

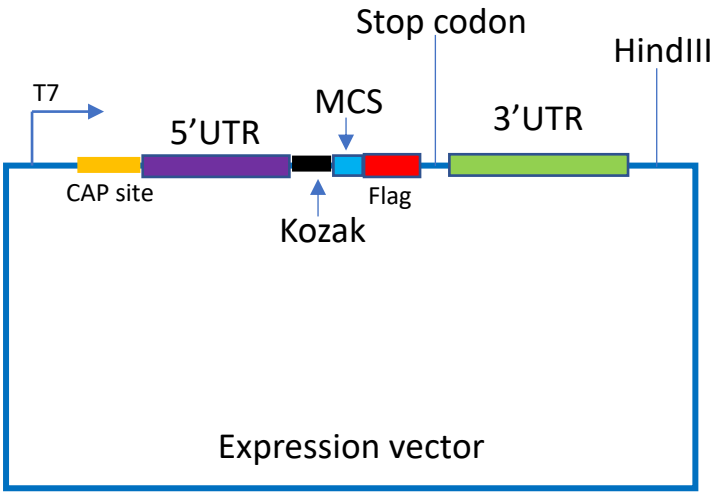


Figure 1. Schematic representation of the expression vector used to clone the ASFV antigens to produce mRNA transcripts.

