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14. ABSTRACT

The overall goal of this proposal is to develop fully synthetic vaccines against respiratory infections using novel nanotechnology platforms based on safe and degradable adjuvant polymer systems that enhance antigen presentation and stimulate immunity. The proposal focuses on achieving the following specific technical objectives: 1) Develop molecular methods for intervention strategies employing novel synthetic nanovaccine platforms encapsulating DNA and protein/peptide antigens that elicit immune response against influenza H5N1; 2) Test the efficacy of nanovaccines-based intervention regimens against influenza H5N1 in animal models. 3) Adopt intervention strategies using most promising nanovaccine platforms to other respiratory infections, such as pulmonary F. tularemia, and test the nanovaccines efficacy in animal models (unfunded option). Focusing on respiratory infections, the leading cause of outpatient illness and a major cause of infectious disease hospitalization in U.S. military personnel, has the potential to develop effective, safe and affordable synthetic vaccines.

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INTRODUCTION

The overall goal of this proposal is to develop fully synthetic vaccines against respiratory infections using novel nanotechnology platforms based on safe and degradable adjuvant polymer systems that enhance antigen presentation and stimulate immunity. Three novel versatile technology platforms will be evaluated: a) biodegradable polyanhydride nanospheres (BPN) carrying a polypeptide/protein antigen (Platform A); b) polymeric micelles of Pluronic block copolymer as DNA vaccine adjuvant (Platform B); and c) block ionomer complexes (BIC) for targeted delivery of DNA (or protein) antigen to the antigen presenting cells (APCs) (Platform C). The central hypothesis is that these polymeric nanoscale delivery systems can provide versatile platforms for development of effective, safe, and cost-efficient vaccines. The materials used in these platforms demonstrated immunomodulatory capabilities and can be targeted to specific populations of immune cells to elicit most efficient immune response. The best technology or their combination will be determined for future development.

The proposal is focusing on a chieving the following specific technical objectives: 1) Develop molecular methods for intervention strategies employing novel synthetic nanovaccine platforms encapsulating DNA and protein/peptide antigens that elicit immune response against influenza H5N1; 2) Test the efficacy of nanovaccines-based intervention regimens against influenza H5N1 in animal models. 3) Adopt intervention strategies using most promising nanovaccine platforms to other respiratory infections, such as pulmonary *F. tularemia*, and test the nanovaccines efficacy in animal models (unfunded option).

BODY OF REPORT

Platform A.

In support of one of the milestone related to synthesis and purification of H5 hemagglutinin (HA) antigen, we expressed the H5 HA0 ectodomain by baculovirus. The following specific steps were carried out and the results obtained are discussed.

<u>Construction of H5 expression vectors (bacmids)</u>

The HA0 ectodomain of HA gene from H5N1 influenza virus (A/Whooper Swan/244/Mongolia/ 05 (clade 2.2)) was cloned and expressed using a baculovirus expression system. A his-tag was added to the C-terminus of the H5 ectodomain to facilitate the purification procedure. Two forms of H5 proteins were expressed. One is a monomer form, which is referred to as H5-M. The other is a trimer form, which was fused with a GCN4pII trimerization module to stabilize the native trimeric structure of H5 protein. The trimer is referred to as H5-T.

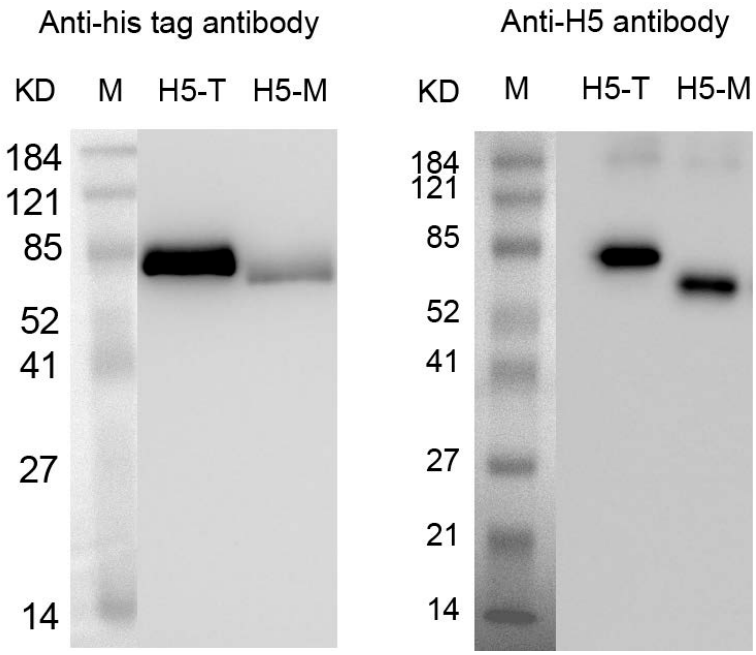


Fig. 1. Western blots of the recombinant H5.

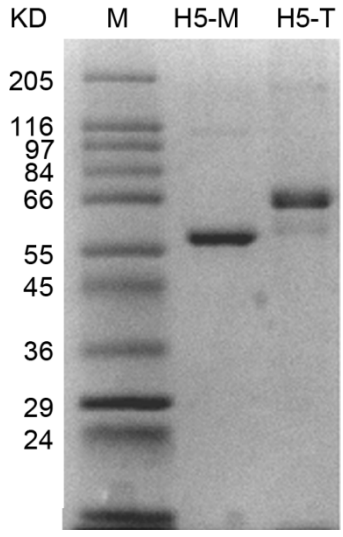


Fig. 2. SDS-PAGE of the purified recombinant H5 proteins.

Making recombinant baculovirus.

Recombinant baculoviruses containing the H5 genes were generated by transfecting insect SF21 cells with bacmid DNA. The baculovirus stocks were amplified for three rounds and the p3 stocks of the recombinant baculovirus were titered and used to produce the H5 proteins.

Expression and purification of H5 proteins

The H5 monomer protein (H5-M) and trimer protein (H5-T) were expressed by infecting the SF21 cell with H5-M or H5-T baculovirus stocks. The supernatant was collected 96 h after infection, and dialyzed to get rid of the EDTA from medium. HA proteins were affinity purified by incubating supernatant overnight at 4^0C with nickel-agarose beads. Non-

specific nickel-bead binding was eliminated by washing with 25 mM imidazole and recombinant protein eluted from the beads with a 300 mM imidazole solution.

Analysis of the H5 proteins

Purified proteins were separated on a 4-10% SDS-PAGE and analyzed by Western blot using anti-H5 (Vietnam 03/04) or anti-6xHis monoclonal antibody. The concentration of the purified protein was measure by the Bradford assay.

The results show that both the expressed proteins (H5-M and H5-T) were recognized by anti-H5 (Vietnam 03/04) antibody and anti-his tag antibody (Figure 1). The purity of the Ni-beads purified protein was >90% (Figure 2). The protein yield for the H5-M monomer is 2 mg/L, and the yield for the H5-T trimer is 5mg/L. Current efforts are focused on expression and processing of 1 L medium per week, which will result in a production rate of 5 mg H5-T protein/week and 2 mg H5-M protein/week.

Milestone #2

In support of the milestone related to the screening of BPNs for immune cell activations, we synthesized libraries of biodegradable polyanhydride nanoparticles of the CPH:SA system as described previously and screened them at high throughput for interactions with immune cells, specifically dendritic cells (DCs). The following specific steps were carried out:

Culture and stimulation of DCs

Bone marrow derived dendritic cells (BMDCs) were prepared from bone marrow cells isolated from the femurs, tibias, humerus' and iliums of C57BL/6 mice (obtained from Harlan Sprague Dawley and housed within the ISU Laboratory Animal Resource Facility, Ames, IA) as previously described (1). Following mouse euthanization and bone excision, attached tissue and muscle were dissected away. The ends of the bone were cut and the marrow was flushed out using a syringe fitted with a 30 gauge needle. Each bone as flushed with 5 mL of RPMI medium containing 1% pen/strep. Large particulates were allowed to settle and then removed. After centrifugation, the cells were resuspended in DC medium (RPMI containing 1% L-glutamine, 1% penicillin-streptomycin solution, 2% HEPES, 0.5% gentamicin, 0.1% β-mercaptoethanol, and 10% heat inactivated fetal bovine serum (FBS) supplemented with GM-CSF (10 ng/mL). The cells were then placed in T75 cell culture flasks in 10 mL of DC medium containing 10

ng/mL GMCSF and incubated at 37 ºC under 5% CO_2 atmosphere. On day 3, 10 mL of fresh DC medium with 10 ng/mL GMCSF was added. On day 6, 10 mL of the culture medium was harvested and placed in a 15 mL centrifuge tube. After centrifugation, the supernatant was decanted and the cells were resuspended into 10 mL of fresh DC medium containing 10 ng/mL GMCSF and re-inoculated into the original flask. On day 8, DCs were removed from the flasks, counted, resuspended in fresh DC medium, and transferred to 24-well plates (1×10^6 cells/well). On day 9, a portion of the cells were stained for CD11c to determine the percentage of DCs. On the same day the remaining DCs were incubated with the different stimulation treatments (films or nanospheres). The DCs were treated with CPH:SA copolymer films and nanospheres of the following chemistries: poly(SA), 13:87 CPH:SA, 25:75 CPH:SA, 37:63 CPH:SA, 50:50 CPH:SA, and 63:37 CPH:SA. Polyanhydride nanospheres were incubated at a concentration of 0.125 mg/mL (2 cm^2 cell growth area). Non-stimulated (NS) DCs and LPS (200 ng/mL) treated DCs were used as negative and positive controls, respectively. After the addition of the stimulants, the DC cultures were incubated for an additional 48 h (37°C, 5% CO_2) at which time the supernatants and DCs were harvested for cytokine production and cell surface marker expression, respectively. Cell viability was assessed by measuring trypan blue exclusion using a hemocytometer and light microscope.

Cell surface marker expression

The expression of cell surface markers including CD11c, CD86, CD40, MHC II, and CD209 were assessed after the 48 h incubation period of the DCs with the stimulation treatments. The adherent DCs were harvested from the culture dishes with vigorous pipetting, placed in polystyrene tubes (BD FALCON™, Franklin Lakes, NJ), centrifuged (250 rcf, 10 min, 4°C), and resuspended in Fc blocking solution consisting of PBS buffer with 0.1% anti-CD16/CD32, 0.1% unlabeled hamster IgG, 1% rat IgG, 1% mouse serum, 0.1% sodium azide, and 1% FBS. After blocking, the DCs were stained and fixed for evaluation of cell surface markers using monoclonal antibodies against CD11c, MHCII, CD86, CD40, and CD209. The samples were analyzed using a Becton-Dickinson FACSCanto flow cytometer (San Jose, CA) and FlowJo (TreeStar Inc, Ashland, OR).

Cytokine secretion

Following incubation of the DCs with the stimulation treatments for 48 h, 200 µL of the supernatants were collected and assayed for the presence of IL-6, IL-10, and IL-12-p40 using the Luminex® Multiplex assay system (Austin, TX).

Statistical Analysis

All data was statistically analyzed by using a one-way model ANOVA with the statistical software JMP® 7 (Cary, NC). Comparisons between treatments were made with Tukey's HSD to determine statistical significance, and p-values of less than or equal to 0.05 were considered significant. All data was log transformed for use of the one-way model ANOVA.

To assess activation of $CD11c^+$ DCs by the CPH:SA nanoparticle libraries, flow cytometry was used to measure levels of cell surface expression of co-stimulatory molecules CD86 and CD40, integrin CD209, and major histocompatibility complex molecule MHC II. Supernatants were also collected and analyzed for cytokine production, including IL-10, IL-12p40, and IL-6. LPS was used as a positive control and a non-stimulated group with medium only acted as a negative control. Prior to use in cell stimulation assays, the BMDC cultures were shown to be >90% positive for CD11c (data not shown).

The results from the analysis of cell surface marker expression following the 48 h

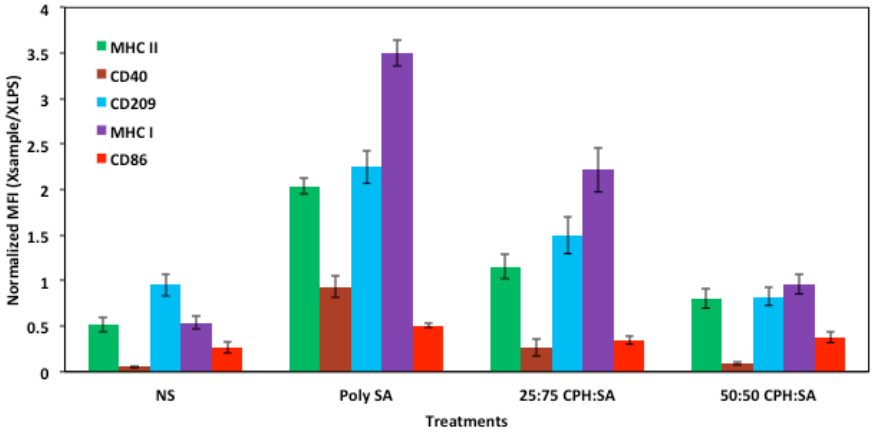


Fig. 3. Analysis of DC activation markers upon treatment with nanoparticles by flow cytometry. Data is presented as MFI of sample normalized by that of LPS (positive control). Data is representative of a minimum of 3 replicates per stimulation group. Errors bars indicate standard error.

stimulation period suggest differential regulation of the selected markers depending on the polymer chemistry and the relative hydrophobicity of each formulation as shown in Figure 3.

The SA-rich (least hydrophobic) chemistries demonstrated the ability to best promote the expression of MHC I, MHC II, CD86, CD40, and CD209. The chemistry-dependent up-regulation of the cell surface markers in comparison to the non-stimulated control is clearly observed with SA-rich nanoparticle compositions. Enhanced expression of these markers clearly correlates with the amount of SA in the nanoparticles, exhibiting a maximum for the poly(SA) nanoparticles. In addition to the MFI measurements, measurements of cells as percent positive for each of the specific markers was performed, and the results were consistent with the observed MFI for each specific cell surface marker (data not shown).

Supernatants were collected and assessed for cytokine concentrations. The results (Figure 4) suggest differential cytokine production (enhancement of IL-6 and regulation of IL-12p40) in response to the composition of CPH:SA nanoparticles. However, the trend for cytokine production is counter to the trend observed with the cell surface marker expression. The more hydrophobic or CPH-rich chemistries appear to better promote production of IL-6 and IL-12p40 as shown in Figure 4. IL-10 was not produced by any of the treatment groups, so the data is not included in the figures or histograms.

To synthesize HA-loaded polyanhydride nanoparticles, the following specific steps were carried out:

Hemagglutinin-loaded Nanoparticle Synthesis

Polyanhydride nanoparticles loaded with two percent HA protein were synthesized with varying molar ratios of sebacic acid (SA), 1,6-bis(*p*-carboxyphenoxy)hexane (CPH), and 1,8-bis(*p*-carboxyphenoxy)-3,6-dioxaoctance (CPTEG). Three copolymers were used for particle fabrication: 20:80 CPH:SA, 20:80 CPTEG:CPH, and 50:50 CPTEG:CPH. Briefly, the polymer was dissolved in chilled methylene chloride at a concentration of 20 mg/mL with 2% HA protein. The solution was sonicated for approximately 30 seconds, and then poured into pentane chilled with a liquid nitrogen bath at a ratio of 1:200 solvent: non-solvent. Protein-loaded particles were then collected via vacuum filtration and characterized by scanning electron microscopy. As shown in Figure 5, scanning electron microscopy images show the size and morphology of HA-loaded nanoparticles to be consistent with previous work in polyanhydride nanoparticles with other proteins.

