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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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Table of Contents

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 was focusing 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.

STATEMENT OF WORK

We are on target or ahead in most of the tasks as described in three previous annual reports (for YEAR 1, 2, and 3) and further accounted for in the present annual report (YEAR 4). This no-cost extension to the project will allow us to finish the critical experiments related to this project. In this time period we will focus mainly on Platform C. We have already identified novel cationic block copolymers that allow preparing stable DNA-containing block ionomer complexes. Click chemistry approach was applied to introduce targeting ligands on the surface of the complex particles that were further characterized for targeted delivery of DNA to the antigen presenting cells. We demonstrated that these polyplexes are less cytotoxic to macrophages as well as stable against thermal challenge. These new promising cationic polymers will be further evaluated in murine model to assess immunogenicity and determine the lead vaccine formulation that protect against H5N1 viral challenge during the no-cost extended period. Finally, we will prepare manuscript and the final report of this project.

Platform A

Recently, mice were immunized with soluble H5 hemagglutinin trimer ($sH5_3$) alone or in conjunction with $H5_3$-loaded, poly I:C–loaded, or blank 20:80 CPTEG:CPH polyanhydride nanoparticles. All mice received a total of 10 μg $H5_3$ per immunization delivered subcutaneously, with booster immunizations at days 21 and 42 as well. At 56 days post-initial immunization, serum was collected from the animals to determine the presence of anti-$H5_3$ antibodies. A fluorescent, multiplex based assay was used to quantify the total serum anti-$H5_3$ IgG. All treatment formulations induced antibodies significantly greater than the saline control; however, there remained little variation among the formulations themselves (Figure 1A). H5-pseudotyped reporter viruses were also used to determine the neutralizing antibody titers. Consistent with total antibody, neutralizing antibody titers were quite similar among formulations (Figure 1B).

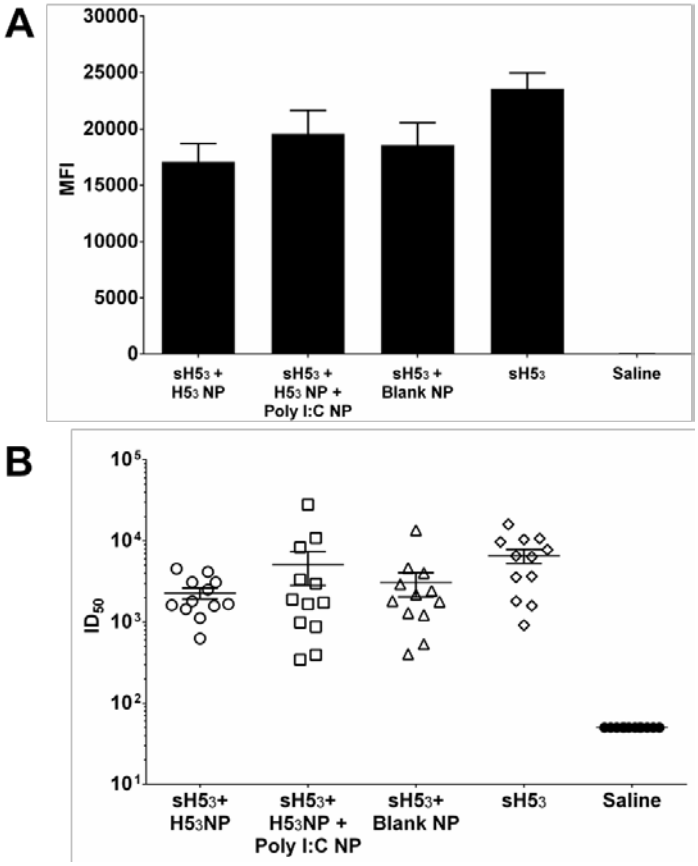


Figure 1: Antibody responses to H53 immunization. Mean fluorescent intensity (MFI) quantifying total IgG serum antibody via a multiplex assay (A). Neutralizing antibody titer determined via H5-pseudotyped reporter viruses (B).

To determine the ability of the total and neutralizing antibodies to provide protection, all mice were intranasally challenged with a live, low pathogenic H5N1 influenza virus 63 days post-initial immunization. Mice receiving saline immunizations began to lose weight 4 days post-challenge and continued to lose approximately 20% of their total body weight before recovering. In contrast, all mice immunized with $H5_3$ and/or polyanhydride nanoparticle formulations maintained their body weight similar to naïve, non-infected control mice. The viral load present in the lung tissue of mice was also determined 3 days post-challenge. As expected, all immunization formulations were able to induce a reduction of viral load upon challenge. Similarly, bronchoalveolar lavage fluid of immunized mice collected 3 days post-challenge showed a large decrease of inflammatory cytokines compared to the saline control (data not shown).

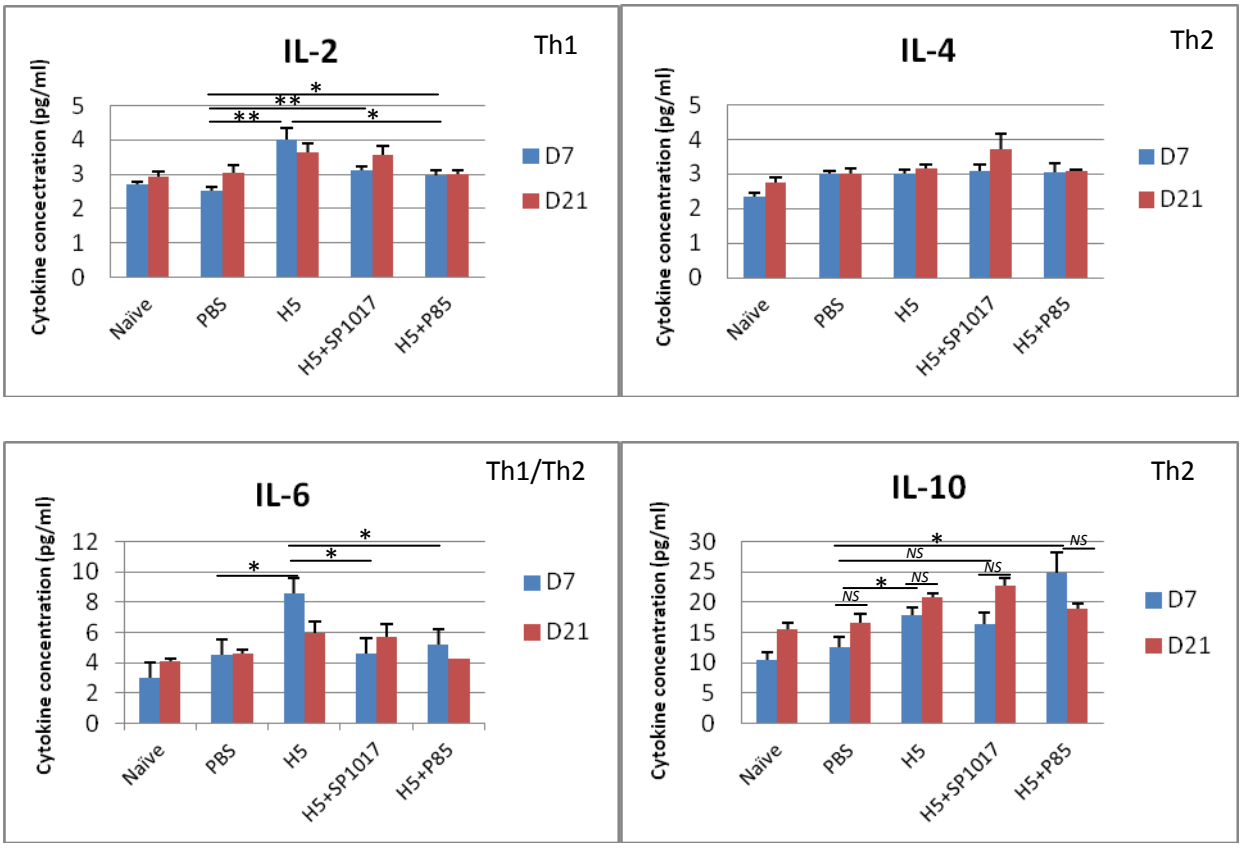
These initial results display a proof-of-concept for this vaccine platform, emphasizing the strength and immunogenicity of the hemagglutinin trimer cloned and expressed. Mice receiving $sH5_3$ alone induced neutralizing antibody titers that were protective against a low pathogenic challenge, similar to mice receiving polyanhydride nanoparticles. Currently, experiments are in progress to examine the advantages of polyanhydride nanoparticles encapsulating $H5_3$ antigen.