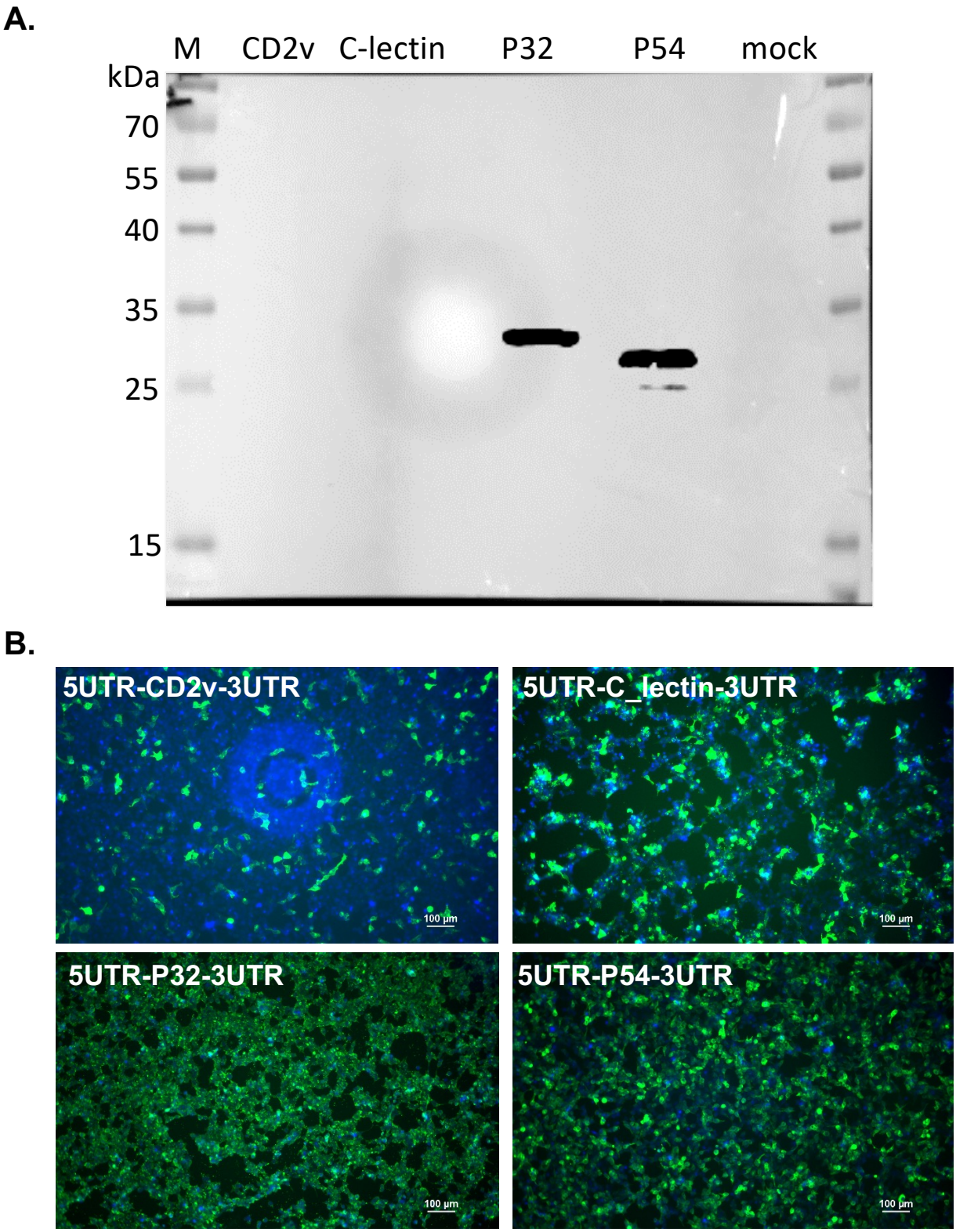


Figure 2. Assessment of the expression levels of ASFV mRNA transcripts in HEK-293T cells. The cells were transfected with the indicated mRNA transcripts using the TransIT®-mRNA according to the manufacturer's instruction. Protein expression was analyzed by Western blotting (A) and by indirect immunofluorescence assay (B).