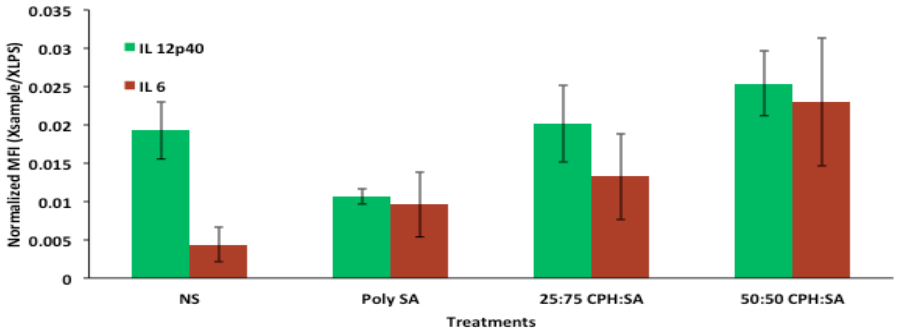


Fig. 4. Analysis of cytokine secretion upon treatment with nanoparticles. Data is presented as concentration of sample normalized by that of LPS (positive control). Data is representative of a minimum of 3 replicates per stimulation group. Errors bars indicate standard error.

Stability and Release of HA from Polyanhydride Nanoparticles

To study the stability and release of HA from polyanhydride nanoparticles, a new release method is being designed. In previous work, protein release via the erosion of polyanhydride particles was observed in 0.5 mL microcentrifuge tubes (2). Protein-loaded particles were incubated in phosphate buffered saline (PBS) with 0.01% w/v sodium azide at 37°C. Samples of the released protein were then taken periodically by completely removing solution and replacing with fresh PBS.

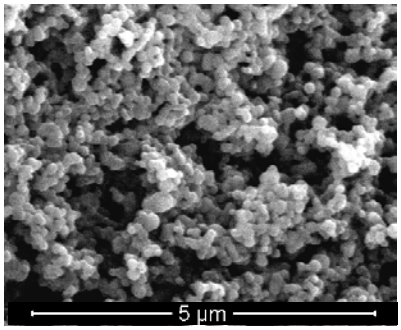


Fig. 5: Polyanhydride nanoparticles loaded with 2% HA.

One shortcoming of this microcentrifuge tube method involves the build-up of polymer degradation products within the buffer between sampling. These degradation products are acidic and the decrease in pH can cause instabilities within many proteins. One solution is to incubate the particles in dialysis cassettes, rather than centrifuge tubes. These dialysis cassettes allow the removal of acidic degradation products and the maintenance of a balanced pH, better mimicking the body's

constant motion of fluid *in vivo*. However, particles within a dialysis cassette tend to sink to the bottom, despite being well dispersed initially. Degradation products as well as protein can become trapped among the settled particles still leading to acidic microenvironments.

We have designed a new release method using 1 mL microcentrifuge tubes with dialysis membranes incorporated into specially designed caps (GE Healthcare, Piscataway, NJ). In our current work, the release from HA-loaded particles takes place in 250 µL of PBS with 0.01% w/v sodium azide loaded into the dialysis tube. This tube is then placed in a 50 mL conical centrifuge tube with additional buffer and then placed on a rocker to flip end over end. This motion allows the constant dispersion of particles as well as allowing dialysis to take place, best mimicking the *in vivo* environment.

Protein Quantification

Currently, a colorimetric micro bicinchonic acid (microBCA) assay (Pierce, Rockford, IL) is being used to identify the concentration of HA protein released from polyanhydride particles. Although many researchers use the absorbance of bovine serum albumin (BSA) or ovalbumin (OVA) to create standard curves, the most accurate results are obtained by using the protein of interest, HA, to create standard curves. However, the HA standard curve has a relatively low slope, which can create misleading results as small changes in the absorbance measured can correlate to large changes in protein concentration. Therefore, an appropriate assay for HA quantification is being optimized through the trials of several other colorimetric techniques (i.e., Bradford assay) as well as fluorescence-based techniques.

Enzyme-linked Immunosorbant Assay (ELISA)

An enzyme-linked immunosorbant assay (ELISA) has been optimized to study the antigenicity of released HA protein. In this case, if the stability of the protein has been preserved then the ability of anti-HA antibodies to bind the protein should be preserved. Briefly, the optimized conditions begin with coating a high-binding 96-well plate with 100 µL/well of released HA protein diluted to 5 µg/mL and 0.5 µg/mL in phosphate buffer saline (PBS). After incubating the plate at 4°C overnight, the wells are emptied and filled with 300 µL/well blocking solution of PBS with 0.05% Tween 20 (PBS-T) and 1% BSA for 2 h at room temperature. The

plate is then washed with PBS-T and incubated with 100 µL/well anti-HA (BEI Resources NR-2731) diluted 1:1000 in PBS-T at 4°C overnight. The following day the wells are washed with PBS-T and incubated with 100 µL/well secondary antibody, alkaline phosphatase conjugated anti-mouse IgG(H+L) (Jackson ImmunoResearch, West Grove, PA), diluted 1:1000 in PBS-T for 2 h at room temperature. Finally, following an additional wash step with PBS-T, 1 mg/mL phosphate substrate (Sigma) is prepared in a substrate buffer of sodium carbonate and magnesium chloride. After adding 100 µL/well of the substrate to each well, the developing plate is read by a microplate spectrophotometer at 405 nm.

Western Blot

We are currently optimizing western blot analysis by performing sodium dodecyl sulfate-polyacrylamide gel electrophoresis for 1 h at 150V to separate the released protein by molecular weight. The protein is transferred from the gel to a polyvinylidene fluoride (PVDF) membrane for 1.5 h at 100V. Following transfer, the PVDF membrane is incubated in blocking solution, tris saline with tween 20 (TBS-T) with 5% milk, on a rocker overnight at 4°C. The next day, the membrane is rocked with 10 mL anti-HA (BEI Resources) diluted 1:1000 in blocking solution for 2 h at room temperature. After rinsing with TBS-T, the secondary HRP-conjugated antibody (Jackson ImmunoResearch) is diluted 1:100,0000 in TBS-T and rocked with the membrane for an additional 2 h. Finally, the membrane is washed an additional time with TBS-T and developed using an ECL Plus substrate (Pierce).

Preliminary dose titration studies with H5 HA antigen. The following specific steps were carried out :

Dose Titration

Full-length HA H5 protein was obtained from Boehringer Ingelheim Vetmedica to perform initial experiments. The protein was produced as a full-length protein and exists as monomers, dimers, and trimers. Using this protein we tested titrating dosages of the protein unadjuvanted or using traditional adjuvants via a parenteral route, subcutaneously, to examine the humoral response curve after a single dose. This was done to gain insight into the optimal dosage i.e. amount of protein that will be needed for designing a vaccine containing our novel polyanhydride nanoparticle platform against H5N1 influenza.

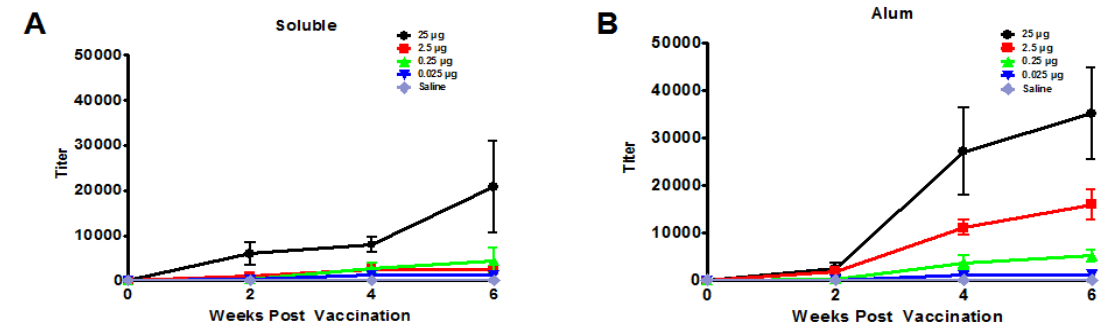


Fig. 6. Total anti-H5 serum antibody response of mice.

Antibody Avidity

The dosage titration experiment has also afforded us the opportunity to optimize and design more sophisticated measures of antibody avidity. Using principles adapted from van Gageldonk et al. (3) and Stenger et al. (4), full length H5 HA was conjugated to carboxylated magnetic beads (Bio-Rad Laboratories, Hercules, CA) according to manufacturer protocols at three different antigen to bead ratios (5, 10, and 12.5 µg/1.25 x 10^6 beads). Three dilutions of serum from the 2.5 µg HA adjuvanted with Alum group seen above, a saline vaccinated negative control, and of a H5 monoclonal antibody NR-2743 (BEI Resources) were incubated 1:1 with conjugated beads prepared from the ratios described previously so that there were 4000 beads per well in a 96 well Bio-Plex Pro Flat-Bottom plate (Bio-Rad). The serum/bead mixture was incubated in the dark for 2 hours at room temperature (RT). The beads were washed three times using a Bio-Plex Pro II magnetic bead washer (Bio-Rad). A biotinylated secondary mouse IgG (eBioscience San Diego, CA) was diluted to 5 µg/mL in buffer and 50 µL was added to the beads. The plate was incubated for 1 h at room temperature. The plate was washed three times and finally 50 µL of a 10 µg/mL Streptavidin-Phycoerythrin (eBioscience) solution was added. Plates were incubated at RT for 30 min, washed, and subsequently mean fluorescence intensities (MFIs) were captured using the Bio-Plex 200 system (Bio-Rad). No appreciable differences in MFIs were indicated between the three formulations of conjugated beads. T herefore we proceeded to adapt the antibody avidity protocol using 5 µg conjugated beads.

The preparation protocol and initial incubation steps remained the same from the above protocol described. After the initial antibody/bead incubation step a chaotropic reagent step is added to dissociate antigen/antibody interactions to measure the strength of those reactions. Urea dissociation was more consistent in the Stenger et al protocol so we optimized those conditions for our assay. Three different concentrations of urea were prepared (3, 6, and 9M) and a PBS control. After washing the beads, 50 µL of the urea solutions or control were added to the wells and incubated for 15 min at RT. The beads were washed and the subsequent secondary steps were performed and MFIs measured. 6M urea proved to be the optimal concentration of chaotropic reagent as it consistently resulted in a reduction of 30-70% MFI from the PBS control.

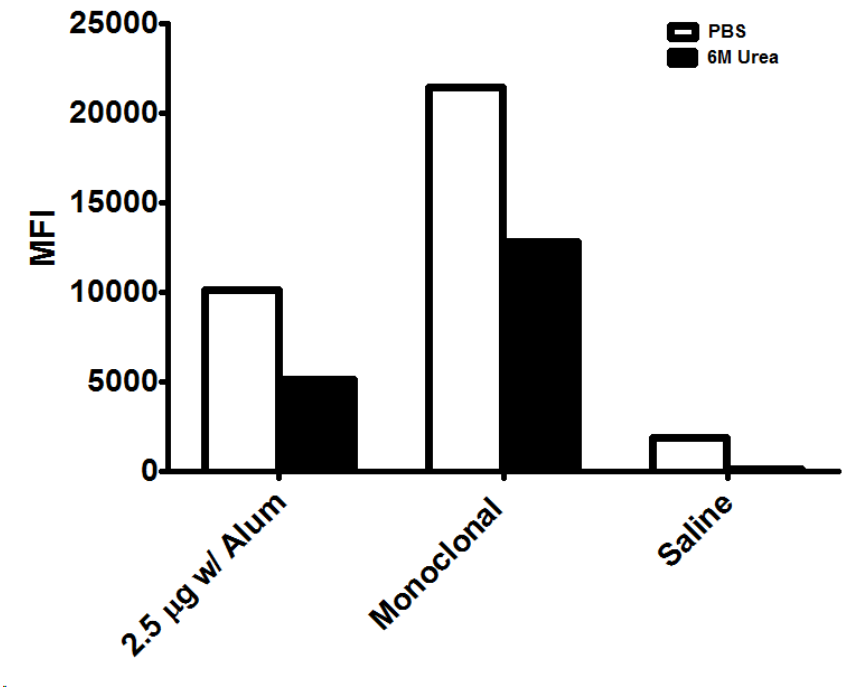


Fig. 7. MFIs from test sera and monoclonal antibody for avidity. Avidity Index reported as (MFI urea treated)/(MFI untreated) * 100%

Figure 6A shows the total anti-H5 serum antibody response of mice vaccinated with soluble protein subcutaneously over a six-week time frame. 25 µg soluble protein only doses elicited measurable titers from baseline levels. 2.5 µg and lower soluble doses remain at relatively baseline levels. When the full length HA protein is adsorbed to alum we are able to detect measurable serum immunoglobulin titer differences in the 25 µg and 2.5 µg groups (Fig. 6B). The antigen dosage titration has provided great insight into the dosage of protein to begin designing vaccine using the novel polyanhydride nanoparticle platform.

Figure 7 is representative histogram of the MFIs obtained from test serum and the monoclonal antibody used. An avidity index can then be calculated using the PBS control and urea treated MFI value where the MFI obtained in the presence of urea is divided by the MFI obtained in the absence of urea (PBS) and multiplied by 100%. The results indicate an avidity index of 50.70 for the 2.5 μg alum group, a 59.62 avidity index for the monoclonal antibody, and 4.71 for the saline negative control.

Current studies are examining dosage titrations experiments via the intranasal route with the newly produced stable H5/Mongolia/Whooper Swan/05 HA protein being produced via baculovirus expression system. Using the serum and bronchiole alveolar lavage (BAL) fluid obtained from those mice we will be able to adapt the multiplex immunoassays described above to measure serum and BAL IgA concentrations after intranasal vaccinations with our novel nanoparticle platform by using IgA specific secondary antibodies.

Platform B.

In support of the design of DNA plasmid encoding influenza protein H5N1 HA, the DNA sequence coding therapeutic gene H5HA has been successfully constructed into the pVAX1 backbone. In order to determine the Luciferase and H5HA expression *in vivo*, 10 μg of pVAX1-Luc (control plasmid) plasmid DNA formulated with/without 0.3% w/v P85 or 0.01% w/v SP1017 were administered i.m. in tibialis anterior muscles in BALB/c mice (5 mice/group). Injection formulations of DNA and DNA with pluronics were injected in left and right tibialis anterior muscles respectively. Mice were sacrificed on day 7 after i.m. injection. Tibialis anterior muscles were harvested, weighed, snap frozen in liquid N_2 and stored at -80°C for isolating mRNA to do RT-PCR for determining the transgene expression of H5HA and Luciferase gene and also the Luciferase assay. The following experiments were performed to determine the Luciferase and H5HA expression *in vivo.*

In order to determine the Luciferase expression *in vivo*, 10 μg of pVAX1-Luc (control plasmid) plasmid DNA formulated with/without 0.3% w/v P85 or 0.01% w/v SP1017 were administered i.m. in tibialis anterior muscles in BALB/c mice (5 mice/group). The animals were subjected to non-invasive bioluminescence detection using the Xenogen In Vivo Imaging System-IVIS 200 on day 4 and day 7 after i.m. administration (Fig. 8 and 9). 10 μg of naked

pVAX1-Luc plasmid DNA (pDNA) produced luciferase expression in skeletal muscles in all 5 mice on day 4 and all 5 mice expressed luciferase with higher intensity on day 7 in P85 group (Fig. 8.A and B). But Fig. 9A and 9B showed that 10 µg of naked pVAX1-Luc pDNA produced luciferase expression in 4 mice out of 5 in SP1017 group. pVAX1-Luc pDNA formulated with 0.3% w/v P85 produced higher luciferase expression in all 5 mice on day 4 and day 7 compared to that of naked pDNA (Fig. 8.A and B). However, plasmid DNA formulated with 0.01% w/v SP1017 produced luciferase expression in 4 mice among 5 mice on day 4 and all mice expressed higher luciferase intensity compared to that of naked pDNA on day 7 (Fig. 9A and B). These results are summarized in Table 1.