Previous work has underlined dose sparing properties of polyanhydride nanoparticles, allowing the enhancement of immune responses towards suboptimal doses of antigen. Likewise, polyanhydride nanoparticles have displayed enhancement of cell-mediated immunity, an important aspect of vaccines targeting viral pathogens such as influenza.

Platform B

Cytokine responses

Cytokines serve as couriers between cells of the immune system and play a crucial role in orchestrating the immune response after vaccination. Therefore, they are an important indicator of effective induction of immune effectors. In order to determine if our Pluronic/pVAX1-H5HA DNA vaccine formulations induce any cytokine responses, in this current study we measured the concentrations of Th1 (IL-2 and INF-γ), Th2 (IL-4 and IL-10) and Th17 (IL-17) cytokines in sera from mice vaccinated with 100 µg of pVAX1-H5HA pDNA formulated with/without 0.01% (w/v) SP1017 or 0.3% (w/v) P85 on day 7 and 21. Th1/Th2/Th17 cytokine concentrations were determined using the Mouse Cytometric Bead Array (CBA) Kit (BD Biosciences, San Diego, CA) following the manufacturer's protocol.



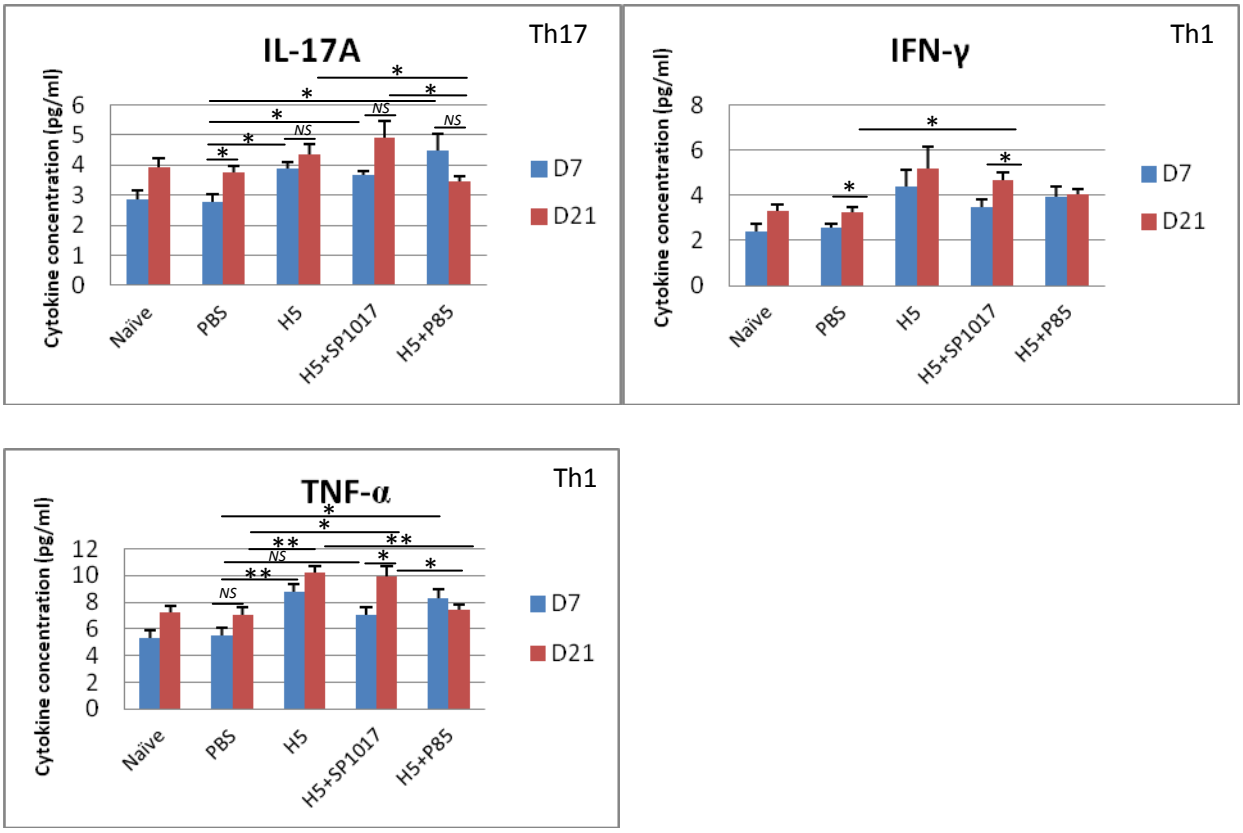


Figure 2. The cytokine response induced in the blood serum after pH5HA vaccination. 100 µg of pVAX1-H5HA pDNA formulated with/without 0.01% (w/v) SP1017 or 0.3% (w/v) P85 in 50 µL of PBS were administered in tibialis anterior muscles (4 mice/group) and boosted with the same formulations on D28. Sera from individual mice were taken 7 and 21days after administration of vaccine formulations and were used to measure Th1 (IL-2, TNF and IFN-g), Th2 (IL-4 and IL-10), Th1/Th2 (IL-6) and Th17 (IL-17) cytokines by Mouse Cytometric Bead Array (CBA). The data is presented as the mean cytokine concentration (pg/ml)±SEM and analyzed with Student's *t*-test (n=4). **P*<0.05, ***P*<0.01 and *NS* is not significant at 0.05 levels.

Pluronic /pVAX1-H5HA pDNA formulated vaccine groups had significantly higher levels of Th1 (IL-2 and TNF), Th2 (IL-10) and Th17 (IL-17) cytokines on day 7 compared to PBS injected mice (Figure 2). However, on day 21, only sera from H5+SP1017 vaccinated mice induced significant increase in TNF and IFN-g (Th1) cytokines. Our data also showed strong Th17 responses. On the other hand, IM vaccination induced significantly higher concentrations of IL-10 (Th2) in H5+P85 injected group of mice on day 7 as compared to other groups but, there was no IL-4 cytokine responses in any of the vaccinated groups. However, only pDNA vaccinated group showed significant increase of IL-6 (Th1/Th2) cytokines on day 7 (Figure 2). These results demonstrated that Pluronics /pVAX1-H5HA pDNA vaccine formulations induced Th1, Th2 and Th17 cytokine responses in mice.

H5HA specific antibody responses

In order to determine if our Pluronics/pVAX1-H5HA DNA vaccine formulations induce antibody responses we performed the ELISA and our data shown in Figure 3. The present study

demonstrated that pH5HA vaccine with/without Pluronics could induce specific IgG antibodies in mice (Figure 3). On the day 21 the IgG antibody level in pH5HA /SP1017 treated groups was higher those in the groups received the naked pH5HA or pH5HA formulated with 0.3% w/v P85. (Figure 3). However, on the day 56 the naked pH5HA exhibited significant increase in IgG antibody compared to pH5HA with Pluronics SP1017 or P85. The mice vaccinated with pH5HA/SP1017 did not show any booster response while the naked pH5HA or pH5HA formulated with 0.3% w/v P85 showed significant booster effect (Figure 3). These data indicated that Pluronic/pVAX1-H5HA DNA vaccine formulations could induce lasting primary antibody responses.