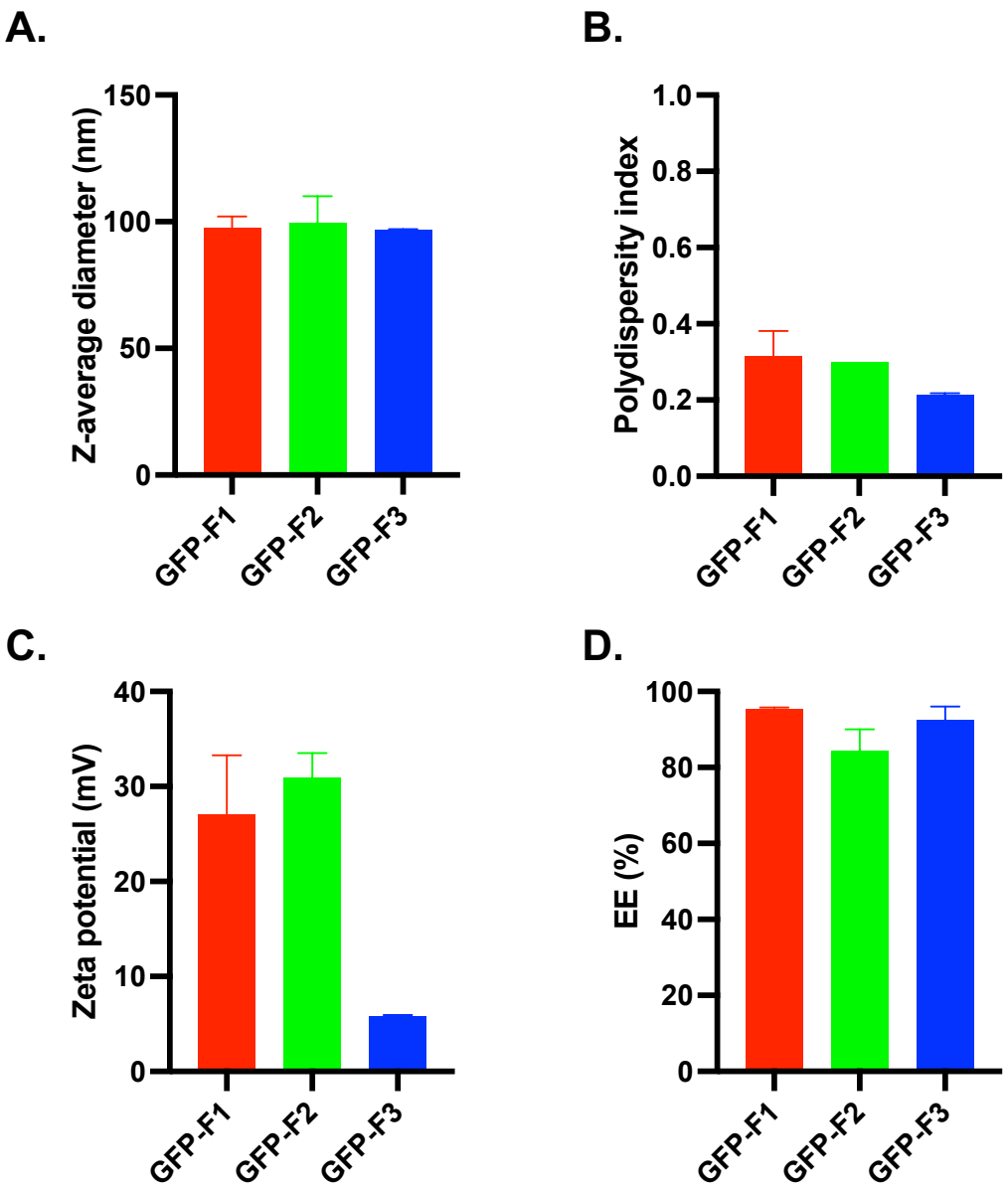


Figure 3. Physical characterization of the LNP encapsulating GFP mRNA. (A) Hydrodynamic size, (B) polydispersity index, and (C) zeta potential measured by using the Zetasizer ZS instrument (Malvern Panalytical). (D) Encapsulation efficiency (EE%) was measured by using the quantified using Quant-iT™ Ribogreen Assay Kit.