Table 1. *In vivo* transgene expression after a single *i.m.* administration of pDNA with/without Pluronics in BALB/c mice (5 mice/group).

	Respondent mice (no.)	
	Day 4	**Day 7**
P85 group		
pVAX1-Luc pDNA	5/5	5/5
pVAX1-Luc pDNA+P85	5/5	5/5
SP1017 group		
pVAX1-Luc pDNA	4/5	5/5
pVAX1-Luc pDNA+SP1017	4/5	5/5

The reasons for variation of transgene expression could be (1) due to the technical error upon injection, could be (2) difference in transcription efficiency among animals. Tibialis anterior muscles were collected from the same animals on day 7 after single i.m. injection and subsequent *in vivo* imaging and used those muscles for analysis of the luciferase activity (Fig. 10B). pVAX1-Luc pDNA formulated with both Pluronics P85 and SP1017 enhanced transgene expression in mice on day 4 and day 7 (Fig. 8, 9 and Fig. 10). To compare the effects of Pluronics on transgene expression of pVAX1-Luc, the average intensity of luminescence were plotted in Fig. 10. pDNA formulated with P85 expressed higher average luminescence intensity compared to that of SP1017 *in vivo* (Fig. 10). Fig. 8, 9 and 10 demonstrate that both Pluronics P85 and SP1017 efficiently enhanced transgene expression of pVAX1-Luc vector in *in vivo*.

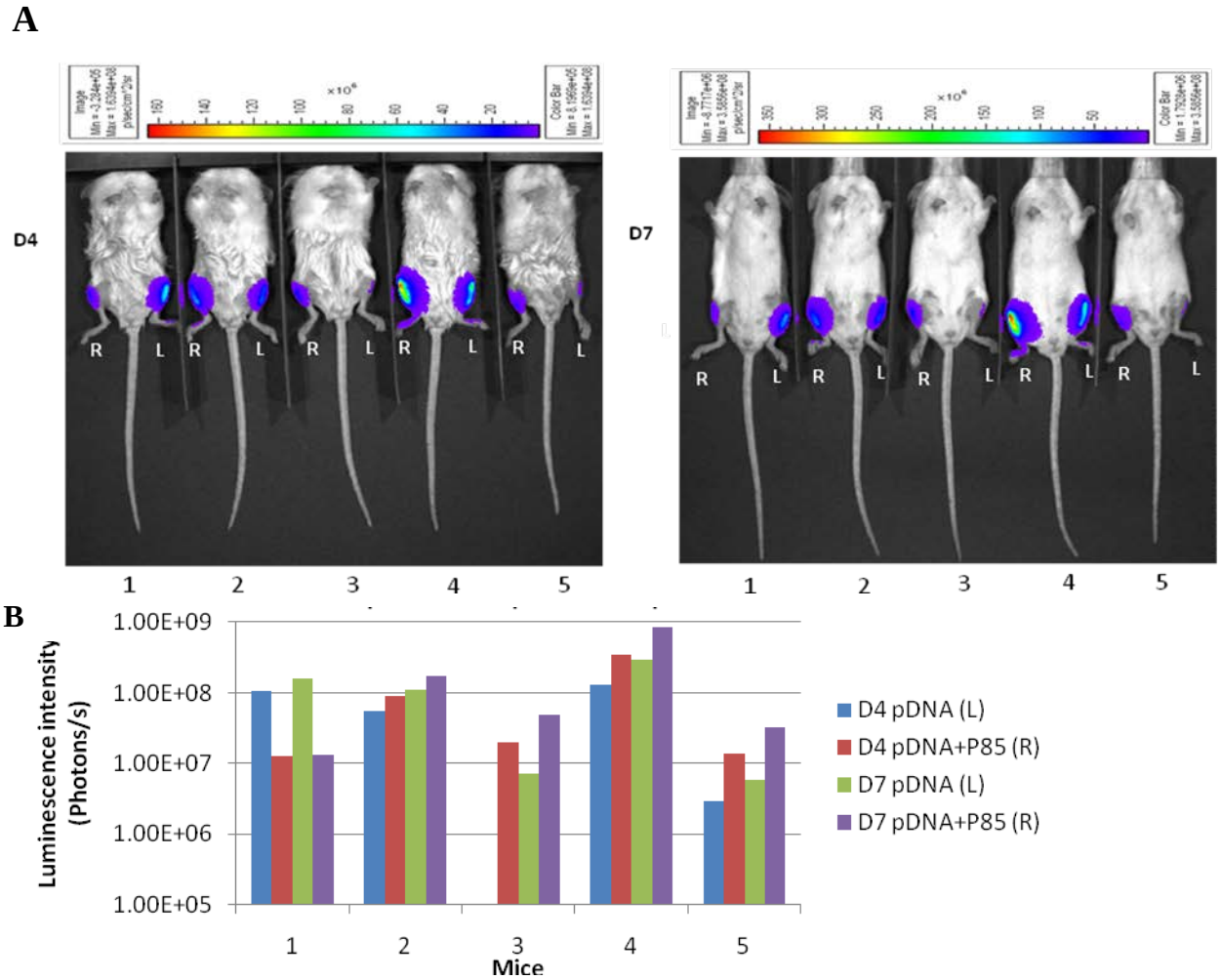


Fig. 8. *In vivo* pVAX1-Luc plasmid expression in bilateral tibialis anterior muscles in BALB/c mice (5 mice/group) after a single administration of 10 µg pDNA formulated with (R, Right tibialis anterior muscles) /without (L, Left tibialis anterior muscles) 0.3% (w/v) P85 in 50 µl PBS. **A**. *In vivo* images were taken on day 4 (D4) and day 7 (D7) after i.m. injection using the Xenogen In Vivo Imaging System-IVIS 200. **B**. Quantitative data of *in vivo* imaging study for each mouse.

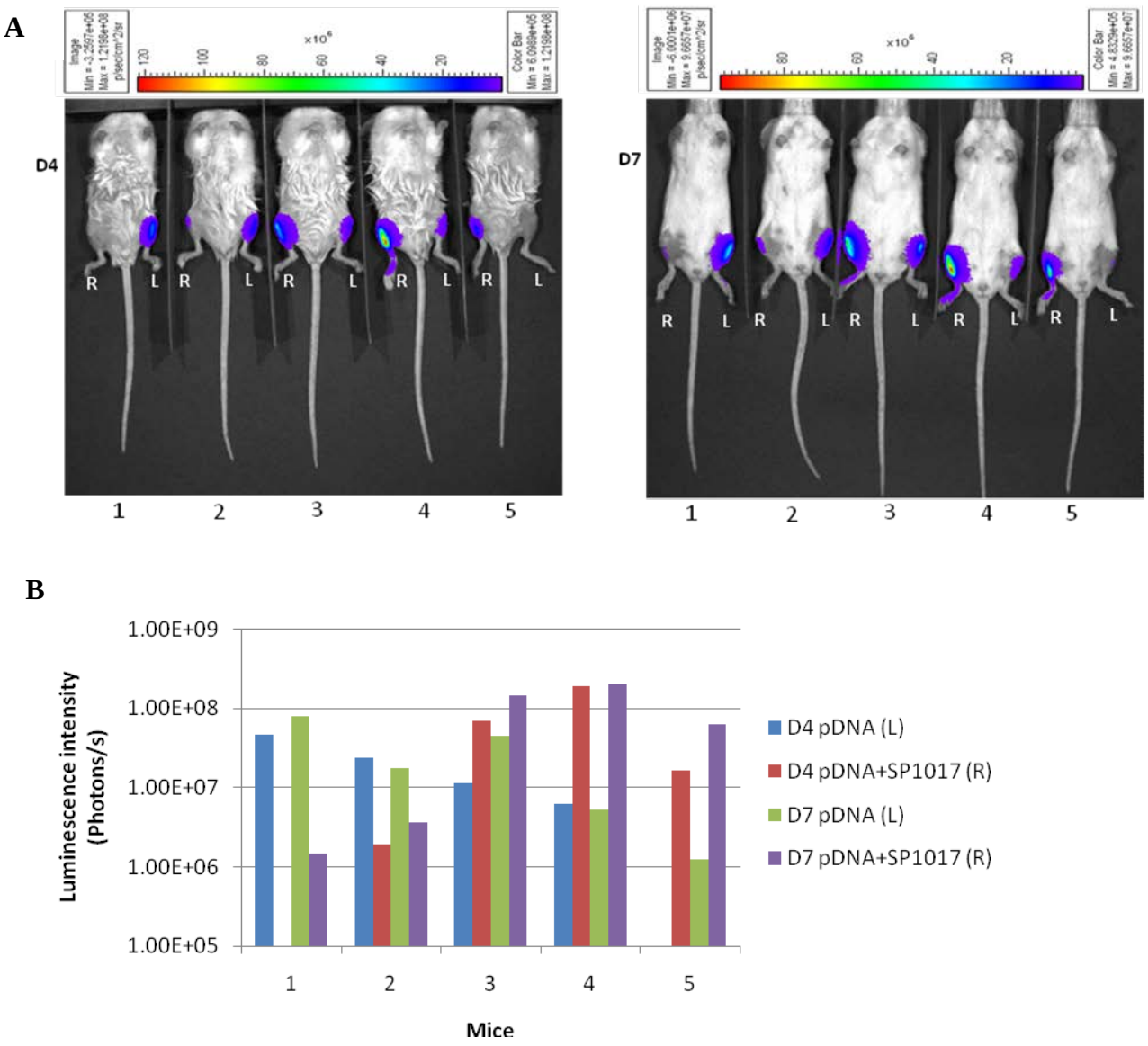
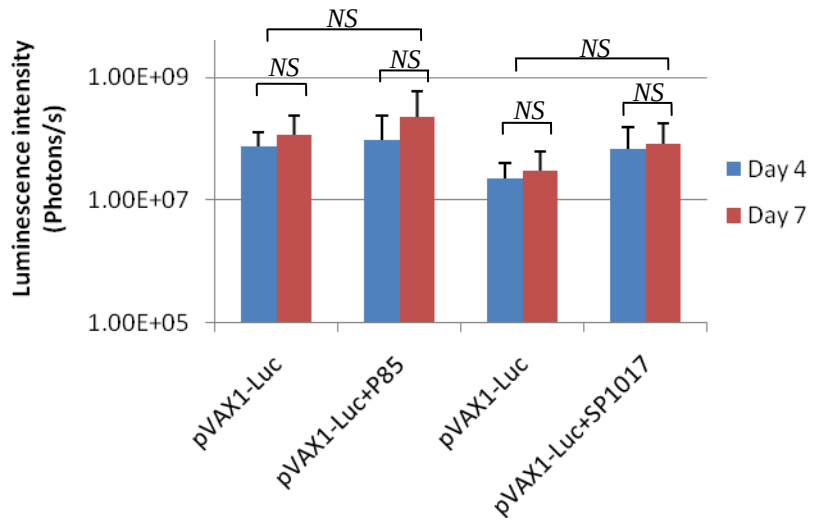


Fig. 9. *In vivo* pVAX1-Luc plasmid expression in bilateral tibialis anterior muscles in BALB/c mice (5 mice/group) after a single administration of 10 µg pDNA formulated with (R, Right tibialis anterior muscles) /without (L, Left tibialis anterior muscles) 0.01% (w/v) SP1017 in 50 µl PBS. **A**. *In vivo* imaging was performed on day 4 (D4) and day 7 (D7) after i.m. injection using the Xenogen In Vivo Imaging System-IVIS 200. **B**. Quantitative data of *in vivo* imaging study for each mouse.

A



B

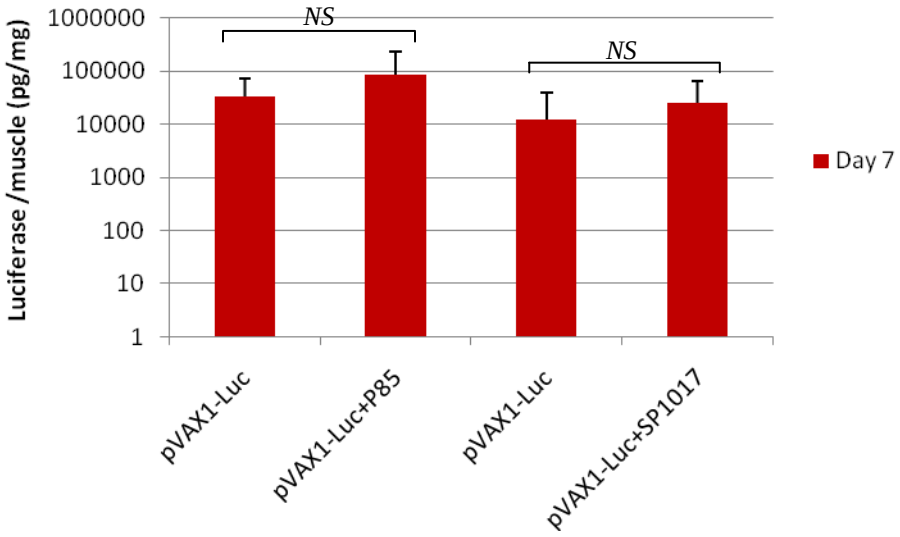
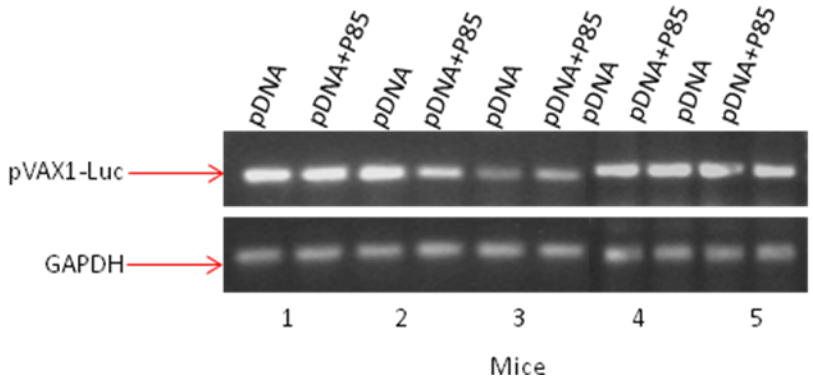


Fig. 10. Average luminescence intensity of mice administrated with 10 µg of pVAX1-Luc pDNA formulated with/without 0.3% w/v P85 or 0.01% SP1017 in tibialis anterior muscles in BALB/c mice (5 mice/ group). Data are mean ±SD (n=5). *P* values were obtained by the means of Student's t test. Each *P* value corresponds to the comparison of naked DNA versus DNA formulated with P85 or 0.01% SP1017 and Day 4 versus Day 7: *NS* is not significant at 0.05 levels. (A) *In vivo* imaging was done on day 4 and day 7 after i.m. injection using the Xenogen In Vivo Imaging System-IVIS 200. (B) Tibialis anterior muscles were collected on day 7 after single i.m. injections and *in vivo* imaging and used for analysis of the luciferase activity.