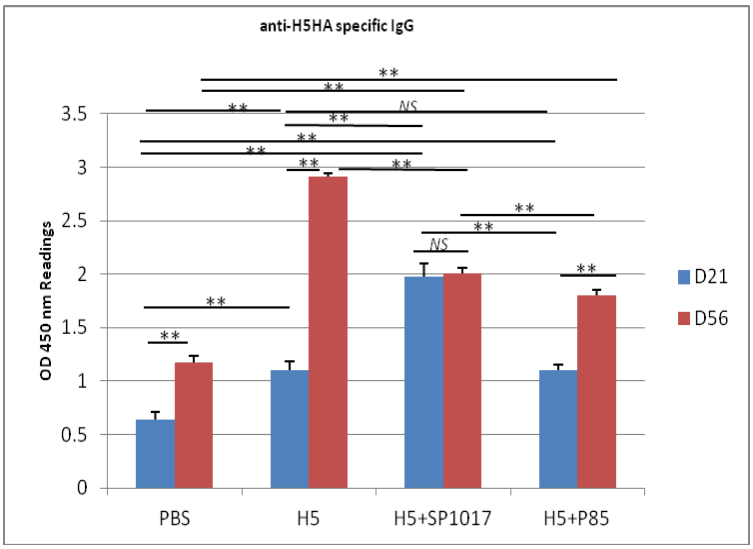


Figure 3. Total serum IgG antibody responses to H5HA. 100 µg of pVAX1-H5HA pDNA formulated with/without 0.01% (w/v) SP1017 or 0.3% (w/v) P85 in 50 µL of PBS were administered in tibialis anterior muscles (4 mice/group) and boosted with the same formulations on D28. Sera from individual mice were taken 21and 56 days after administration of vaccine formulations and were tested for binding to H5 hemagglutinin by ELISA. All data are presented as means ±SEM and analyzed with Student's *t*-test (n=4) at 1:31250 serum dilution. ***P*<0.01, **P*<0.05 and *NS* is not significant at 0.05 levels.

Hemagglutination inhibition assay

The hemagglutination inhibition (HAI) assay was adapted from the CDC laboratory-based influenza surveillance manual (1).To inactivate non-specific inhibitors, sera were treated with receptor destroying enzyme (RDE) (Denka-Seiken, Tokyo, Japan) prior to being tested. Briefly, three parts RDE was added to one part sera and incubated overnight at 37°C. RDE was inactivated by incubation at 56°C for ~60 min. RDE-treated sera were two-fold serially diluted in v-bottom microtiter plates. An equal volume of virus iVN04 (NR-4143, Biodefense and Emerging Infections Research Resources Repository, NIAID, NIH, Bethesda, MD), adjusted to approximately 4 HAU/25 µl was added to each well. The plates were covered and incubated at room temperature for 30 min followed by the addition of 1 % turkey erythrocytes (TRBC) (Innovative Research, MI, USA). The plates were mixed by agitation, covered, and the TRBC were allowed to settle for 60 min at room temperature. The HAI titer was determined by the reciprocal dilution of the last well, which contained non-agglutinated TRBC. Positive and negative serum controls were included for each plate.

Mice received 2 doses of pH5HA had higher serum HAI Ab titers against the A/Vietnam/1203/2004 (H5N1) virus (Figure 4). A very similar level of serum HAI Ab titers were observed in the groups of mice those primed and boosted with pH5HA/0.01% (w/v) SP1017 or 0.3% (w/v) P85 (Figure 4).

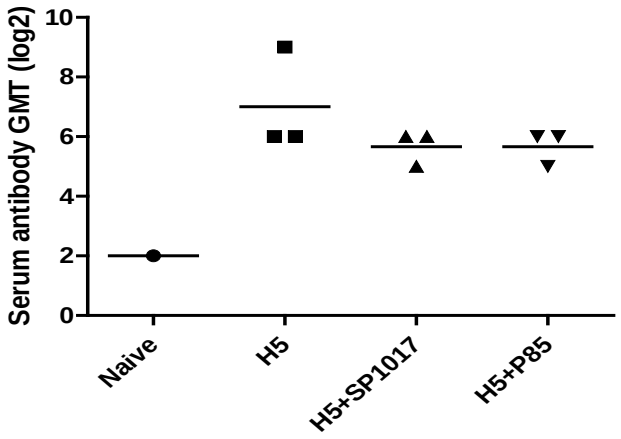


Figure 4. The hemagglutination inhibition (HAI) antibody response induced after pH5HA vaccination. 100 µg of pVAX1-H5HA pDNA formulated with/without 0.01% (w/v) SP1017 or 0.3% (w/v) P85 in 50 µL of PBS were administered in tibialis anterior muscles (3 mice/group) and boosted with the same formulations on D28. Sera from individual mice were taken 56 days after administration of vaccine formulations and were used to measure HAI antibody response against A/Vietnam/1203/2004 (H5N1). The geometric mean titer obtained from 3 mice ±SD.

Viral challenge study

Next, we determine whether immunization with Pluronic/pVAX1-H5HA DNA vaccine formulations can protect the mice against Low Pathogenicity H5N1 viral challenges.

100 µg of pVAX1-H5HA 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 (9 mice/group) and boosted on day 28 (D28) with the same formulations. On day 64 after the first vaccination the animals were challenged via intranasal administration of 30 µl of 1:10 diluted $1.9x10^6$ $TCID_{50}$ Low pathogenicity influenza virus A/H5N1 (VNH5N1-PR8CDC-RG. Four mice from each group were euthanized on day 3 for determination of viral load in lung while the remaining 5 mice in each group were monitored for development of clinical signs and loss of body weight for 14 days (Figure 5).

All mice in the control group (PBS treatment) survived the challenges while exhibiting higher weight loss and delayed recovery compared to the vaccinated groups (Figure 5 A and B). All the animals in Pluronic/pVAX1-H5HA DNA vaccinated groups showed less discomfort. H5+SP1017, H5+P85 and pDNA alone vaccinated group of animals exhibited a weight loss of only 14%, 19% and 17% respectively of their average pre-challenge body weight on day 7 and regained their original body weight on day 14 (Figure 5 A). Though, Pluronic/pVAX1-H5HA DNA vaccine formulation significantly protected the weight loss compared to PBS control group, there was no

significant difference in weight loss observed among the Pluronic/pVAX1-H5HA DNA vaccinated groups (Figure 5 B).

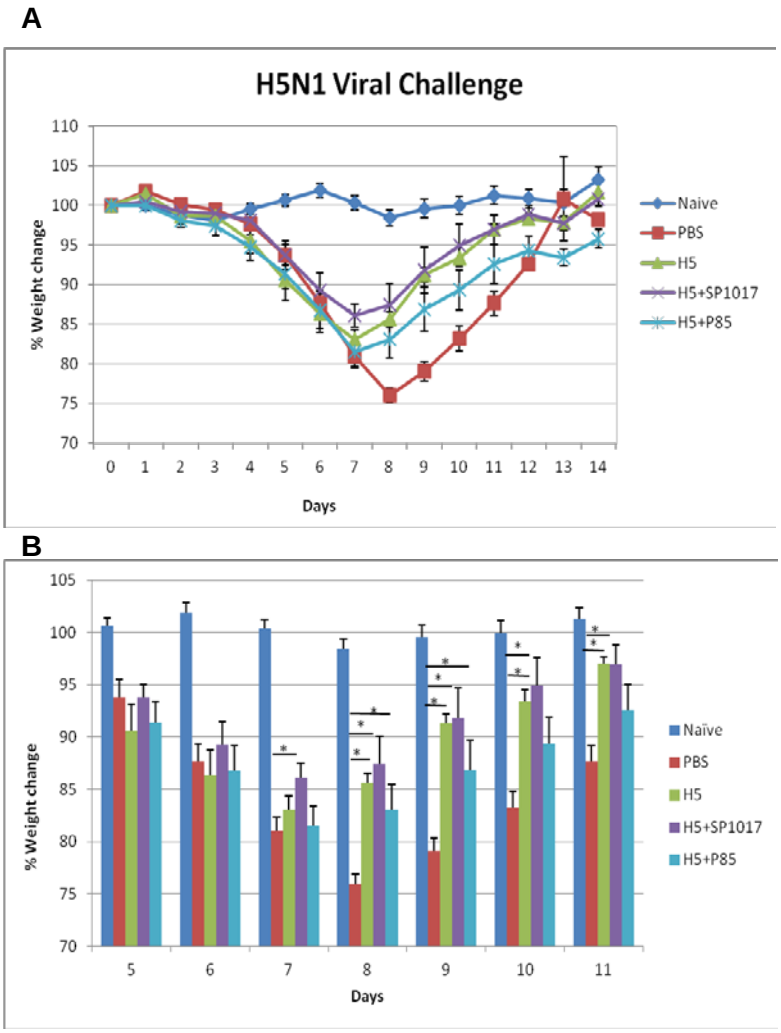


Figure 5. 100 µg of pVAX1-H5HA pDNA formulated with/without 0.01% (w/v) SP1017 or 0.3% (w/v) P85 in 50 µl of PBS were administered in tibialis anterior muscles of BALB/c mice (5 mice/group) and boosted with the same formulations on D28. Mice were challenged intranasally on D64 with 30 µl of 1:10 diluted $1.9x10^6$ $TCID_{50}$ Low pathogenicity influenza virus A/H5N1 (VNH5N1-PR8CDC-RG). A, Body weight changes data expressed as a percentage of the pre-challenged body weight and presented as mean ± SEM (*n* =3-5) and B, data analyzed with Student's *t*-test (n=5), **P*<0.05.