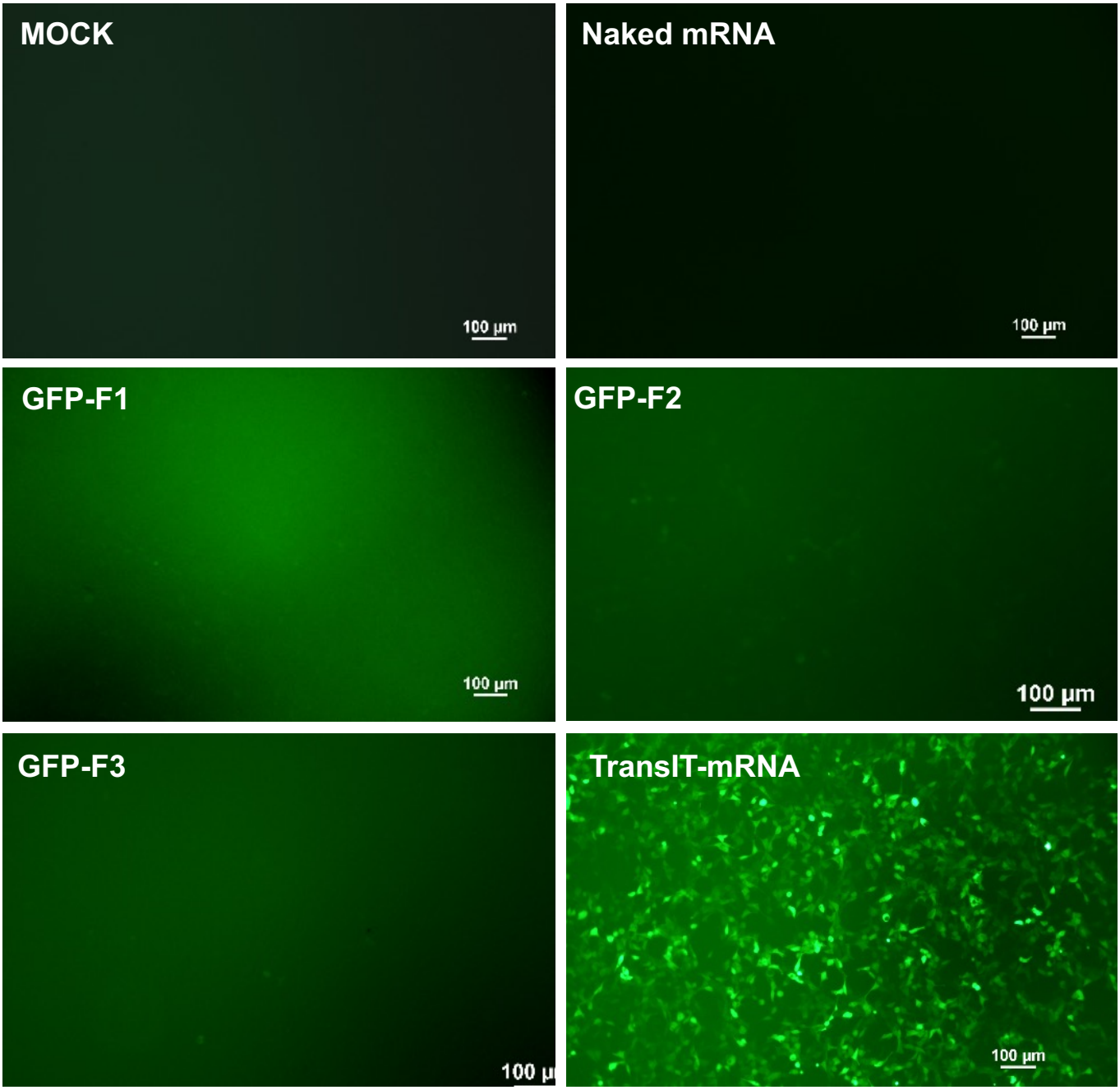


Figure 4. Transfection efficiency in the LNPs encapsulating GFP mRNA transcripts in HEK-293T cells. GFP mRNA transcripts were encapsulated using three different LNP formulations (designated as GPF-F1, GFP-F2, and GFP-F3). 500 ng of each GFP-LNP preparation was added directly to the culture medium of HEK-293T cells in a 24-well plate. An equal amount of naked mRNA or mRNA mixed with the commercial transfectant TransIT®-mRNA (Mirus Bio) was added to separate wells containing HEK-293T cells to serve as controls. The fluorescent signals were observed at 24 hrs post-transfection.