To determine the transgene expression of Luciferase and H5HA at mRNA level, 10 µg of pVAX1-H5HA and pVAX1-Luc (control plasmid) pDNA formulated with/without 0.3% w/v P85 or 0.01% w/v SP1017 were administered in the tibialis anterior muscles of BALB/c mice (5 mice/ group). On day 7, mice were sacrificed and tibialis anterior muscles were collected to perform the RT-PCR. mRNA levels were normalized to that of GAPDH. mRNA levels of Luciferase were determined in all 5 mice in both groups treated with/without P85 or SP1017 (Fig. 11.A and B). However, the group treated with/without 0.01% w/v SP1017 expressed higher level of mRNA of luciferase compared to that of P85 (Fig. 11). Expression levels of luciferase at mRNA levels also varied in all 5 mice in both groups. The similar results also observed in case of H5HA transcription. H5HA expression was observed in all 5 mice in both groups treated with/without 0.3% w/v P85 or 0.01% w/v SP1017 (Fig. 12). The variations in transcription of H5HA were also observed in both groups (Fig. 12A and B). However, expressions of H5HA at mRNA levels were higher in the group treated with/without SP1017 compared to that of P85. Co-administration of both Pluronics with pDNA increased the transcription of H5HA at mRNA levels compared to the naked pDNA (Fig. 12).

A



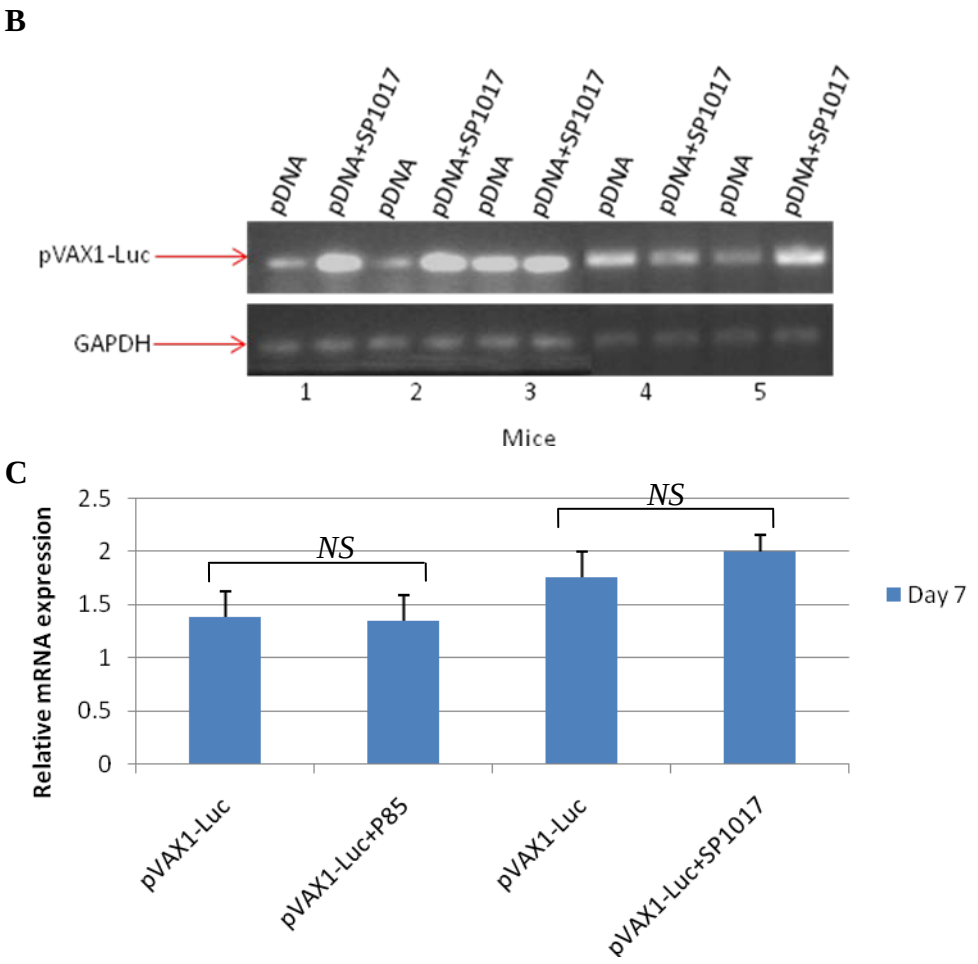


Fig. 11. Effects of Pluronics on mRNA levels of Luciferase gene. 10 µg of pVAX1-Luc pDNA formulated with/without 0.3 % w/v P85 or 0.01% w/v SP1017 were administered in tibialis anterior muscles of BALB/c mice (5 mice/group). Tissue samples were collected on day 7 and used for the RT-PCR. RT-PCR products of Luciferase (230 bp) (n=5) from P85 treated group (**A**) and from SP1017 group (**B**) were run by electrophoresis on a 2% agarose gel containing ethidium bromide. The images were acquired by using Gel Doc (Bio-Rad) and analyzed by ImageJ software (NIH). (**C**) mRNA levels for Luciferase gene expressison normalized with respect to GAPDH and expressed as arbitrary units. Data are reported as means ±SD (n=5). *P* values were obtained by the means of Student's t test. Each *P* value corresponds to the comparison of naked DNA versus DNA formulated with P85 or SP1017: *NS* is not significant at 0.05 levels.

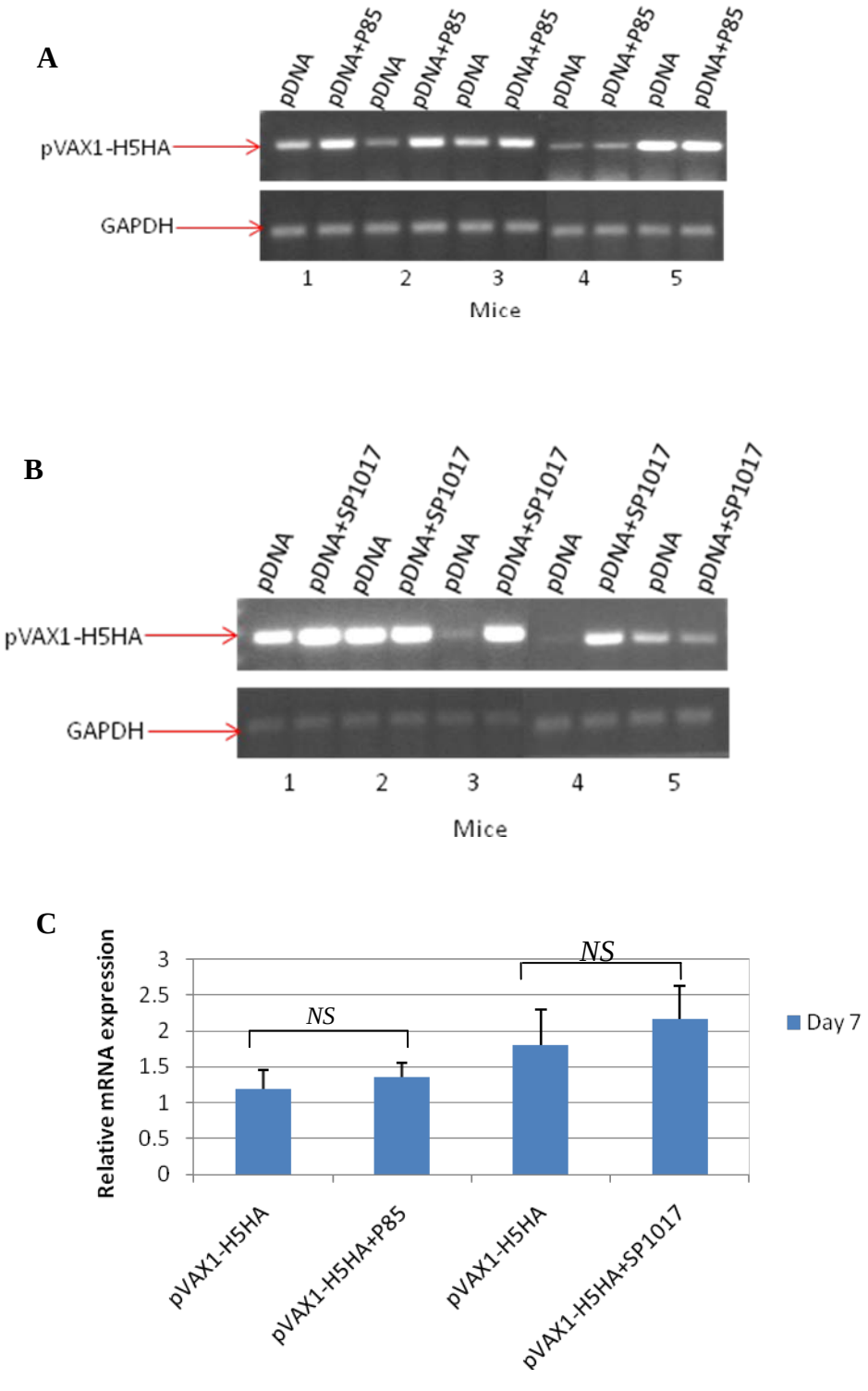


Fig. 12. Effects of Pluronics on m RNA levels of H5HA gene expression. 10 µ g of pVAX1-H5HA pDNA formulated with/without 0.3 % w/v P85 or 0.01% w/v SP1017 were administered

in tibialis anterior muscles of BALB/c mice (5 mice/group). Tissue samples were collected on day 7 and used for the RT-PCR. The RT-PCR products of H5HA expression (230 bp, n=5) from P85-treated (**A**) and SP1017-treated (**B**) groups were run by electrophoresis on a 2% agarose gel containing ethidium bromide. The images were acquired by using Gel Doc (Bio-Rad) and analyzed by ImageJ software (NIH). (C) mRNA levels for H5HA expression normalized with respect to GAPDH and expressed as arbitrary units. Data are reported as means ±SD (n=5). *P* values were obtained by the means of Student's t test. Each *P* value corresponds to the comparison of naked DNA versus DNA formulated with P85 or SP1017: *NS* is not significant at 0.05 levels.

The effects of 0.3% w/v P85 and 0.01% w/v SP1017 formulated pDNA of Luciferase and H5HA on transgene expression after i.m. administration in the tibialis anterior muscles in mice are summarized on Table 2.

Table 2. Effects of 0.3% w/v P85 and 0.01% w/v SP1017 formulated pDNA of Luciferase and H5HA on transgene expression after i.m. administration in tibialis anterior muscles in BALB/c mice on day 7.

	In vivo luciferase expression Luminescence intensity (Photons/s)		*Ex vivo* luciferase expression Luciferase/muscle (ng/mg)		Luciferase expression at mRNA level Luciferase/GAPDH (Arbitrary units)		H5HA expression at mRNA level H5HA/GAPDH (Arbitrary units)	
	pDNA	pDNA +Pluronic	pDNA	pDNA +Pluronic	pDNA	pDNA +Pluronic	pDNA	pDNA +Pluronic
P85 group	1.15E+08	2.26E+08	33.88	86.51	1.38	1.34	1.19	1.36
(Fold Increase)	(1.0)	(1.97)	(1.0)	(2.56)	(1.0)	(0.97)	(1.0)	(1.15)
Significance		*NS*		*NS*		*NS*		*NS*
SP1017 group	2.96E+07	8.37E+07	12.43	25.97	1.76	2.0	1.81	2.17
(Fold Increase)	(1.0)	(2.83)	(1.0)	(2.09)	(1.0)	(1.14)	(1.0)	(1.2)
Significance		*NS*		*NS*		*NS*		*NS*

NS: data are not statistically significant

To avoid the combined effects of plasmid DNA and Pluronics on transgene expression in mice, plasmid DNA formulations with/without Pluronics block copolymers were administered in only right tibialis anterior muscles of BALB/c mice.

In a first set of experiments, 10 µg of pVAX1-Luc (control plasmid) plasmid DNA formulated with/without 0.3% w/v P85 or 0.01% w/v SP1017 were administered i.m. in tibialis anterior muscles in BALB/c mice (5 mice/group). The animals were imaged on day 4 and day 7 after i.m. administration (Fig. 13 and 14). Luciferase expression in skeletal muscles was detected on day 4 in all 5 mice after single injection of pVAX1-Luc pDNA. The level of expression was further elevated on a day 7 after injection (Fig. 13A and 14A). Similar results were also obtained with pDNA formulated with 0.3% w/v P85 or 0.01% w/v SP1017 (Fig. 13B, 13C and 14A). Tibialis anterior muscles were collected on day 7 and luminescence assay was performed on muscle tissue to determine the luciferase activity (Fig. 14B).

As it seen from the presented data, formulation of pVAX1-Luc pDNA with Pluronic copolymers (either Pluronics P85 or SP1017) enhances transgene expression in mice (Fig. 13 and 147). To compare the effects of Pluronics on transgene expression of pVAX1-Luc, the average intensity of luminescence were plotted in Fig. 14.Transfection with pDNA in combination with P85 led to higher expression of encoded gene compared to pDNA formulated with SP1017 *in vivo* (Fig. 14).

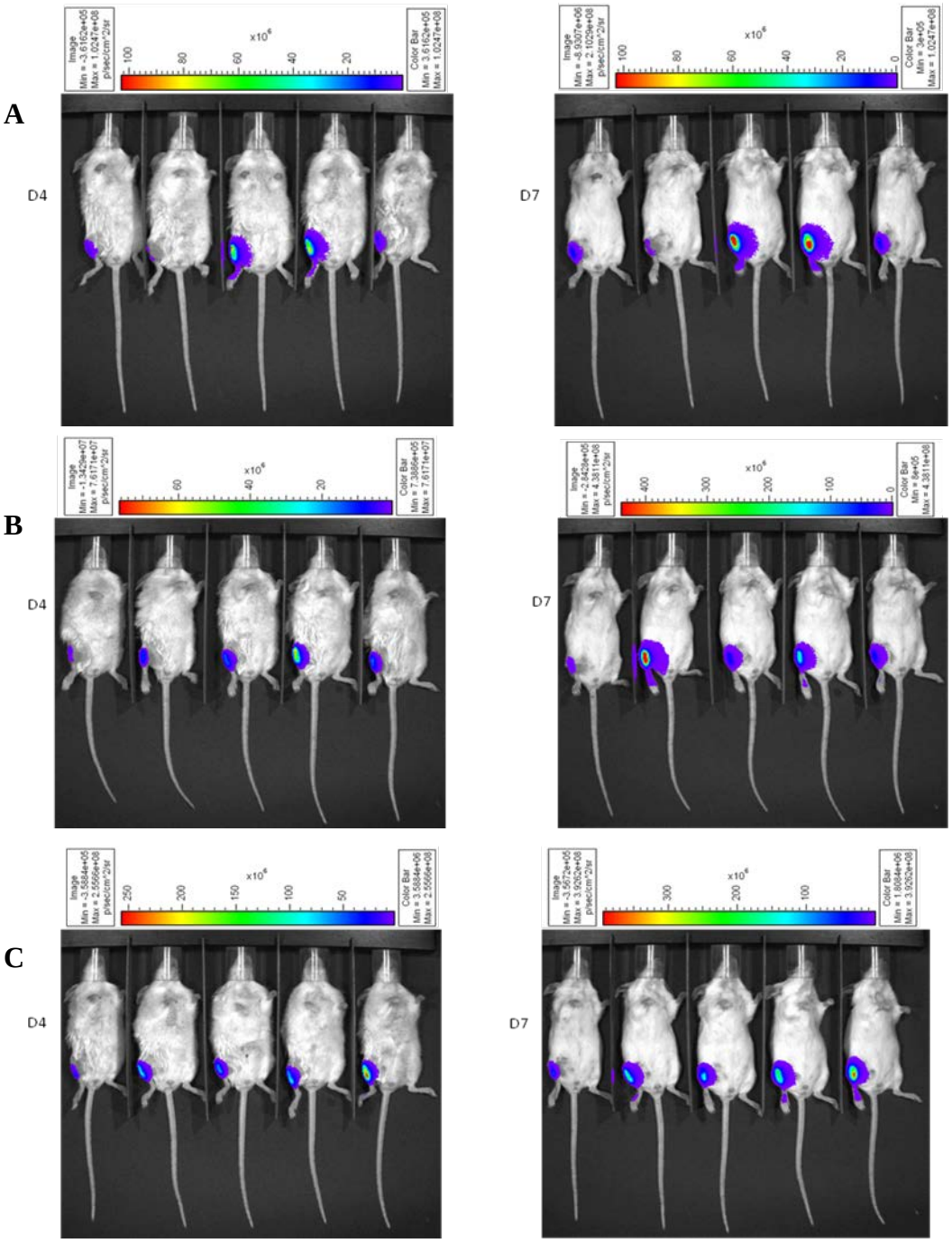


Fig. 13. *In vivo* pVAX1-Luc plasmid expression in tibialis anterior muscles in BALB/c mice (5 mice/group) after a single administration of 10 µg pVAX1-Luc pDNA formulated with /without 0.01% (w/v) SP1017 or 0.3% (w/v) P85 in 50 µl PBS **A**. pDNA only, **B**. pDNA with 0.01% SP1017; and **C**. pDNA with 0.3% (w/v) P85. *In vivo* imaging was performed on day 4 (D4) and day 7 (D7) after i.m. injection using the Xenogen In Vivo Imaging System-IVIS 200.