In order to determine the viral load in the challenged mice lungs, lung tissues were collected from the vaccinated groups of mice on 3 days post-challenge. Lung tissues were then tested by Real-time RT-PCR to determine viral load in each group of mice. Lung tissues were processed and analyzed following the modified procedure of Alsharifi, et. al., 2009 (2). PCR was performed on an Applied Biosystems 7500 Fast Real-Time PCR System. For the standard curve, normal, non-influenza challenged mice lungs (PBS controls) were used. RNA from Stock influenza A H5 virus was extracted using the Qiagen QiAmp Viral RNA Kit Mini Kit (Qiagen, Carlsbad, CA). For the standard curve, the H5 extracted viral RNA were standardized to the normal mouse lungs. Standard curves were performed with each set of PCR reactions (Figure 6 A).

Quantitation of viral genetic loads in lungs of Pluronic/pVAX1-H5HA DNA vaccinated groups demonstrated protective effect of the vaccine formulations compared to PBS controlled mice on day 3 post-viral challenge (Figure 6). Pluronic P85/ pVAX1-H5HA DNA vaccinated groups had

significantly less viral RNA in the lungs compared to PBS vaccinated mice. (Figure 6 B). However, viral loads determined for Pluronic/pVAX1-H5HA DNA vaccinated groups did not show any significant differences among them.

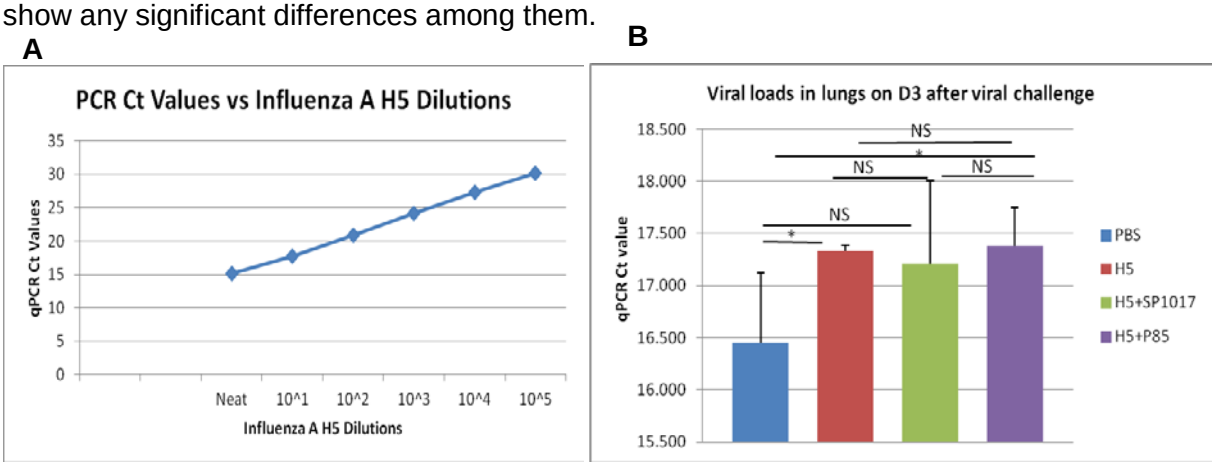


Figure 6. Viral loads in lungs on day 3 post-viral challenge. 100 µg of pVAX1-H5HA pDNA formulated with/without 0.01% (w/v) SP1017 or 0.3% (w/v) P85 in 50 µl of PBS were administered in tibialis anterior muscles of BALB/c mice (4 mice/group) and boosted with the same formulations on D28. Mice were challenged intranasally on D64 with 30 µl of 1:10 diluted $1.9x10^6$ $TCID_{50}$ Low pathogenicity influenza virus A/H5N1 (VNH5N1-PR8CDC-RG). A. Threshold (Ct) values for H5 standard control dilutions. B. Viral Loads in lungs on D3 post-viral challenge as expressed mean qPCR Ct values ± SD and data analyzed with Student's *t*-test (n=4). **P*<0.05, *NS* is not significant at 0.05 levels.

Overall, these results demonstrated that Pluronic/pVAX1-H5HA DNA vaccine formulations have significant protective effective over PBS control treatment against Low pathogenicity influenza virus A/H5N1 (VNH5N1-PR8CDC-RG).

We also continued studies in the task aimed to maximize levels and duration of transgene expression after administration in skeletal muscle in a mouse. We previously reported an increase in gene expression as a result of distal inflammation (Ischemia) only when DNA was formulated with Pluronic P85 and not DNA alone. We studied another model of acute distal inflammation (i.e. peritonitis) to explain the generality of increase in gene expression because of inflammation. In peritonitis distal inflammation model, *i.p* injection of filter-sterilized λ-Carrageenan (1mg/200µl PBS) was performed on 0 day and 2nd day followed by *i.m.* injections of plasmid DNA in healthy leg (t.a.) a day after generation of inflammation. The level of inflammation in peritonitis was studied by quantifying percent WBC's in blood by vetscan hematology analysis of serial blood samples (Figure 7C) from tail vein and compared to blood samples from healthy animals. DNA injections were performed with/without Pluronic P85 (0.3% w/v) and compared to healthy animals. In order to separate the effect of Pluronic on gene expression, Pluronic and DNA were injected separately in left and right hind limb, respectively. Luciferase reporter protein expression was monitored by injecting D-luciferin (150 mg luciferin/kg body weight) *i.p.* followed by non invasive *in vivo* bioluminescent imaging using Xenogen IVIS 200 imaging system..

Similar to the previous results, in DNA+P85 group, an increased gene expression was observed in the presence of distal inflammation (in this case peritonitis) compared to healthy animals. Again this increase was limited to DNA/Pluronic group and was not observed in animals injected

with naked DNA (Figure 7B black bars and white bars of DNA group). Interestingly, Pluronic when separated by space (DNA and P85 injected in separate legs) significantly increased gene expression both in healthy and peritonitis animals compared to DNA alone (Figure 7B and D). Infiltration of immune cells (e.g. neutrophils, macrophages etc) have long been corelated with repair and regeneration of muscle tissue. Increase in gene expression in the presence of P85 could be explained by increased immune cell infiltration and their role in increasing the transgene expression and tissue repair. Therefore, we further attempted to understand the role of macrophage in gene transfer in *in vivo* (mice hindlimb ischemia model, MHLIM) and *in vitro* models (co-culture of macrophage with myoblast and myotubes).

Colocalization of GFP expression and cell specific markers was studied to determine what cells are expressing the transgene after intramuscular injection of DNA. Plasmid DNA encoding GFP with/without Pluronics was injected into the ischemic *t.a.* muscle of MHLIM. The muscle specimens were harvested 4th day after injection and individually processed for immunohistochemistry. The results of this experiment suggest that in the presence of P85/SP1017 the plasmid DNA is also taken-up by antigen presenting cells (Figure 8) in addition to muscle cells.