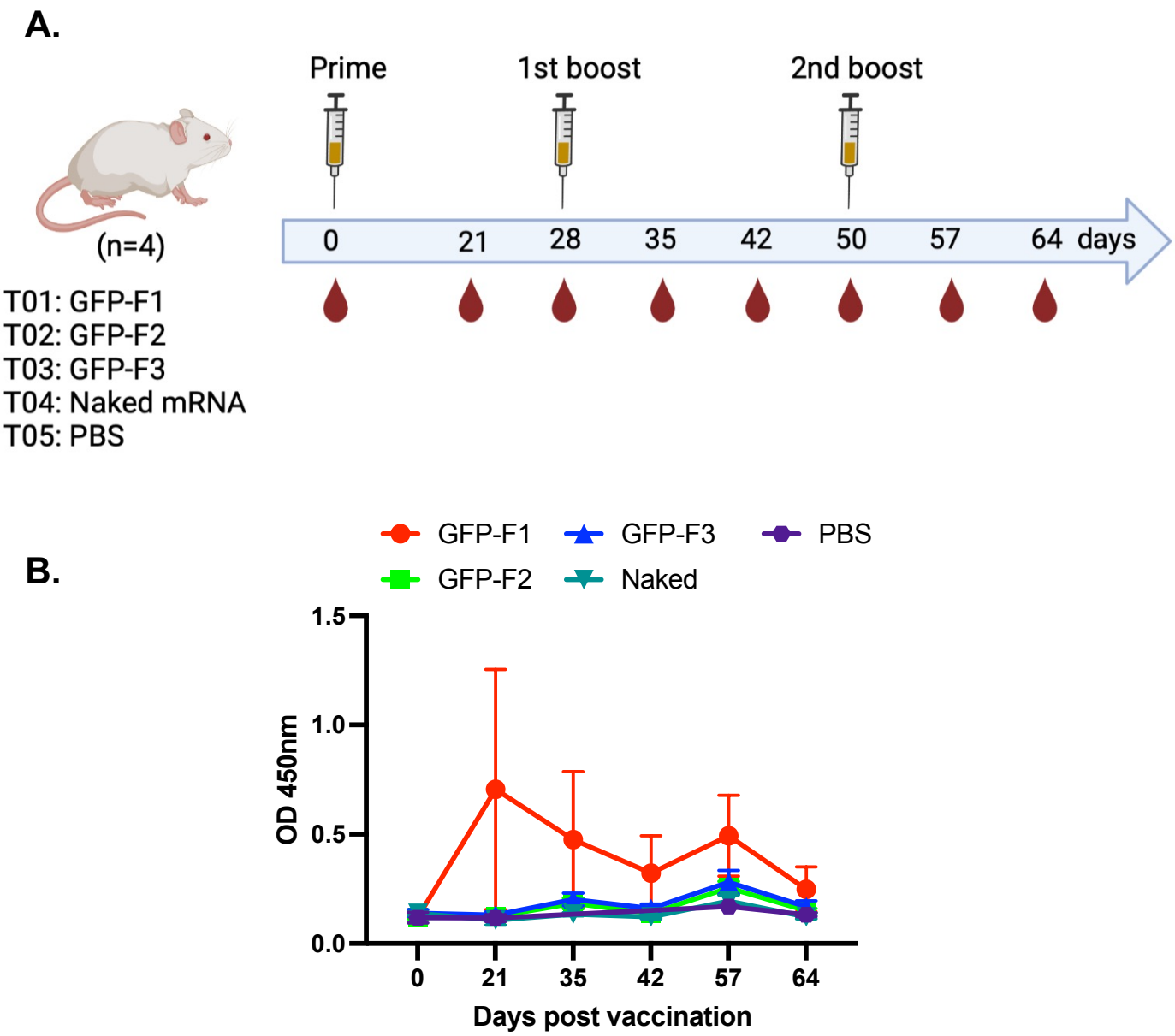


Figure 5. Assessment of immune response in mice following immunization with the LNPs encapsulating GFP mRNA transcripts. (A) Experimental design. (B) Anti-GFP antibodies as measured by an indirect ELISA.