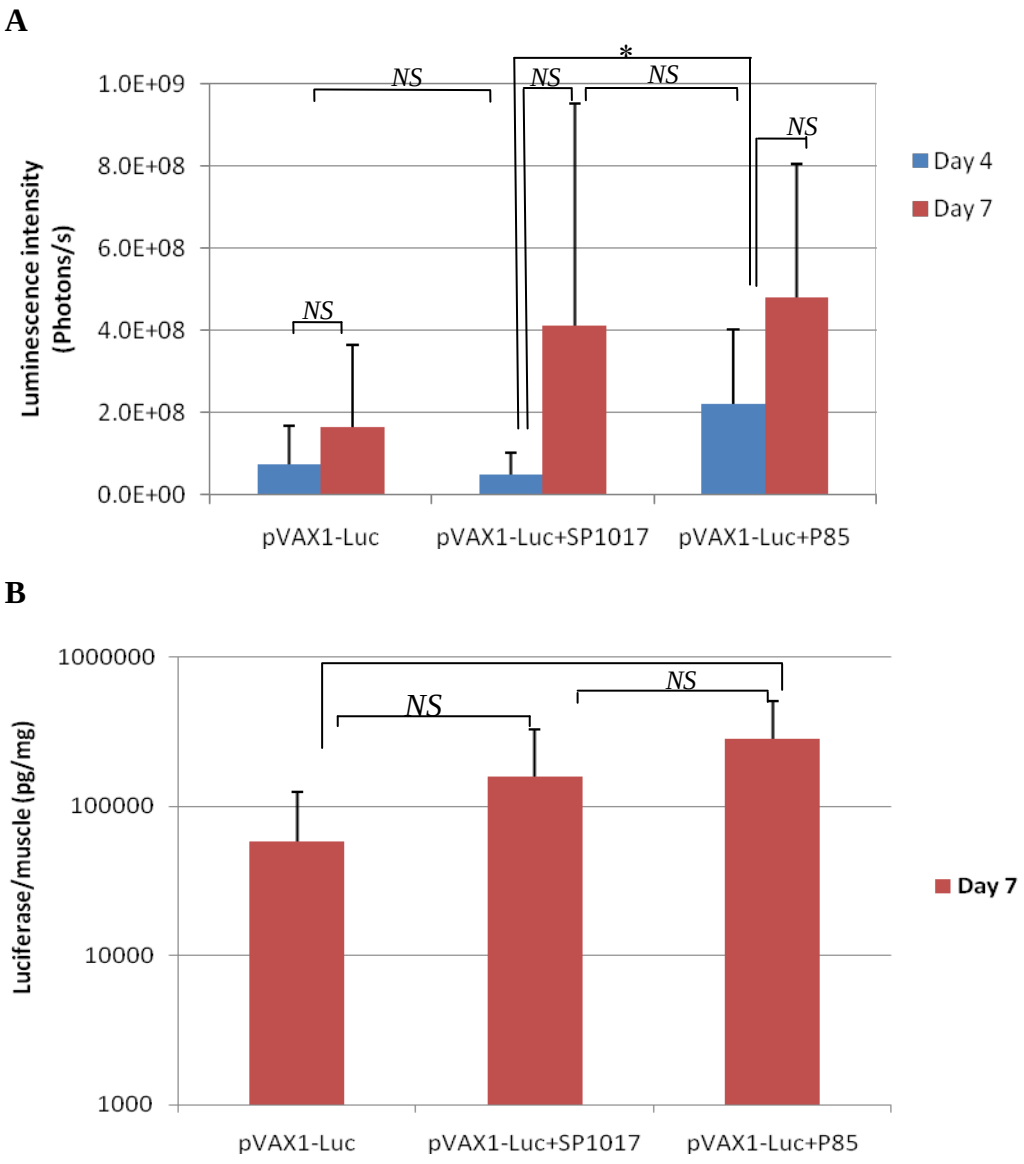


Fig. 14. (**A**) Average luminescence intensity in the muscle of mice injected with pVAX1-Luc pDNA formulated with/without 0.3% w/v P85 or 0.01% SP1017. *In vivo* imaging was performed on day 4 and day 7. (**B**) Average luciferase activity in muscle tissue on day 7 after single i.m. injections. Data are presented as mean ±SD (n=5). *P* values were obtained by the means of Student's t test. *$P<0.05$, *NS* : the differences are not statistically significant.

To determine the transgene expression of luciferase and H5HA at mRNA level, 10 µg of pVAX1-H5HA and pVAX1-Luc (control plasmid) pDNA formulated with/without 0.3% w/v P85 or 0.01% w/v SP1017 were i.m. injected to BALB/c mice (5 mice/ group). On day 7, mice were sacrificed and tibialis anterior muscles were collected to perform the RT-PCR. mRNA levels were normalized to that of GAPDH. Transgene mRNA levels were detected in all mice injected with pVAX1-Luc pDNA or its formulations with Pluronic copolymers. However, H5HA mRNA levels varied in the groups treated with pVAX1-H5HA plasmid (Fig. 15.A and B). pVAX1-Luc pDNA formulated with 0.3% w/v P85 or 0.01% w/v SP1017 produced significantly higher luciferase expression at mRNA level in all 5 mice on day 7 compared to that of naked pDNA (Fig. 15A and C). The similar results were also observed in case of H5HA transcription (Fig. 15B and D). Expressions of H5HA at mRNA levels were higher in the group treated with P85compared to that of SP1017. These results suggest that co-administration of both Pluronics with pDNA increased the transcription of Luciferase and H5HA at mRNA levels compared to that of naked pDNA (Fig. 15).

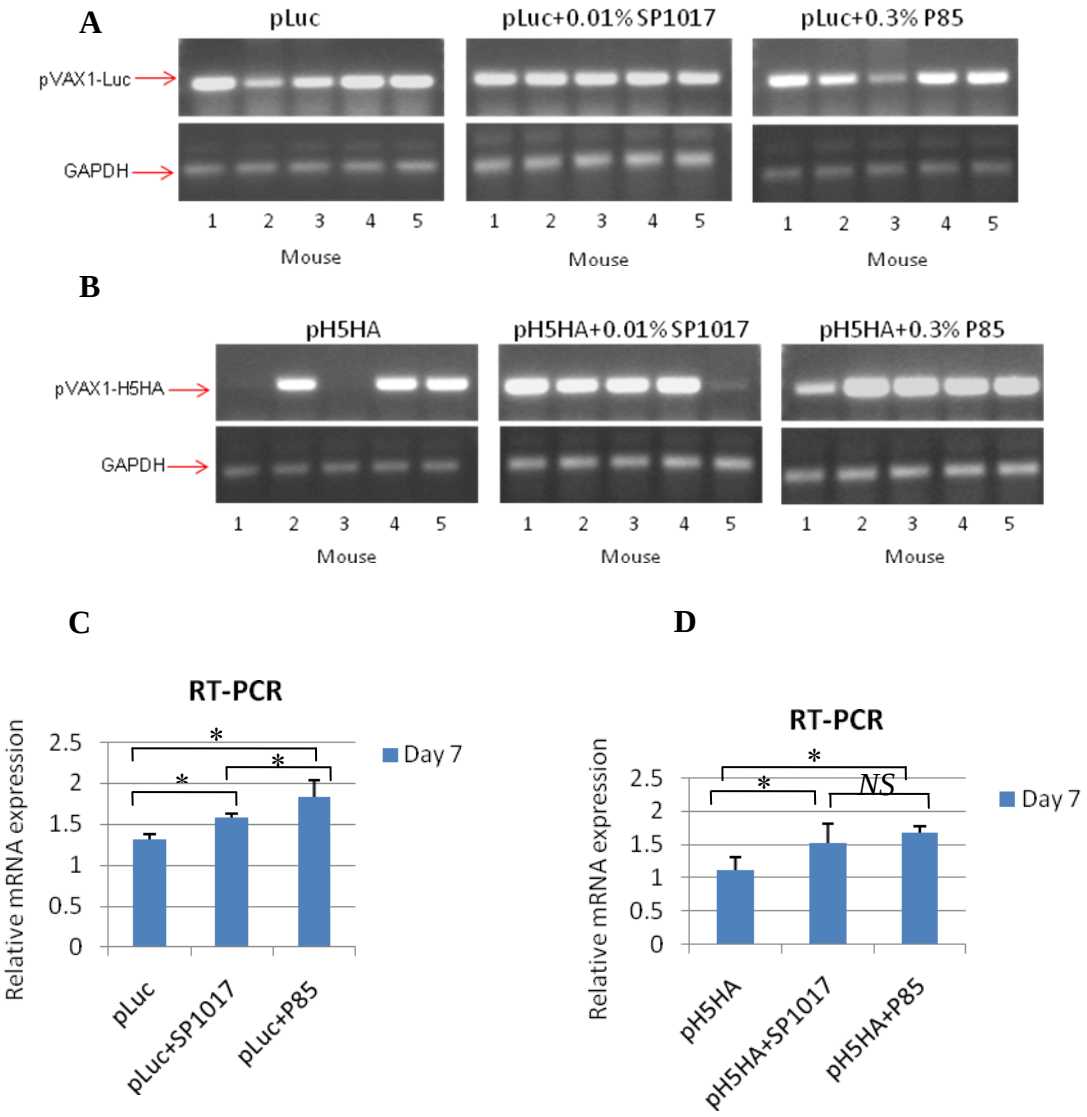


Fig. 15. Effects of Pluronics on mRNA levels of Luciferase and H5HA gene expression. RT-PCR products of (**A**) Luciferase (230 bp) and (**B**) H5HA (230 bp) gene expression were run on 2% agarose gel containing ethidium bromide. The images were acquired by using Gel Doc (Bio-Rad) and analyzed by ImageJ software (NIH). (**C**) and (**D**) mRNA levels for luciferase and H5HA gene normalized with respect to GAPDH and expressed in arbitrary units. Data are presented as mean ±SD (n=5). *P* values were obtained by the means of Student's t test. **P*<0.05, *NS* : the differences are not statistically significant.

The effects of 0.3% w/v P85 and 0.01% w/v SP1017 on transgene expression after i.m. administration in mice are summarized in Table 3.

Table 3. Effects of 0.3% w/v P85 and 0.01% w/v SP1017 formulated pDNA of Luciferase and H5HA on transgene expression after i.m. administration in tibialis anterior muscles in BALB/c mice on day 7.

	In vivo luciferase expression Luminescence intensity (Photons/s)	*Ex vivo* luciferase expression Luciferase/muscle (ng/mg)	Luciferase expression at mRNA level Luciferase/GAPDH (Arbitrary units)	H5HA expression at mRNA level H5HA/GAPDH (Arbitrary units)
pDNA only group	1.63E+08	57.77	1.32	1.12
(Fold increase)	(1.0)	(1.0)	(1.0)	(1.0)
pDNA/SP1017 group	4.11E+08	158.90	1.58	1.52
(Fold increase)	(2.52)	(2.75)	(1.2)	(1.36)
Significance	*NS*	*NS*	**P*<0.05	**P*<0.05
pDNA/P85 group	4.79E+08	280.18	1.83	1.68
(Fold increase)	(2.93)	(4.85)	(1.4)	(1.5)
Significance	*NS*	*NS*	**P*<0.05	**P*<0.05

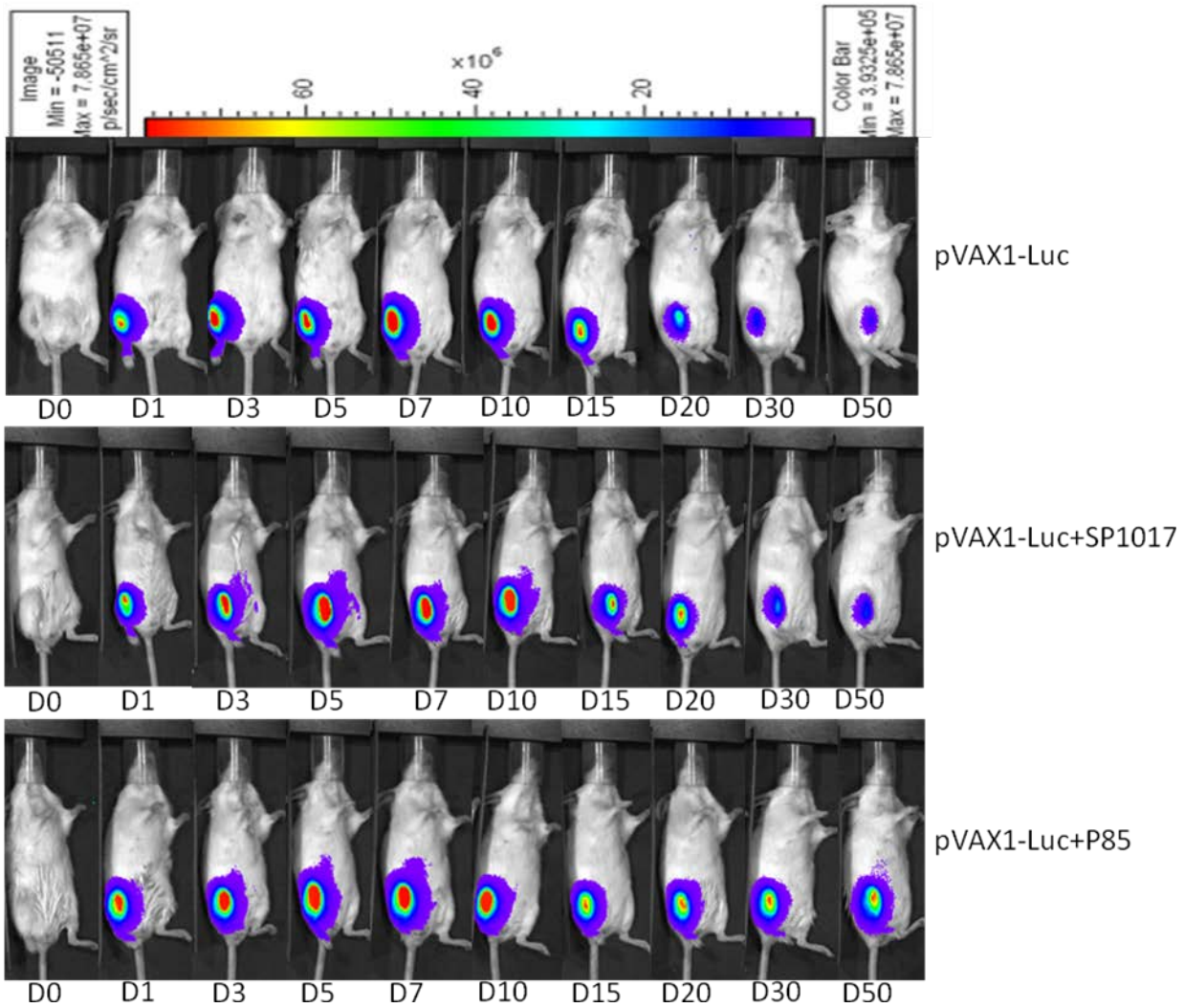
NS: data are not statistically significant