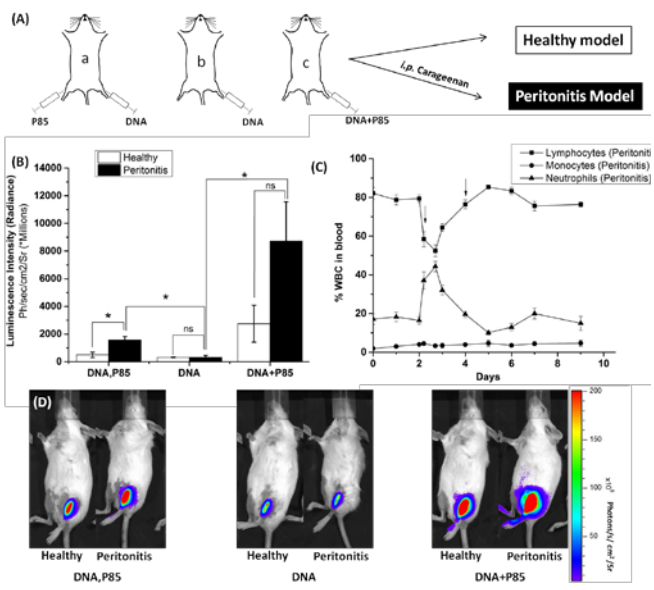


Figure 7. Scheme of experiment (A) Involves 50ul injection of P85 (0.3% wt/vol) and DNA alone (10 µg) in separate muscles (a), DNA alone (b), and DNA+Pluronic P85 (c) in *tibialis anterior* muscle of healthy and peritonitis inflammation model. ***In vivo* gene expression after 4 days of *i.m.* injections (quatified by bioluminescene imaging) (B):** DNA injections were performed in *tibialis anterior* muscle of healthy mice (white bars) and peritonitis model (black bars). **Kinetics of WBC count (C)** in peritonitis model (solid lines) and healthy mice (dotted lines B). 30-50µl peripheral blood was collected at different time points by tail clip method and percent white blood cells was determined by VetScan HM5 hematology system. Data are mean ±SEM (n=5). . **Representative IVIS images (D).** Data are mean ±SEM (n=3). ns is non significant at p value of 0.05 * p < 0.05

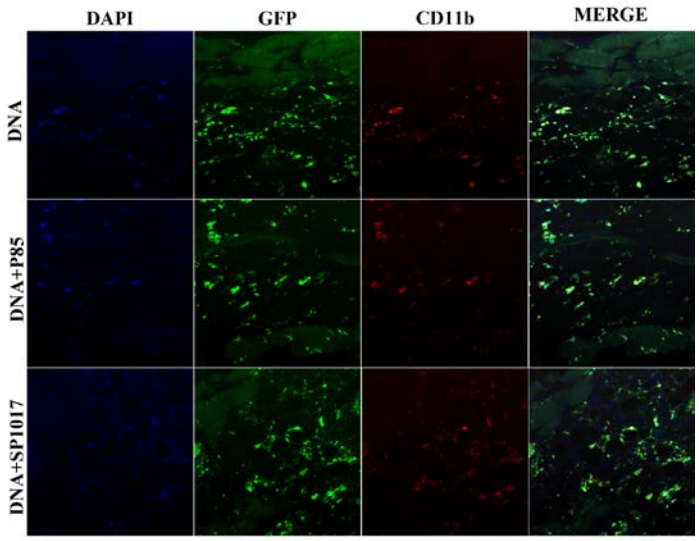
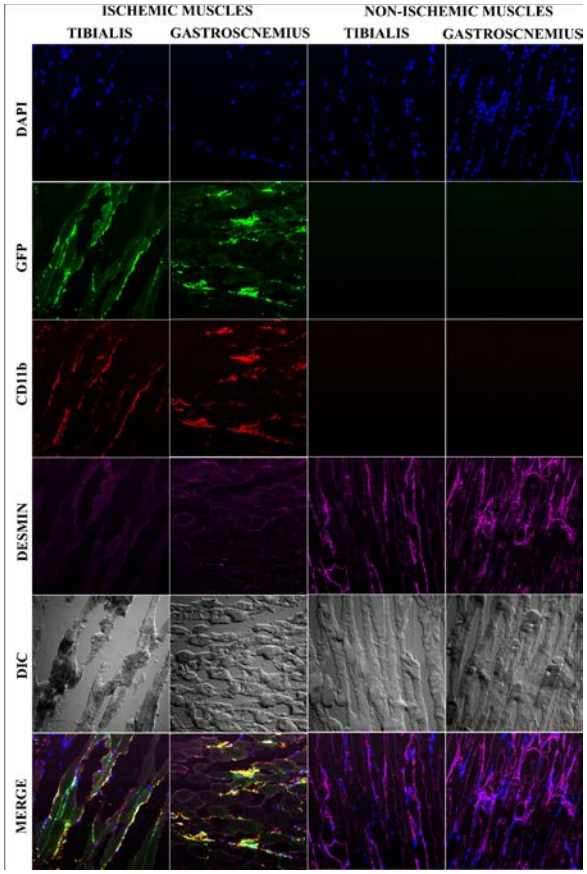


Figure 8. Confocal imaging of GFP expression in ischemic muscle 4 days after single injection of DNA alone, DNA+P85 (0.6% w/v) or DNA+SP1017 (0.1% w/v). The color staining corresponds to nucleus (blue); macrophage/myeloid cells (Anti Cd11b) (red); and GFP (green). The GFP expression was assayed by immunohistochemistry using polyclonal rabbit anti-GFP primary antibody. The last panels in each row present digitally superimposed images of three preceding panels to visualize the colocalization (yellow or white). The images were taken with Ziess 710 confocal laser scanning microscope using 20x objective.

We further studied the possibility of horizontal transfer of nucleic acids (DNA/mRNA/protein) from APC's to adjacent muscle cells upon *i.m.* Pluronic mediated gene delivery. To test this hypothesis, macrophages transfected *ex vivo* with GFP encoding plasmid were adoptively transferred to ischemic mice 48 hours post ischemic surgery by *intrajugular vein* (*i.j.v)* injection. Ischemic and non-ischemic muscles (i.e. *tibialis anterior* and gastroscnemius) were isolated 3 days post DNA injection and processed for immunohistochemistry. 10 µm sections were studied for co-localization of GFP expression with macrophages (CD11b) and muscle cells (desmin) using Ziess LSM 710 confocal laser scanning microscope. As expected, GFP expressing



Fgure 9. Confocal imaging of ischemic and non ischemic muscles of ischemic mice: RAW 264.7 macrophages were transfected ex vivo and injected in balb/c mice 48 post ischemia surgery by *i.j.v* injection. Tissues were isolated 3 days post injection and 10um sections of frozen tissues were processed for immunohistochemistry. The color staining corresponds to nucleus (blue), GFP expression (green), CD11b cell marker (red), desmin muscle marker (cyan), phase contrast image (DIC). The last panels in each row present digitally superimposed images of five preceding panels to visualize the colocalization (yellow or white). The images are representative of 3 sections per muscle and 3 mice per group. The images were taken with Ziess 710 confocal laser scanning microsope using 20x objective. Scale bar represents 50um.