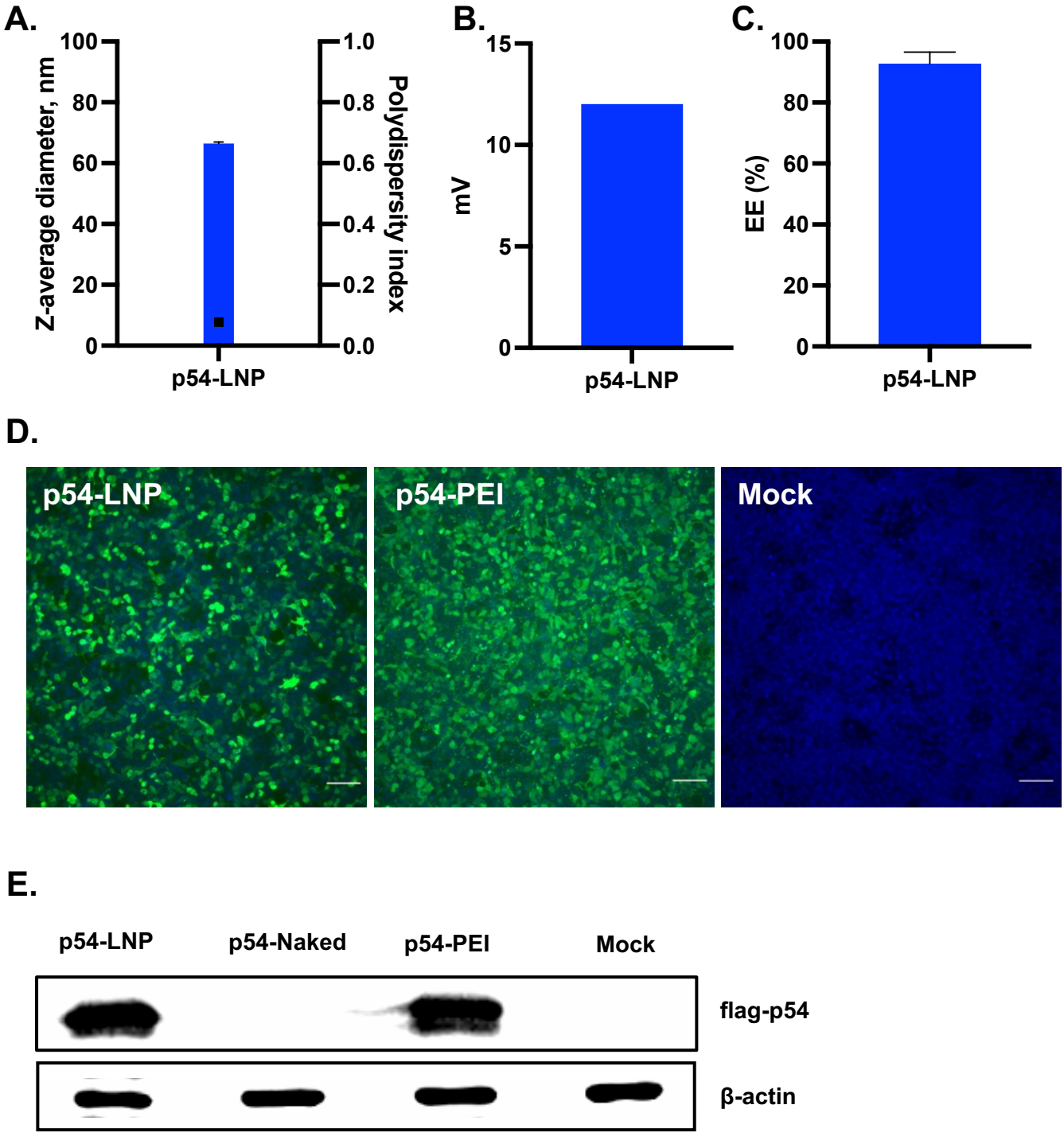
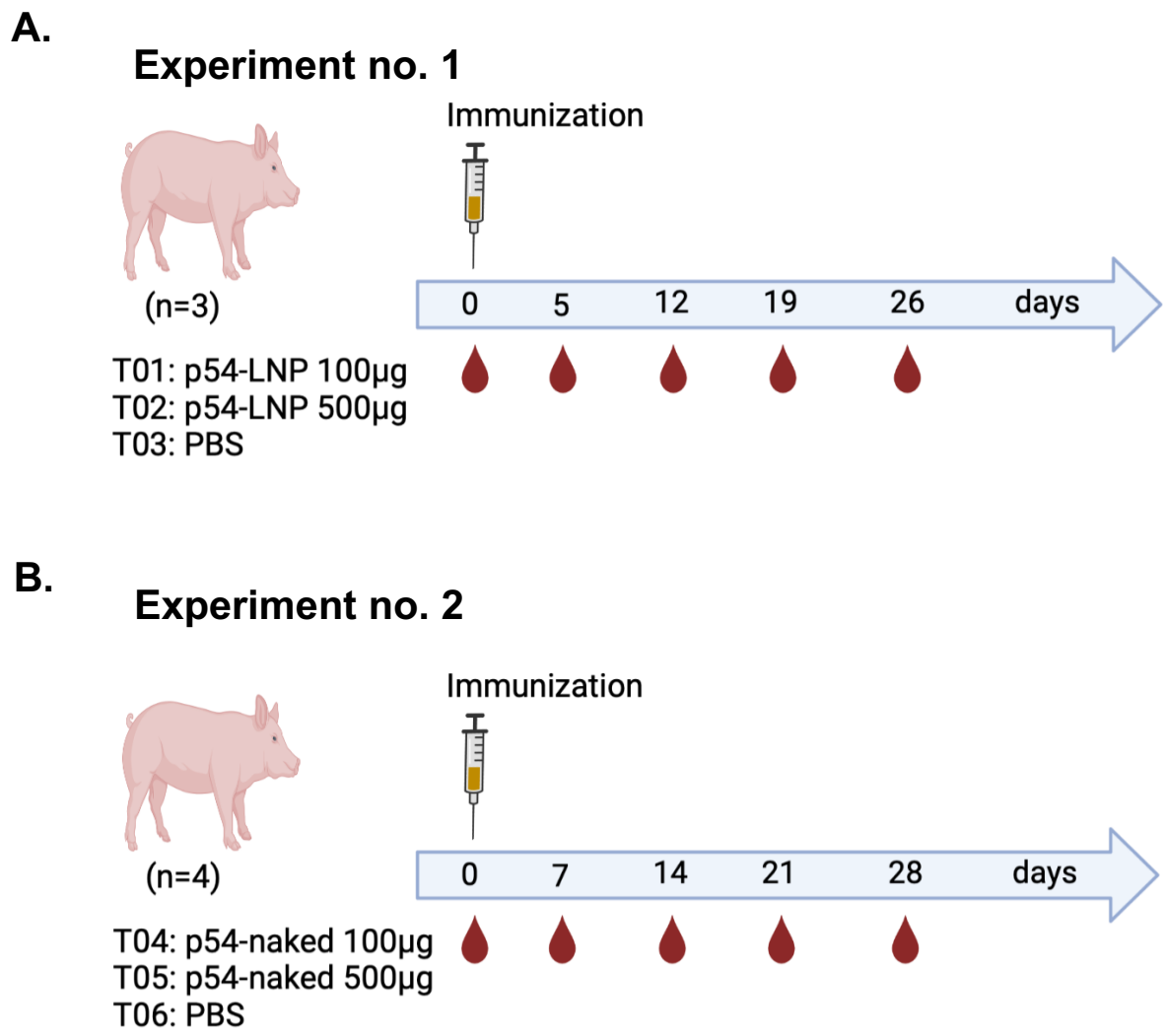