Next, in order to compare the effect of Pluronics SP1017 with P85 on the level and prolongation of transgene expression of H5HA and Luciferase gene in tibialis anterior muscles in BALB/c mice, 10 μg of plasmid DNA formulated with/without 0.3% w/v P85 or 0.01% w/v SP1017 were administered. The following experiments were performed to determine the Luciferase and H5HA expression *in vivo.*

In a first set of experiments, 10 μg of pVAX1-Luc (control plasmid) plasmid DNA (pDNA) formulated with/without 0.3% w/v P85 or 0.01% w/v SP1017 were administered i.m. in tibialis anterior muscles in BALB/c mice (10 mice/group). The animals were subjected to non-invasive bioluminescence detection using the Xenogen In Vivo Imaging System-IVIS 200 on day 0, 1, 3, 4, 5, 7, 10, 15, 20, 30, and 50 after i.m. administration (Fig. 16A). Transgene expression was greatly prolonged and lasted for at least 50 days for Luciferase gene (Fig. 16A). 10 μg of naked pVAX1-Luc pDNA produced luciferase expression in skeletal muscles with higher intensity on day 10 however as it seen from the presented data, formulation of pVAX1-

Luc pDNA with Pluronic copolymers (either Pluronics P85 or SP1017) enhances transgene expression in mice on day 7 (Fig. 16A and B). Tibialis anterior muscles were also collected on day 4 and luminescence assay was performed on muscle tissue to determine the luciferase activity (Fig. 17). P85 and SP1017 significantly enhanced transgene expression of Luciferase reporter gene in mice by 6- and 5-fold respectively, on day 4 (Fig. 17). To compare the effects of Pluronics on transgene expression of pVAX1-Luc, the average intensity of luminescence were plotted in Fig. 17. Transfection with pDNA in combination with P85 led to higher average luminescence expression of encoded gene compared to pDNA formulated with SP1017 *in vivo* (Fig. 16 and 17). Fig. and 17 demonstrated that both Pluronics P85 and SP1017 efficiently enhanced transgene expression of pVAX1-Luc vector in *in vivo*.

A



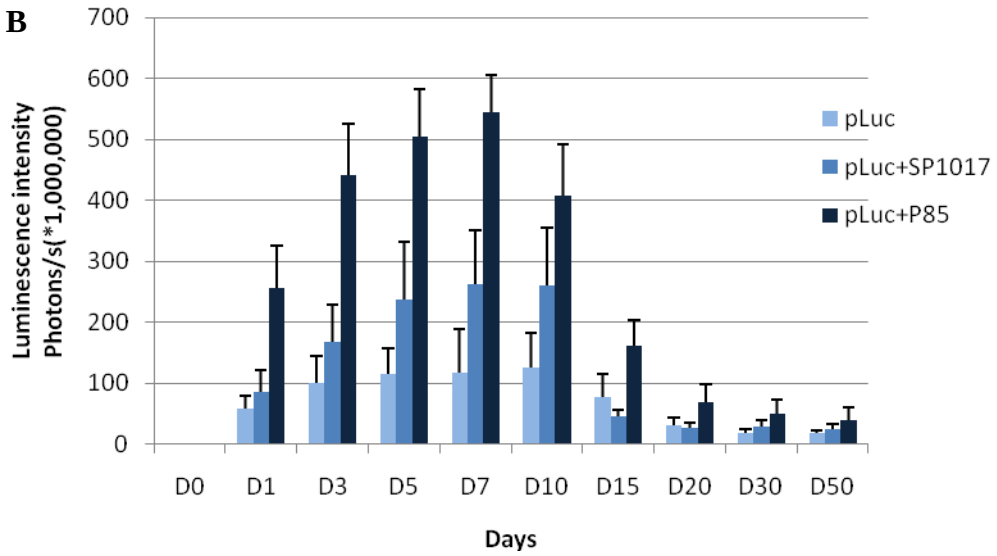


Fig. 16. A. *In vivo* pVAX1-Luc plasmid expression in tibialis anterior muscles in BALB/c mice (10 mice/group) after a single administration of 10 µ g pVAX1-Luc pDNA formulated with /without 0.01% (w/v) SP1017 or 0.3% (w/v) P85 in 50 µl. *In vivo* imaging was done on until day 50 (D50) after i.m. injection using the Xenogen In Vivo Imaging System-IVIS 200. B. Average luminescence intensity of mice. Data are mean ±SEM (n=10).

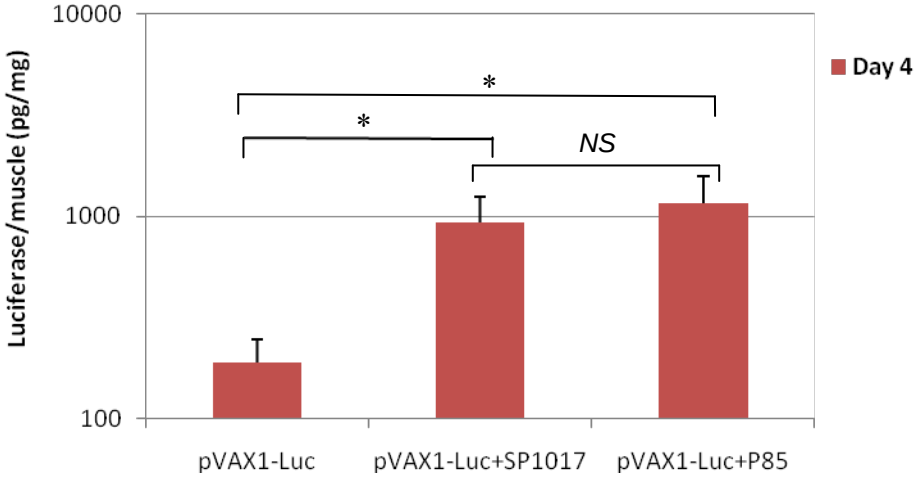


Fig. 17. Average luminescence intensity of mice administrated with 10 µg of pVAX1-Luc pDNA formulated with/without 0.3% w/v P85 or 0.01% SP1017 in tibialis anterior muscles in BALB/c mice (10 mice/ group). Data are mean ±SEM (n=10). *P* values were obtained by the means of Student's t test. Each *P* value corresponds to the comparison of naked DNA versus DNA formulated with P85 or 0.01% SP1017 on Day 4. * *P*<0.05, *NS* is not significant at 0.05

levels. Tibialis anterior muscles were collected on day 4 and used for analysis of the *ex-vivo* luciferase activity.

Expression of H5HA gene at mRNA levels: To determine the transgene expression and prolongation of H5HA at mRNA level, 10 µg of pVAX1-H5HA pDNA formulated with/without 0.3% w/v P85 or 0.01% w/v SP1017 were administered in tibialis anterior muscles of BALB/c mice (5 mice/ group). To determine the level and prolongation of H5HA expression, tibialis anterior muscles were collected to perform the Real-time RT-PCR on day 7, 14 and 28 after administration of the formulations. RNA were extracted from the harvested muscles using RNeasy Fibrous Tissue Mini kit according to the manufacturer's protocol (QIAgen, Valencia, CA). RNA samples were quantified by NanoDrop 2000 spectrophotometer (Thermo Fisher, Waltham, MA). 3.5 µg of total DNase-treated RNA from each sample were used for cDNA synthesis. Reverse transcription to prepare cDNA was performed with Oligo(dT) primers (Invitrogen, Carlsbad, CA) and Superscript II (Invitrogen, Carlsbad, CA). RNAseOUT (Invitrogen, Carlsbad, CA) was used to inhibit ribonuclease activity during reaction sequences. Efficient cDNA synthesis was accomplished by incubation of total RNA for 90 min at 42°C followed by 5 min denaturation at 94°C. The cDNAs were further used for the Real-time RT-PCR. HA specific influenza virus H5 and GAPDH primers and probes were designed for the H5 and GAPDH TaqMan Real-time assay by using Primer Express software (Applied Biosystems, Branchburg, NJ). Primers and probes were H5 Forward primer 5'GAG ATT GTA GCG TAG CTG GAT GG 3', H5 Reverse primer 5'CAT TCC GGC ACA TTG ATG AA 3' , TaqMAN H5 probe 5' TCC TCG GAA ACC CAA TGT GTG ACG A 3', GAPDH Forward primer 5' ACT GGC ATG GCC TTC CG 3', GAPDH Reverse primer 5' CAG GCG GCA CGT CAG ATC 3' and TaqMan GAPDH probe 5' TTC CTA CCC CCA ATG TGT CCG TCG T 3' (Sigma, St. Louis, MO). Two microliters of cDNA samples were used per 20 µL reaction volume containing 5 µL 2X Taqman Universal PCR Master Mix (Applied Biosystems, Branchburg, NJ), and 250 nM of each primer and probe. All samples and controls were tested in triplicate. The PCR thermal profile consisted of 50°C for 2 minutes, 95°C for 10 minutes, and then 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. Amplification, detection, and data analysis were performed on an ABI Prism 7000 Real-time thermal cycler (Applied Biosystems, Branchburg, NJ) with "FAM- Tamra quencher" selected as the detector. Relative expression of H5HA was

normalized to expression of GAPDH. Relative quantification of gene expression was calculated based on the comparative *C*T (threshold cycle value) method (Δ*C*T = *C*T gene of interest – *C*T housekeeping gene). Comparison of gene expression in different samples was performed based on the differences in Δ*C*T of individual samples (ΔΔ*C*T) and the fold difference was calculated as 2^ ΔΔ*C*T. All data are presented as means ± standard error (SEM) and analyzed with Student's *t*-test (n=3). Differences were accepted as statistically significant at $p < 0.05$. H5HA gene transcription were determined in all 5 mice in all 3 groups through day 28 though transcriptions of H5HA varied in those groups. In this study, we incorporated 3 most positive reactions in Real-time RT-PCR assay to calculate the data from 3 out of 5 mice per group. pVAX1-H5HA pDNA formulated with 0.3% w/v P85 or 0.01% w/v SP1017 produced significantly higher H5HA gene expression at mRNA levels on day 7 compared to naked pDNA (Fig. 18). Compared to naked pDNA, 0.01% SP1017 and 0.3% P85 increased the transcription of H5HA by 5.3 and 40.6 fold on day 7 in tibialis anterior muscles. However, H5HA gene transcription levels were decreased by days. On day 15, SP1017 and P85 increased the mRNA levels by 1.8 and 11.9 fold respectively. Whereas, fold induction by SP1017 and P85 were by 0.4 and 0.8 fold respectively compared to naked pDNA on day 28. Expression of H5HA at mRNA levels was always higher in the group treated with P85 compared to SP1017. These results suggest that administration of pVAX1-H5HA pDNA prolonged the transcription until day 28 and co-administration of both Pluronics with pDNA increased the transcription levels compared to naked pDNA (Fig. 1811).

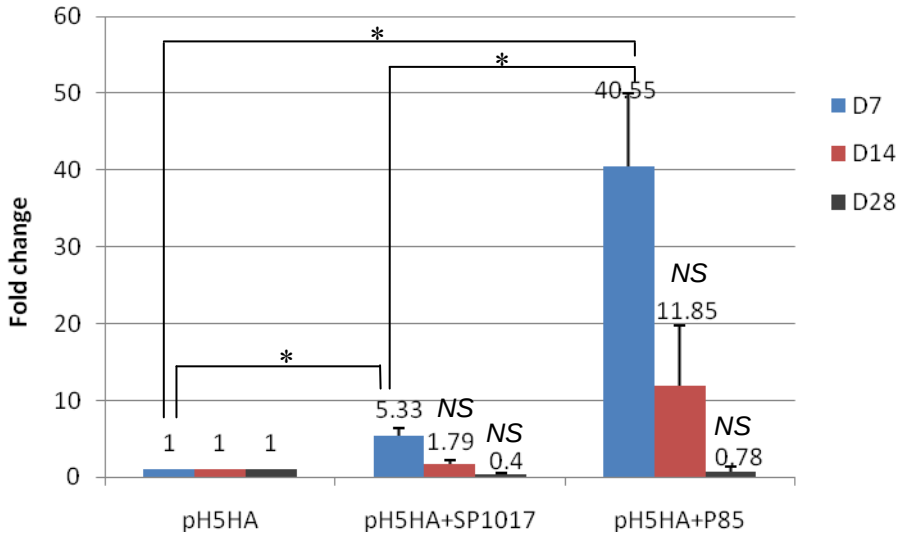


Fig. 18. mRNA levels of H5HA gene determined by Real-time RT-PCR. Balb/c mice administrated with 10 μg of pVAX1-H5HA pDNA formulated with/without 0.3% w/v P85 or 0.01% SP1017 in tibialis anterior muscles (5 mice/ group). Tibialis anterior muscles were collected on day 7 (D7), day 14 (D14) and day 28 (D28) after administration of the formulations and performed Real-time RT-PCR. Induction ratios of H5HA gene (fold change) by Pluronics were calculated using expression level, normalized to the level of the naked pDNA group. Data are mean ±SEM (n=3). *P* values were obtained by the means of Student's t test. Each *P* value corresponds to the comparison of naked DNA versus DNA formulated with P85 or 0.01% SP1017. **P*<0.05, *NS* is not significant at 0.05 levels.

Now, we are focused on evaluating whether a single immunization with Pluronic/pVAX1-H5HA DNA vaccine can produce an immune response.

In order to determine the immune response, 10 μg of pVAX1-H5HA pDNA formulated with/without 0.3% w/v P85 or 0.01% w/v SP1017 were administered i.m. in tibialis anterior muscles in BALB/c mice (5 mice/group). H5HA specific antibodies in blood sera were detected by enzyme-linked immunosorbent assay (ELISA). Blood sera were collected on day 7, 14 and 28 after i.m. injection. H5HA protein was purified from HEK-293T cells transfected with pH5HA using MagneHis Protein Purification System according to manufacturer's protocol (Promega, Madison, WI). 96-well microtiter plates (Nunc Life Technollogies, Rochester, NY) were coated with 2 μg/mL purified H5HA in PBS overnight at 4°C. Plates were blocked for 1 h a t room temperature with 1 % BSA/ PBS. 100 μL of serum dilutions were added to the plates and incubated for 2 h at room temperature. Plates were washed five times, and HRP conjugated mouse IgG detection antibody (Bethyl Laboratories,Inc. Montgomery,TX) diluted 1:50,000 was added to wells followed by 1 h incubation at room temperature. TMB peroxidase substrate (Bethyl Laboratories,Inc. Montgomery,TX) then added to the wells and allowed to develop for 15 min at room temperature. Optical density measurements were done at 450nm.