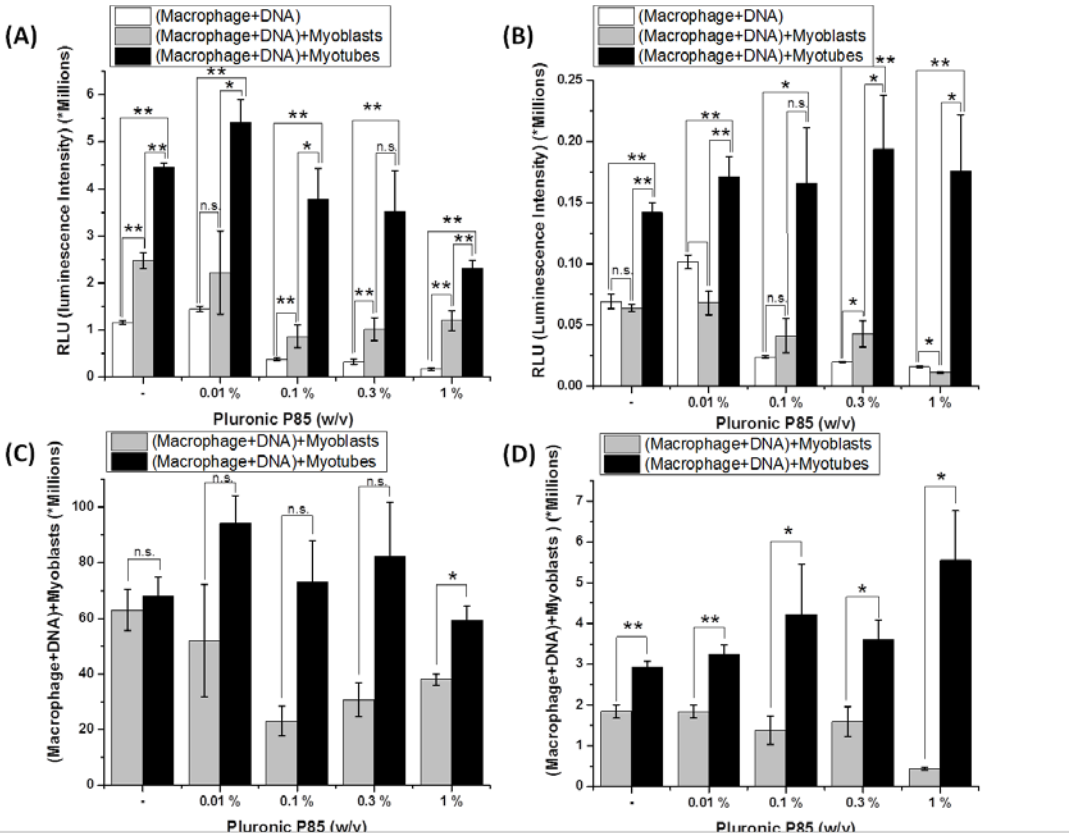
macrophages were recruited to ischemic muscles and not non-ischemic muscles and GFP expression colocalized with CD11b+ve macrophages in ischemic tissues (Figure 9). Interestingly, GFP expression was also observed in muscle fibers and it co-localized with muscle cell marker (Desmin). This would imply that the APCs exchange and transfer the expressed protein/nucleic acids across the muscle membranes and thus collectively represents a reservoirs and conduits for the gene expression. Notably there were lower levels of muscle specific marker (desmin) in ischemic muscle compared to non-ischemic muscle which is a characteristic feature of ischemia that results in depletion of desmin marker and was reported in earlier studies too (3, 4). In an important way, macrophages were localized on the close proximities/edges of muscle fibres characterized by intense colocalization of GFP and CD11b marker. This cell-to-cell contact may thus help assist exchange of repackaged nucleic acids or proteins from macrophages across the muscle membranes of otherwise hard to transfect muscle fibers.

We developed a co-culture model to demonstrate the importance of cell-to-cell contacts whereby *in vitro* transfected macrophages (Mac) were co-cultured on monolayer of post-mitotic differentiated myotubes (MT) and precursor myoblasts (MB). Our aim was to understand whether transfer of nucleic acids can occur from transfected macrophages to host cells (myoblasts or myotubes). We utilized two different plasmids with different promoters: gWIZ-luc plasmid with a constitutive promoter CMV for macrophage transfection and pDesmin-lucia plasmid with cell specific (myoblast and myotube) promoter desmin. When the nucleic acids are transferred, the gene expression should increase upon co-culture, if not there will be no increase in expression. Moreover, if the nucleic acids are transferred and promoter is constitutive, reporter gene will be expressed in both macrophages and co-culture monolayer (MB /MT) so the fold increase in reporter protein expression would be smaller than if the promoter is myoblast/myotube specific (Desmin promoter). In the latter case only MT or MB cells will express the genes (promoter specific expression) and not macrophages, so the fold increase would be more pronounced compared to former. Moreover, because desmin expression increases upon differentiation of myoblasts to myotubes, an increase in gene expression would be even higher in Mac+MT compared to Mac+MB co-culture. Comparison of these two systems served as an internal control to determine the increase in gene expression and affirm the mechanism of gene transfer.

Macrophages were transfected with commercial lipoplex kit genePORTER3000 and membrane bound lipoplexes were removed by rinsing transfected macrophages twice with 1mg/ml heparin sulphate. Macrophages were resuspended in complete media or CM (DMEM supplemented with 10% FBS) and 100,000 cells/well were cultured on top of 50,000/well C2C12 myoblasts or C2C12 myoblasts derived myotubes (day 8 post differentiation). After 2 hours when macrophages adhere to myoblast/myotube monolayer, the co-culture was rinsed thrice with serum-free media to remove traces of serum. The co-culture was then exposed to increasing concentrations of Pluronic P85 (0.01, 0.3, 1.0% w/v) for 2 hours.

In CMV plasmid co-culture model total gene expression increased 2 times in Mac+MT compared to Mac+MB and this fold increase remained the same with increasing concentrations of Pluronic P85 (both total and normalized gene expression) (Figure 10 A and C). Total gene expression was always higher in co-culture compared to Mac alone when CMV plasmid was used (Figure 10A). On the other hand, in desmin plasmid co-culture model total increase in gene expression was 2 times in Mac+MT compared to Mac+MB, and these folds increase in gene expression was significantly higher with increasing concentration of Pluronic P85 (16- and 13- times higher for total and normalized gene expression, respectively). As expected, the gene expression levels in macrophage alone were always similar to Mac+MB and, hence, confirm the desmin promoter dependent expression in Mac+MT (Figure 10B). Increase in gene expression in Mac+MT co-culture can only be explained by desmin specific expression and happened only when nucleic acids were transferred horizontally from Mac to MT.

Although, total gene expression was used to compare the levels of gene expression in co-culture (Mac+MB/MT) compared to Mac alone (Figure 10A and B), little fold increase in CMV and significant high fold increase in desmin specific gene expression was also observed in normalized data (Figure 10C and D), which further increased in P85 concentration-dependent manner. We also observed lower levels of normalized reporter protein upon co-culture compared to macrophage alone. This observation can be explained by the Fas-L Fas-R mediated apopotosis induced upon co-culture of macrophages on myoblast/myotube which is an evolutionary mechanism to reduce the inflammatory macrophages after the inflammation is resolved in muscle tissue.



Pluronic P85	Fold increase (total expression)		Fold increase (total expression/total protein)	
	(Macrophage+DNA)+Myoblast v/s (Macrophage+DNA)+Myotube	(Macrophage+DNA)+Myoblast v/s (Macrophage+DNA)+Myotube	(Macrophage+DNA)+Myoblast v/s (Macrophage+DNA)+Myotube	(Macrophage+DNA)+Myoblast v/s (Macrophage+DNA)+Myotube
	CMV promoter	Desmin promoter	CMV promoter	Desmin promoter
-	2	2	1	2
0.01 %	2	3	2	2
0.1 %	4	4	3	3
0.3 %	3	5	3	2
1.0 %	2	16	2	13

Figure 10. Coculture of macrophages transfected with gWIZ-luc plasmid (constitutive CMV promoter) (A,C) and pDesmin-lucia (muscle specific desmin promoter) (B,D) with precursor muscle cells and differentiated mytubes. The coculture was exposed to increasing concentration of pluronics for 2 hrs and gene expression was determined 24 hrs later. Fold increase in total gene expression (left two colums) and normalized gene expression to total protein in cell lysates (right two colums) represents cell specific expression of reporter genes and hence transfer of nucleic acids among macrophages to myoblasts or myotubes. Data are mean±SEM (n=6) and statistical significance was calculated using student's t test at $p<0.05$.

PLATFORM C

Formation, morphology and stability of Cationic Poly(2-Oxazolines) HEZ008/pDNA polyplexes

Previous report described the formation and characterization such as particle size and polydispersity of HEZ008/pDNA polyplexes, as well as the stability of polyplexes against physiological saline dilution and refrigerated storage. In this report we demonstrated spherical morphology and estimate diameter around 100nm of polyplexes exemplified by the Transmission Electron Microscopy (TEM) image of HEZ008/pDNA (N/P ratio 10) (Figure 11a). HEZ008 non-targeted polymer was able to condense DNAs and form polyplexes with pDNA, thus retarding pDNA migration on the agarose gel if N/P ratios above 2 (Figure 11b).

Stability of HEZ008/pDNA polyplexes is critical factors regarding the translation of this gene delivery platform into *in vivo* application. We subsequently performed a number of stability studies as following. First, we measured temperature stability of HEZ008/pDNA polyplexes at N/P ratio of 5 and 10 by plotting the Z-average diameter and PDI as a function of increasing temperatures from 25°C to 70°C (Figure 11c). Interestingly, the Z-ave diameter and PDI of polyplexes were found to be stable throughout the entire analyzed temperature both for 5:1 and 10:1 N:P ratios. We then investigated the stability of polyplexes against DNase I degradation. As shown in Figure 10d, pDNAs without complexing was degraded by DNase I while polyplexes at N:P ratio 5 or 10 preserved pDNAs from enzymatic degradation. When adding heparin, protected pDNAs were released from the complexes and then visualized on the agarose gel under UV light.