Figure 6. Physical characterizations of the LNP encapsulating the DNA plasmid encoding ASFV p54 gene. (A) Particle size and polydispersity index. (B) Zeta potential. (C) Encapsulation efficiency (EE%). (D&E) Transfection efficiency in HEK-293T cells as measured by indirect immunofluorescent assay (D) and by western blotting (E).



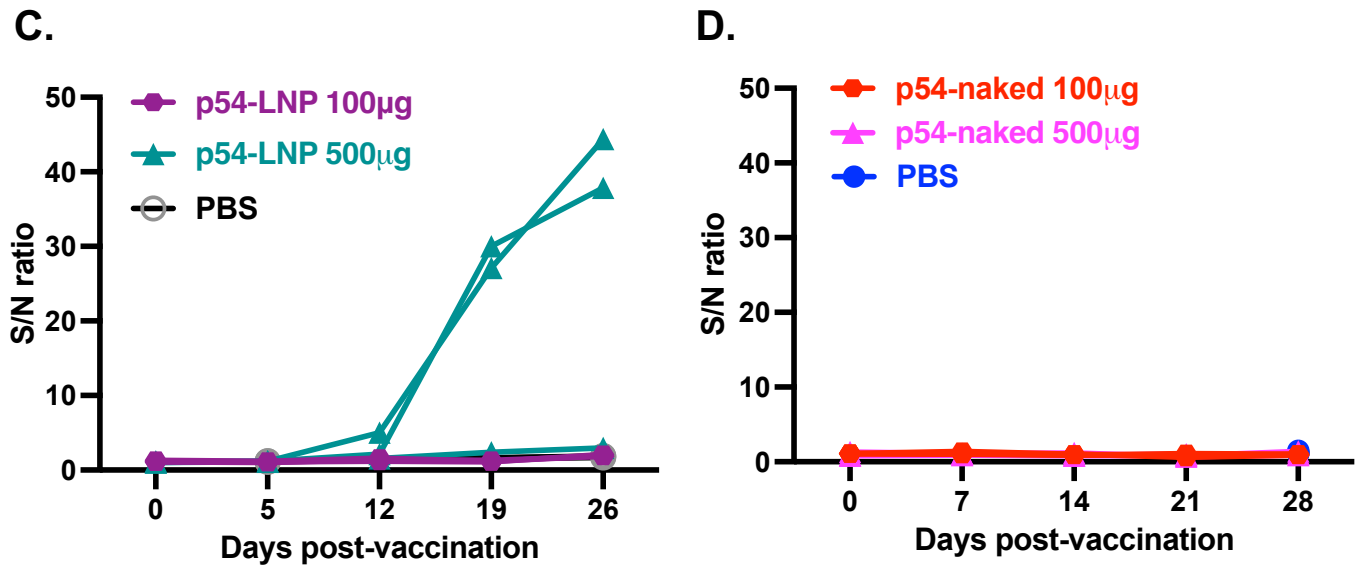


Figure 7. Assessment of immune response in pigs followed immunization with naked or LNP encapsulated p54 DNA plasmid. (A & B) Experimental design. (C & D) Anti-p54 antibodies as measured by the LIPS assay. Data are expressed as the sample to negative (S/N) ratio.

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