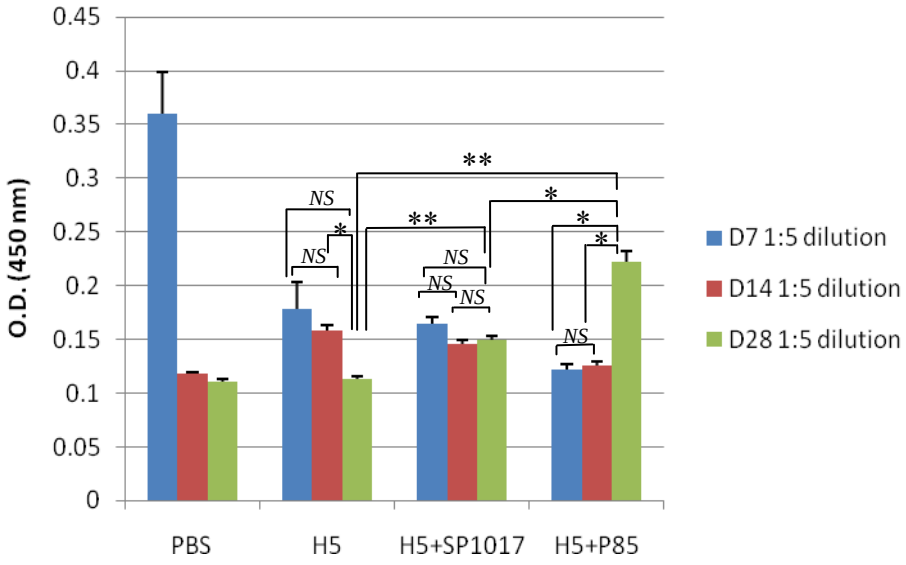


Fig. 19. Total serum IgG antibody responses to H5HA.10 µg of pVAX1-H5HA pDNA formulated with/without 0.01% (w/v) SP1017 or 0.3% (w/v) P85 in 50 µL of PBS were administered in tibialis anterior muscles in BALB/c mice (5 mice/group). Sera from individual mice were taken 7, 14 and 28 days after single administration of vaccine formulations and were tested for binding to H5 hemagglutinin by ELISA. All data are presented as means ± standard error (SEM) and analyzed with Student's *t*-test (n=5). Each *P* value corresponds to the comparison of naked DNA versus DNA formulated with P85 or 0.01% SP1017 ***P*< 0.01 significant at 0.01 levels. **P*<0.05 and *NS* is not significant at 0.05 levels.

In order to determine H5HA specific antibody levels in sera after single i.m. administration of pH5HA in combination with Pluronics in the tibialis anterior muscles of BALB/c mice (5mice/group) were determined by enzyme-linked immunosorbent assay (ELISA). The single immunization of mice with pH5HA formulations induced low but detectable levels of total IgG antibodies as measured by ELISA plates coated with H5HA protein (Fig. 19). The levels of virus-specific antibodies were significantly increased after 28 days in the groups immunized with Pluronics SP1017 and P85 compared to naked DNA (Fig. 19A). The non-specific anti-H5HA IgG were also detected in PBS group probably due to the presence of highly conserved amino acid sequences of pocket on the stem of HA (Fig. 19).

As H5HA pDNA vaccines are poorly immunogenic, to induce the robust humoral antibody responses against H5HA, the following measures will be taken:

1. Mice will be immunized once with H5HA pDNA with increased doses (50 or 100 µg /mouse).
2. Prime-boost immunization regimen will be carried out with lower doses of H5HA pDNA.

Platform C

In support of studies in Platform C we successfully synthesized and characterized a series of targeted BIC/DNA compositions. BICs were prepared using mannose-capped poly(ethylene glycol)-block-poly(l-lysine) diblock copolymer m annose-PEG_{114}-PLL_{62} (the numbers in subscripts show the relative number of monomer units in the blocks) and gWIZ-LUC plasmid DNA (Luc-DNA) via our previously identified method (Figure 20).

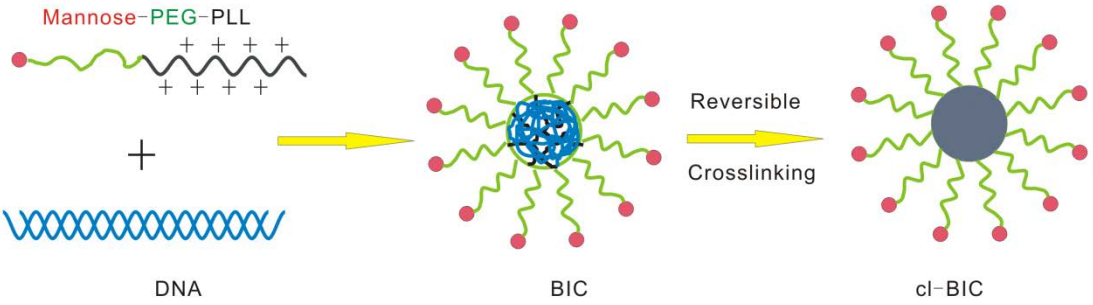


Fig. 20. Schematic representation of the synthesis of crosslinked (cl) -BICs

To verify the formation of BICs and condensation DNA by mannose-PEG_{114}-PLL_{62}, ethidium bromide (EtBr) exclusion assay was used. Briefly, DNA was mixed with ethidium bromide (DNA: EBr= 5:1 w/w) prior to particle formation. Then cationic block polymer mannose-PEG_{114}-PLL_{62} were added to the mixture at predetermined N : P ratio(the molar ratio of amine groups of PLL to DNA phosphate groups), then vortexed for 20 seconds immediately, and incubated for 30min at R.T. Upon mixing this systems remained transparent and no precipitation was observed over entire range of N:P ratios examined. The resulting solutions were added to two wells on a white opaque 96 well plate and fluorescence was measured with 360 nm excitation and 590 nm emission. As shown in Figure 21**A**, addition of cationic mannose-PEG_{114}-PLL_{62} copolymer to DNA-EB complex resulted in quenching of the EB fluorescence due to the displacement of EB by the copolymer. Fluorescent intensity decreased gradually with the increase of N:P ratio, and leveled off when the N:P ratio exceeded 1:1. These data suggest that maximal quenching of EB fluorescence was observed at equivalency of the polycation (PLL) and DNA charges (N:P = 1).

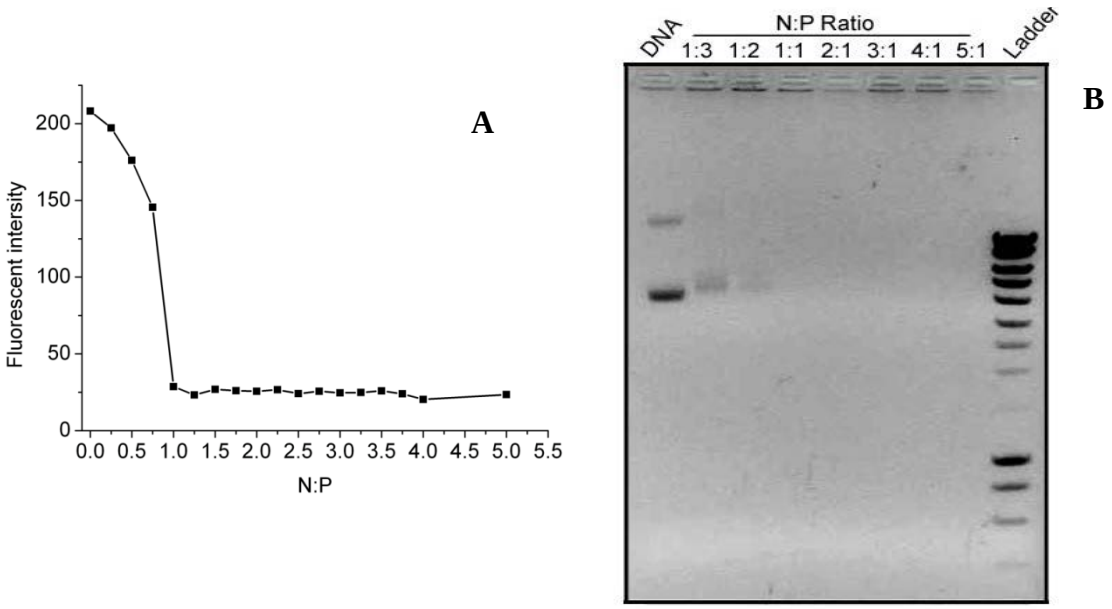


Fig 21. A. Binding of mannose-PEG_{114}-PLL_{62} with plasmid DNA examined by ethidium bromide exclusion assay. Data are presented as mean fluorescence intensity of the samples. **B**. Agarose gel retardation assay. The final concentration of DNA was 15ug/ml

Formation of BIC between DNA and mannose-PEG-PLL was also investigated by retardation assay using agarose gel electrophoresis (Fig. 21**B**). Migration of the DNA in the gel was retarded as the amount of the copolymer was increased. This demonstrated that mannose-PEG-PLL copolymer was binding to DNA, neutralizing its charge. At stoichiometric composition (N : P = 1) complete neutralization of DNA by the copolymer was achieved and no DNA bands were observed at N: P ratio larger than 1, c onsistent with the results from EB exlusion assay (Fig.21**A**). Thus, both methods confirmed that cationic block copolymer mannose-PEG-PLL can form complexes with DNA..

BICs were further cross-linked using dithio-bis-(succinimidyl propionate), DSP, a reducible crosslinker containing disulfide bonds. Complexes were prepared in the presense of the excess of block copolymer at N:P ratio equal 3. Predetermined amounts of DSP dissolved in DMSO were added to the formed BIC to induce the cross-linking. The amount of DSP was calculated on the basis of the targeted cross-linking ratio defined as the total amount of reactive groups in DSP solution versus total number of Lys residues in mannose-PEG-PLL copolymer. It is important to note that the extent of targeted cross-linking represents the maximum theoretical amount of cross-linking that can take place, rather than the precise extent of cross-linking, which is expected to be lower. Mixtures were incubated for 1 h at room temperature followed by dialysis

against 10mM HEPES buffer (pH 7.4) to remove excess amount of cationic polymers. Hydrodynamic diameter and zata-potential of BICs were determined by dynamic light scattering (Malvern Zetasizer, Malvern Instruments Ltd., Malvern, UK). All measurements were performed in automatic mode, at 25^{o}C. Software provided by the manufacturer was used to calculate the size, polydispersity indices and zeta-potential of BICs (Table 4). All measurements were performed at least in triplicate. All examined BICs formed small particles with averaged diameters in the range of 90-100 nm with relatively narrow size distribution (polydispersity indices were below 0.2) and nearly neutral surface charge (Table 4).

Table 4. Physico-chemical characteristics of mannose-PEG$_{114}$-PLL$_{62}$/Luc-DNA BICs (N:P 3:1)

Degree of crosslinking (cl) with or without dialysis	Particle Size (nm)	Polydispersity Index	Zeta Potential (mV)
0%	99.0 ± 1.4	0.198±0.008	1.70±0.65
25% non-dialyzed	114.3±1.4	0.208±0.010	0.09±0.32
50% non-dialyzed	101.4±0.5	0.163±0.010	-6.23±0.07
75% non-dialyzed	103.8±0.4	0.204±0.010	-7.58±0.51
25% with dialysis	83.6±0.6	0.243±0.022	2.13±0.84
50% with dialysis	169.8±4.7	0.290±0.032	-8.26±0.33
75% with dialysis	103.6±0.7	0.147±0.007	-6.72±0.31

The morphology of cl-BICs was further examined by atomic force microscopy (AFM) using a Multimode NanoScope IV system operated in a tapping mode. For sample preparation, BICs were deposited on mica surface for 2 min, followed by surface drying under argon atmosphere. The AFM images were processed and the widths and heights of the particles were determined by using Femtoscan software (Advanced Technologies Center, Moscow, Russia). A representative AFM image of cl-BIC with 25% targeted degree of cross-linking is shown in Figure 22 and indicated the formation of slightly elongated complex particles.

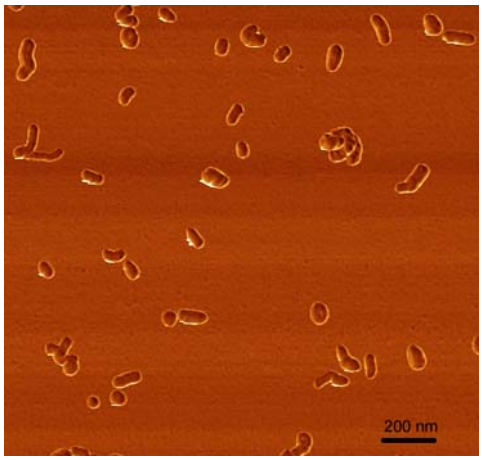


Fig. 22. AFM image of mannose-PEG_{114}-PLL_{62}/Luc-DNA BICs (N:P = 3) with targeted degree of cross-linking of 25%. Bar is equal 200 nm.

Stability of cross-linked BIC was studied by agarose gel electrophoresis after co-incubation with heparin for 1 hour. Heparin, an anionic polysaccharide with highl charge density, can strongly interact with the mannose-PEG-PLL cationic copolymer and may substitute DNA from non-crosslinked BICs which could subsequently be visualized by EtBr staining by gel electrophoresis (Figure 23**A**). As expected, DNA was substituted from the non cross-linked BICs by heparin. On the contrary, the formation of cross-links between the polycation chains in BIC rendered the DNA release from the cross-linked BICs even in the presence of high concentrations of heparin suggesting that the stability of the BIC was greatly improved (Figure 23**B**).

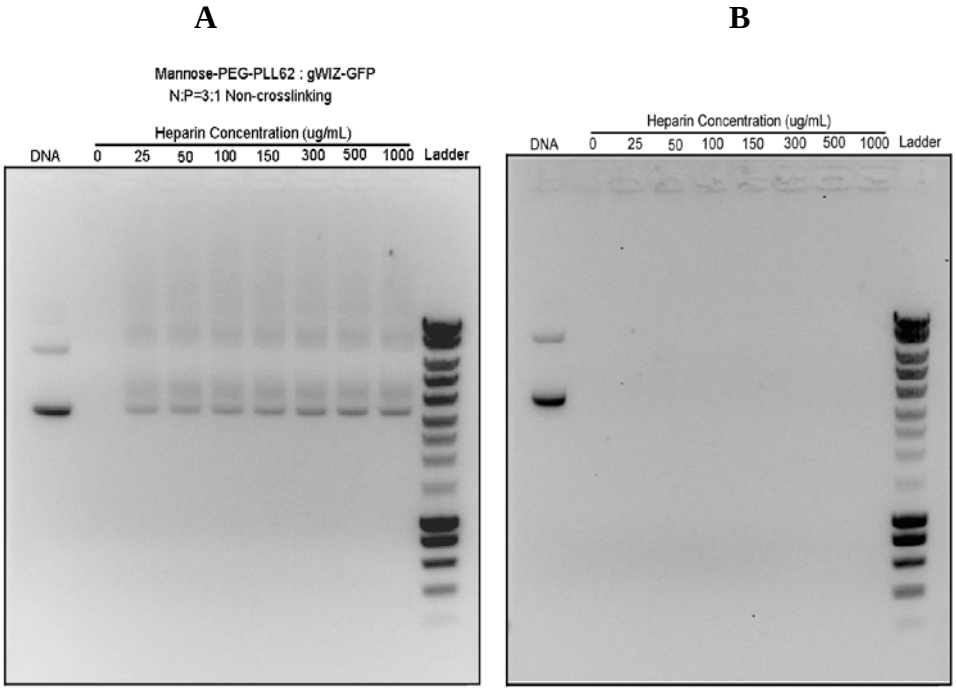


Fig. 23. Stability of non-crosslinked BICs (**A**) and crosslinked clBICs (**B**) (N:P =3:1). Both types of BICs were incubated with heparin for 1 hour prior to agarose gel electrophoresis; the values represent the concentration of heparin (ug/ml).

The introduced cross-links in clBIC contained reversible disulfide bonds that are relatively stable in extracellular environment but degrade in reducing environment inside the cells. Redox sensitivety of cross-linked BIC (complexes were prepared using DNA plasmid encoding GFP protein) and was confirmed by incubating complexes with dithiothreitol, DTT, for 30 m in at 37oC followed by challenging them with heparin and analyzing by gel electrophoresis (Figure 24). The bands corresponding to DNA were observed in case of complexes treated with DTT confirming reduction of disulfide bonds.