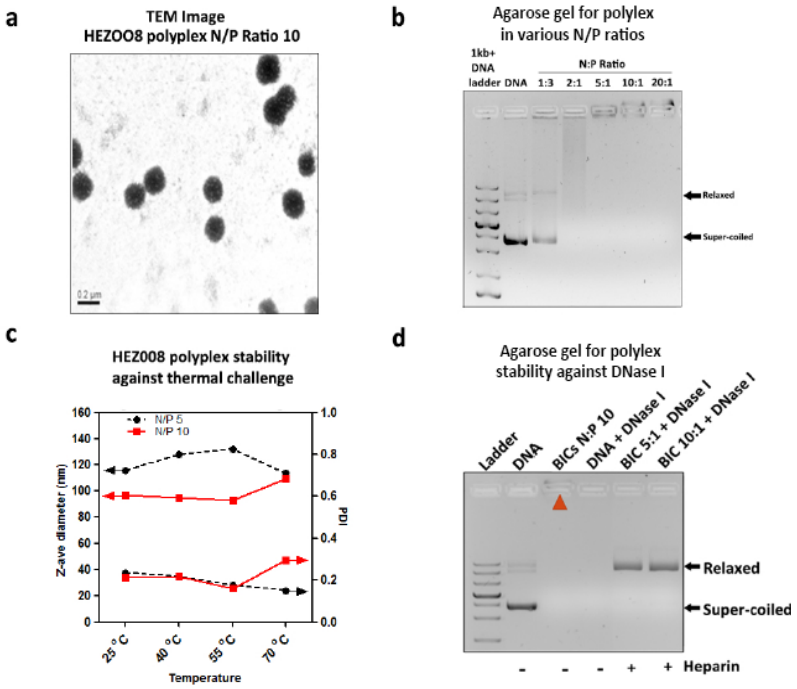


Figure 11. Formation, morphology and stability of HEZ008/pDNA polyplexes. (a) TEM image of polyplex at N:P ratio of 10 as a representative result; (b) agarose gel electrophoresis of polyplexes (N:P ratios ranged from 1:3 to 20:1); (c)The Z-ave diameter and corresponding PDI of polyplexes formed at N/P charge ratios 5 and 10 against temperature increase from 25 to 40, 55 and 70°C; (d) agarose gel electrophoresis of polyplexes (N/P ratios of 5 and 10) against DNase I digestion: lanes include DNA ladder, DNA, Polyplex N/P 10 without DNase I and Heparin, DNA plus DNase I, polyplex N/P 5 with DNase I plus DNA released by heparin, and polyplex N/P 10 with DNase I plus DNA released by heparin.

Serum protein binding of HEZ008/pDNA polyplexes

Nanoparticles binding to serum proteins are directly linked to their uptake and further clearance by mononuclear phagocyte system (MPS) *in vivo*. The hydrophilic blocks of poly(2-Oxaoline) [i.e. pMeOx and pEtOx homoblocks] have been previously discovered with anti-fouling or so called “stealth” properties due to the unique chemical structure of POx backbones. This prompted us to evaluate our newly synthesized HEZ008 in this regards when forming polyplexes. We utilized SDS-PAGE gel with SYPRO Ruby staining to show serum proteins that bound to various N:P ratios of polyplexes and found that very low protein binding to our polyplexes (all 5, 10, and 20 N:P ratios) as compared to the complexes formed by poly(ethylene gylcol)-b-poly(L-Lysine) (PEG-PLL)/pDNA or poly(L-Lysine) (PLL)/pDNAs (Figure 12b). We further quantified serum protein binding via microBCA assay and confirmed that very low serum protein binding by HEZ008/pDNA polyplexes in contrast to other control complexes (Figure 12a), in agreement with the intensity of bound serum protein bands visualized by SYPRO Ruby staining on the SDS-PAGE gel. The major bound proteins were identified from LC-MS analysis as IgG heavy and light chains, complementary C3, C4, and C5, and β and γ chain of fibrinogen isoforms.

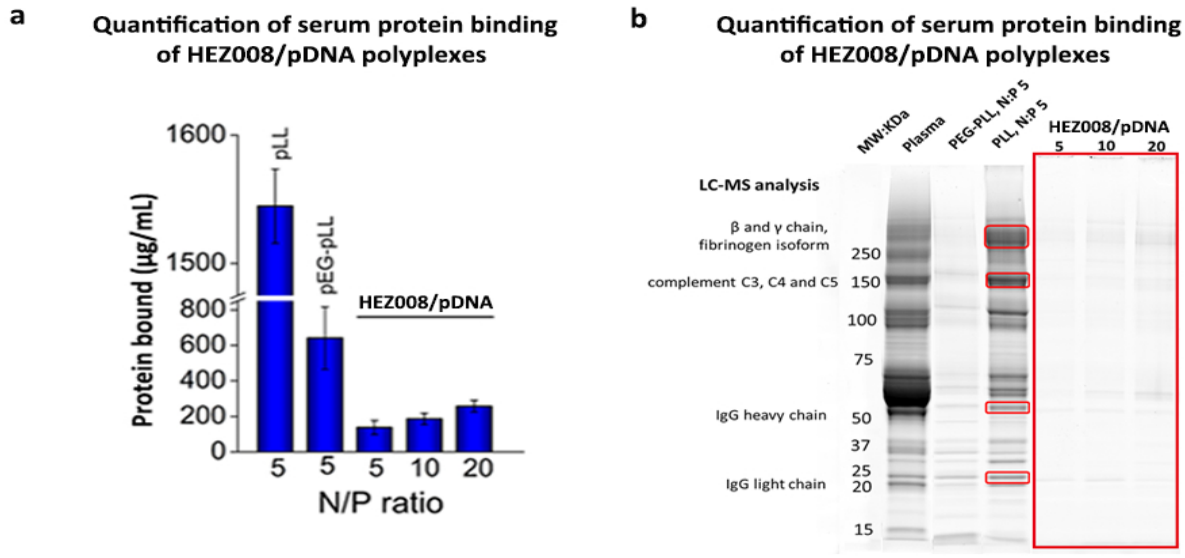


Figure 12. Serum protein binding of HEZ008/pDNA polyplexes. (a) Quantification of bound serum proteins to polyplexes corresponding to (b); (b) rat serum protein binding to HEZ/008 polyplexes (large red box) visualized on SDS-PAGE protein gel as compared to PEG-PLL or PLL/pDNA complexes. Small red box indicating major bound proteins identified by LC-MS as β and γ chain of fibrinogen isoforms, complementary C3, C4, and C5, and IgG heavy and light chains.

Determination of *in vitro* cytotoxicity of HEZ008 Polymers

Many cationic polymer based delivery systems were associated with toxicity issues. In order to condense DNA forming stable complexes, large excess of cationic polymers were often used, which may induce cytotoxicity due to their direct interaction with cell membranes. In this respect,

we studied *in vitro* cytotoxicities of HEZ008 polymers in RAW264.7 murine macrophage and B16 melanoma cell lines. Cytotoxicities of PEG-PLL were tested in parallel as a comparison. Interestingly, up to 500μg/mL of HEZ008 polymers showed minimum influence on RAW cells viability (Figure 13a) while PEG-PLL induced obviate cytotoxicity with an approximate IC_{50} value equal to 50μg/mL. Similar results were observed in B16 cells (Figure 13b).