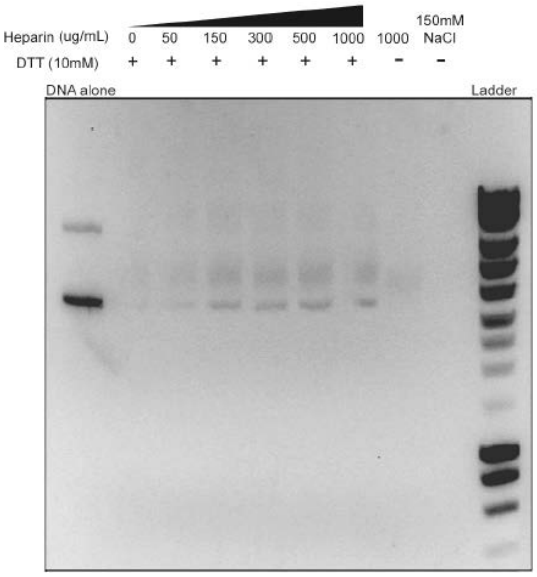


Fig. 24. Stability of cross-linked BICs in in the presence of 10 mM DTT (C). BIC samples (mannose-PEG_{114}-PLL_{62}/GFP-DNA) were incubated with heparin for1 hour prior to agarose gel electrophoresis. The values represent the concentration of heparin (ug/ml). Complexes were prepared at N:P = 3 with 25% targeted degree of cross-linking and purified by dialysis.

The cl-BIC were also incubated with NaCl solution for 30min, and ran on the agarose gel. According to Figure 24 (second lane from the right), no D NA was substituted by heparin, suggesting that cross-linked BICs were stable at physiologically relevant concentrations of salt (NaCl, 150mM).

Possible cytotoxicity of mannose-PEG-PLL/DNA complexes in both NIH/3T3 cells and mouse bone marrow derived macrophage (BMM) cells was evaluate using standard MTT assay (Figure 25). For macrophages, both cl-mannose-PEG-PLL BICs (black bars) and cl-PEG-PLL BICs (blue bars) exhibited much lower cytotoxicity as compared to non-crosslinked mannose-

PEG-PLL BICs (red bars). This might ascribe to the removal of excess of cationic polymers from cl-BICs by dialysis. Furthermore, we observed significantly lower toxicity of our BICs than that of commercial transfection polymeric reagent Exgene 500 (green bars).

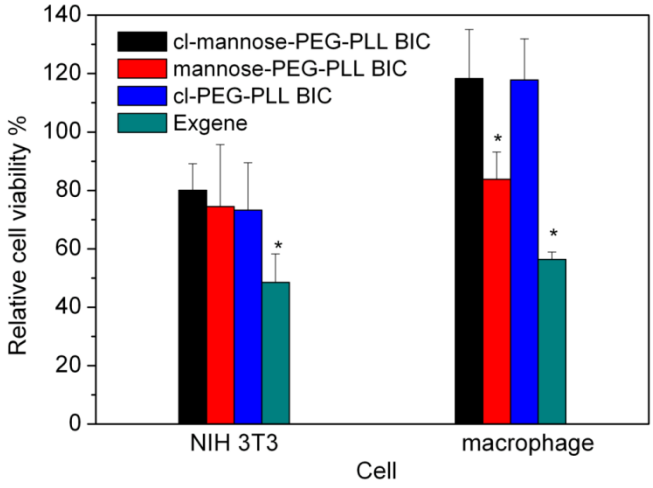


Fig. 25. Cytotoxicity of BICs to NIH/3T3 cells and BMMs.

For further in vitro transfection experiments, BMMs cells were used as the model cell line corresponding to high mannose receptor expression while NIH/3T3 fibroblasts with low level of mannose receptor expression were used as a negative control. The physicochemical characteristics of BICs used for transfection experiments are presented in Table 5.

Table 5. Physicochemical characteristics of of various BICs used in transfection experiments

Polymers in Luc-DNA complex	Particle Size (nm)	PDI
EXGENE 500 (PEI)	336.0±18.4	0.344±0.028
Mannose-PEG_{114}-PLL_{62}	91.7±2.4	0.156±0.010
PEG_{114}-PLL_{50}	87.3±0.4	0.173±0.028
Mannose-PEG_{114}-PLL_{62} (cl)	106.1±3.9	0.168±0.009
PEG_{114}-PLL_{50} (cl)	87.9±1.6	0.148±0.013
Mannose-PEG_{114}-PLL_{62} (cl and dialysis)	95.5±2.4	0.202±0.011
PEG_{114}-PLL_{50} (cl and dialysis)	92.2±1.8	0.213±0.015

As it seen from Figure 26, tranfection efficiency of BIC was low compared to Exgene 500/DNA complexes and no difference was observed between targeted and nontargeted BICs in both cell models. However, mouse BMM cells did not express considerable level of mannose receptors as was determined by FACS analysis via staining mannose receptors with specific antibody (only 2.3% of BMM cells were positive for mannose receptor). Therefore, selecting appropriate

macrophage cells that express considerable amount of mannose receptors is important for testing the targeted gene delivery strategy.

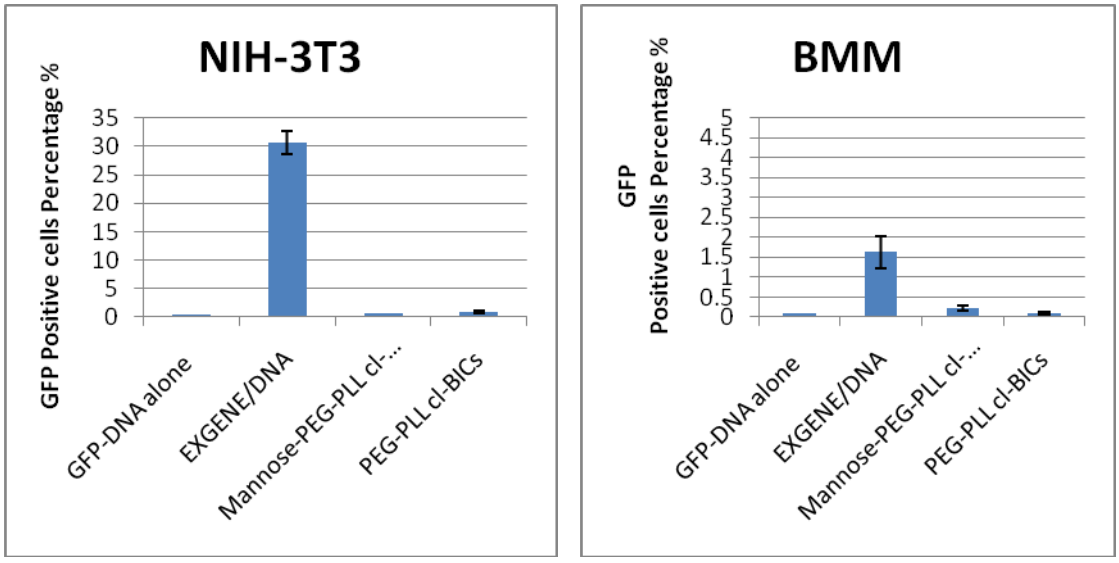


Fig. 26. Transfection of NIH-3T3 cells and BMMs with various BICs.

An interesting option is human monocyte-derived macrophage cells. These macrophage cells were differentiated from human monocytes in the culture medium supplemented with macrophage colony-stimulating factor (MCSF). Primary mouse monoclonal mannose receptor antibody was applied at 1:20 dilution and goat-anti-mouse IgG conjugated with Alexa 488 dye (green color) were used as secondary antibody. A strong staining of mannose receptors was observed on the surface of these human macrophage cells (Figure 27). However, there was no significant difference of luciferase expression between the cells transfected with mannose-functionalized BICs and non-targeted BICs (Figure 28).

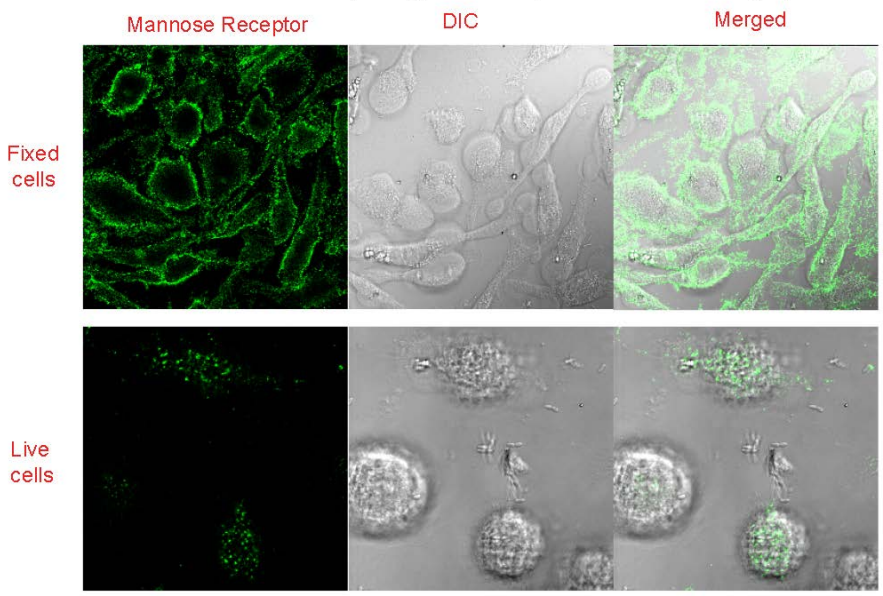


Fig.27. Confocal images of human monocyte-derived macrophage cells stained for mannose receptors (green).

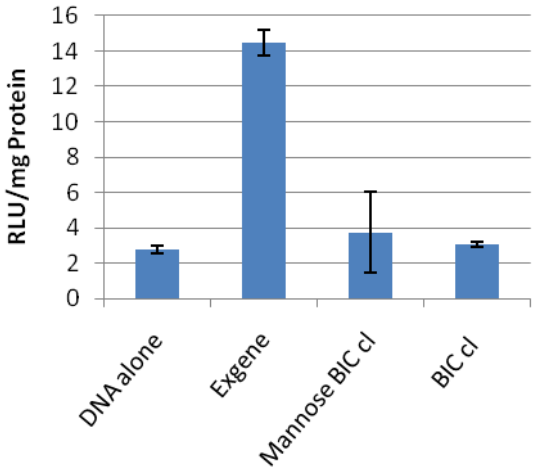


Fig.28. Transfection of human monocyte-derived macrophages with Luc-DNA plasmid incorporated into cross-linked mannose-decorated BIC or into BIC without mannose targeting moieties.

For the future studies a mouse macrophage cell line RAW264.7 may be used as mannose receptor positive cells. Furthermore, BICs formed by cationic copolymers with different lengths of PLL block and various density of surface mannose targeting moietieswill be tested.

KEY RESEARCH ACCOMPLISHMENTS

- Synthesis and purification of H5 HA antigen has been completed.
- The effect of polymer chemistry on immune cell activation has been quantified via cell surface marker expression and cytokine secretion profiles.
- Initial *in vivo* studies have established the required doses for HA administration in mice.
- DNA plasmids encoding H5N1 HA protein, pVAX1-H5HA and plasmid encoding reporter gene for luciferase (Luc), pVAX1-Luc, have been efficiently expressed the transgenes *in vivo* post in tramuscular injection as was determined by non-invasive animal optical imaging, real time RT-PCR as well as reverse-trancriptase PCR .
- Co-administration of pVAX1-Luc/pVAX1-H5HA plasmid with Pluronics block copolymers (0.01% SP1017/ 0.3% P85) significantly increased the level of transgene expression in skeletal muscle of mouse.
- Transgene expressions were also greatly prolonged and lasted for at least 50 and 28 days for Luc and H5HA gene respectively, in skeletal muscle.
- DNA plasmids were formulated intowell-defined BICs using mannose-modified cationic block copolymers, mannose-PEG-PLL.
- Stability of BICs was reinforced by introduction of reductively cleavable crosslinks.
- In vitro cytotoxicity studies revealed that crosslinked BICs were less toxic compared to non-crosslinked complexes.
- Transfection efficiency was preliminarily evaluated in murine and human macrophage cells.

REPORTABLE OUTCOMES

1. Shaheen Ahmed, Tatiana K. Bronich, Alexander Kabanov. “Development of DNA Vaccine against Avian Influenza A Virus (H5N1)”, *Molecular Therapy* **19**, 1360-1398 (July 2011) and in Abstract book of the 14th Annual Meeting of the American Society of Gene & Cell Therapy, May 18-21, 2011, Seattle, WA.

2. J.H. Wilson-Welder, L. Huntimer, K.A. Ross, B. Carrillo-Conde, L. Pruisner, B. Narasimhan, M.J. Wannemuehler, "Encapsulation of Antigens into Microparticles Results in Dosage Sparing Capabilities," *Trans. Soc. Biomater*., **34**, 257 (2011)

3. Shaheen Ahmed, Caroline Roques,Tatiana K. Bronich, Alexander Kabanov. "Adjuvented DNA Vaccine against Avian Influenza A Virus (H5N1)", In book of abstracts of 8th International NanoDDS, Omaha, NE, Oct. 2010, p.115.

Conference Presentations

1. J.H. Wilson-Welder, L. Huntimer, K.A. Ross, B. Carrillo-Conde, L. Pruisner, B. Narasimhan, and M.J. Wannemuehler, "Encapsulation of Antigens into Microparticles Results in Dosage Sparing Capabilities," Annual Meeting of the Society of Biomaterials, Orlando, FL, April 14, 2011

2. J. Wilson-Welder, L. Huntimer, K.A. Ross, B. Carrillo-Conde, L. Pruisner, B. Narasimhan, and M.J. Wannemuehler, "Encapsulation of Model Antigens Into Microparticles Results in Dosage Sparing Capabilities," Annual AIChE Meeting, Salt Lake City, UT, November 11, 2010

3. S. Ahmed, T.Bronich, A. Kabanov, "Development of DNA Vaccine against Avian Influenza A Virus (H5N1)," 14th Annual Meeting of the American Society of Gene & Cell Therapy, Seattle, WA, May 18-21, 2011.

Invited Presentations

1. "Pathogen-Mimicking" Nanoparticles for Prevention and Treatment of Respiratory Infectious Diseases," California Nanosystems Institute, UCLA, Los Angeles, CA, May 3, 2011
2. "Pathogen Mimicry: A Viable Design Strategy for Prevention and Treatment of Respiratory Infectious Diseases," Osborn Club, Iowa State University, Ames, IA, April 11, 2011
3. "Nanoparticle-Based Platforms for Biodefense Pathogens and Global Public Health," Global Biosurveillance: Enabling Science and Technology, 2nd B iothreat Nonproliferation Conference, Santa Fe, NM, January 19, 2011

4. "Nanovaccine Platforms for Infectious Respiratory Diseases," FAN-IITB Conference on Biosciences and Bioengineering Applications in Healthcare, Santa Clara, CA, September 26, 2010

5. "Nanovaccine Platforms for Biodefense Pathogens," Department of Chemical and Biochemical Engineering, University of Iowa, Iowa City, IA, September 16, 2010

CONCLUSIONS

- The robust procedures for the synthesis and purification of H5 HA antigen were developed.
- The methods for the preparation of targeted, stable, and well-defined BICs based on mannose-capped poly(ethylene glycol)-block-poly(l-lysine) diblock copolymers and plasmid DNA were developed.
- Mannose functionalized BICs provide a safe and stable platform for gene delivery to macrophages.
- We demonstrated that co-administration of DNA (pVAX1-Luc and pVAX1-H5HA vaccines with Pluronic block copolymers (Pluronic P85 or SP1017) significantly enhance gene expression and prolongation of both Luciferase reporter gene and therapeutic H5HA gene in the skeletal muscle of mouse.

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