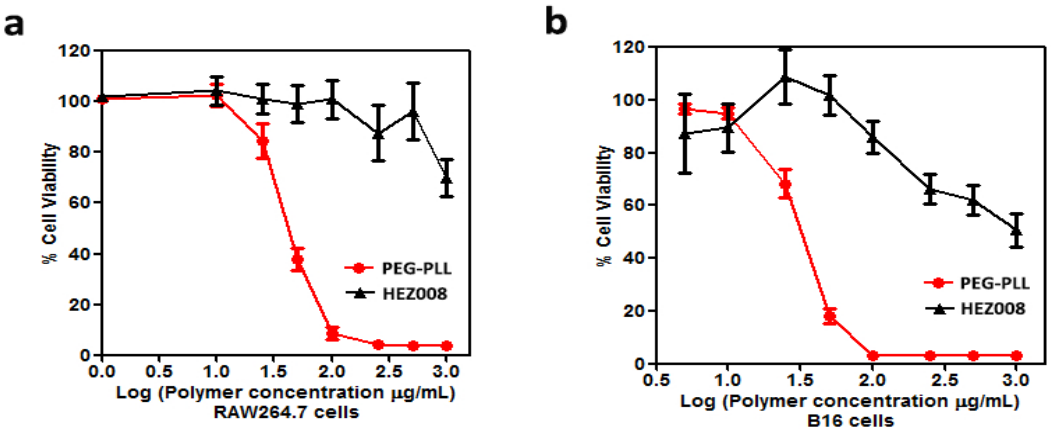


Figure 13. *In vitro* cytotoxicity of HEZ008 polymers in RAW264.7 murine macrophage cells (a) and B16 melanoma cells (b). Cell monolayers were incubated with respective polymer for 24 h before cell viability measurement by MTT assay. Data represents average ± S.D. of hexaplicate samples. Red line, PEG-PLL; Black line, HEZ008.

Analysis of cellular uptake of HEZ008/pDNA polyplex in macrophage cells

Cellular uptake of HEZ008/pDNA polyplex (pDNA was gWIZ-Luc plasmid DNA, N/P 5) were studied in RAW264.7 macrophage cells using confocal microscopy. For this experiment, the HEZ008 polymer was fluorescence labeled with Carboxyrhodamine. 110 (Figure 14, green) using click chemistry while pDNA was visualized using TOTO-3 fluorescenc dye (Invitrogen) (Figure 14, red). Figure 14 displayed the incubation of polyplex with cells at 0.5, 2, 4, 6, 8, 10 and 24 h time points, respectively. We found that the uptake process in RAW cells was rather slow, and till 4h no significant amount of polyplex were taken up but after 6 h and 8 h they started to enter the cells, and majority of polyplex were taken up after 10 h. Furthermore, polyplex were found very concentrated in the perinuclear region of the cell after 24 h incubation (Figure 14); however, significant loss of cell viability was also evidenced for 24 h time point.

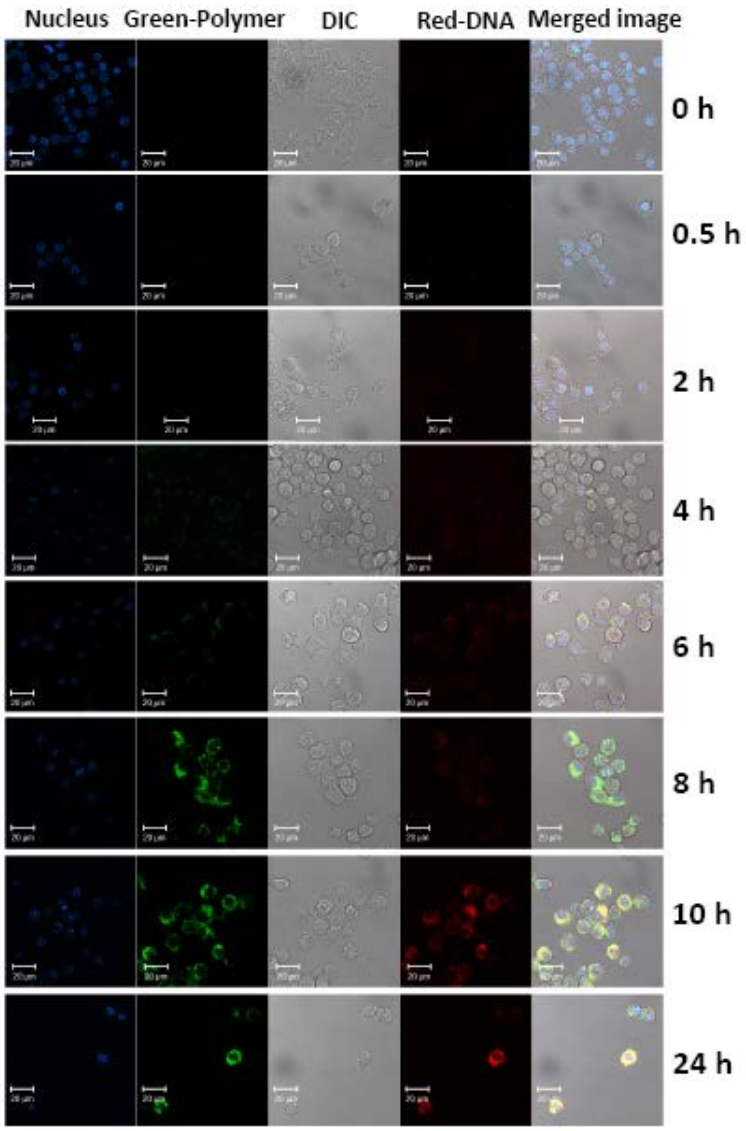


Figure 14. Cellular uptake of BICs in RAW macrophage cells. Blue, nuclear staining (Hoechst); Green, HEZ008; Red, DNA with TOTO-3 dye.

Future directions

In this study we demonstrated that the cationic polymer HEZ008 exhibited extremely minimum cytotoxicity and very resistant to serum protein binding when complexed with pDNA. We also found that the HEZ008/pDNA polyplexes were slowly uptaken by RAW macrophage cells (10 h as optimum). The safety and anti-fouling properties encouraged our exploration of HEZ008 as a new gene delivery platform. The HEZ008/pDNA polyplexes showed excellent stability against enzymatic DNase-I digestion as well as thermal challenge. Furthermore, the polyplexes were very stable upon dilution in buffers and salines as well as storage at 4 degrees for at least 2 weeks as indicated in previous report. The stability of polyplexes we demonstrated here is crucial particularly when applying this technology in biological systems.

The slow uptakes in macrophage cells also offer an opportunity to minimize non-specific uptake *in vivo* but provide a time window to target specific cell populations via attaching affinity ligands on alkyne terminal group via click chemistry tool. The clickable property of the polymer was evidenced by successfully attaching a fluorescence dye and could be further developed extensively for targeting purpose. In future studies, the stability, uptake and targeting property of mannose-clicked HEZ008 (Man-HEZ008) can be explored both *in vitro* and *in vivo* by using mannose receptor overexpressed macrophage cell models.

KEY RESEARCH ACCOMPLISHMENTS

- **Platform A:** Soluble H5 hemagglutinin trimer ($sH5_3$) alone or in conjunction with polyanhydride nanoparticles induced total serum anti-$H5_3$ IgG and neutralizing antibodies significantly greater than the saline control.
- **Platform A:** All mice immunized with $H5_3$ and/or polyanhydride nanoparticle formulations were protective against a low pathogenic Influenza virus A/H5N1 (VNH5N1-PR8CDC-RG) challenge.
- **Platform B:** DNA-based vaccinations induced Th1, Th2 and Th17 cytokine responses in mice.
- **Platform B:** Pluronic/H5HA DNA vaccine formulations could induce primary antibody response.
- **Platform B:** Pluronic/H5HA DNA vaccines induced serum HAI Ab against the A/Vietnam/1203/2004 (H5N1) virus.
- **Platform B:** Pluronic/pVAX1-H5HA DNA vaccine formulations significantly protected the weight loss compared to PBS control group when the animals are challenged with the mouse infectious dose of low pathogenic Influenza virus A/H5N1 (VNH5N1-PR8CDC-RG).
- **Platform B:** Viral genetic loads in lungs of Pluronic/pVAX1-H5HA DNA vaccinated groups demonstrated protective effect of the vaccine formulations compared to PBS controlled mice on day 3 post-viral challenge. Pluronic P85/ pVAX1-H5HA DNA vaccinated groups had significantly less viral RNA in the lungs compared to PBS vaccinated mice.
- **Platform B:** Presence of distal inflammation (peritonitis) significantly enhanced the muscle gene expression only when DNA was injected with Pluronic P85 and not DNA alone.
- **Platform B:** Effect of inflammation on gene expression did not diminish though it was less pronounced when DNA and P85 were separated by space (Left and right hind limb).
- **Platform B:** Gene expression (GFP) in ischemic muscle injected with DNA alone, DNA+P85 and DNA+SP1017 colocalized with CD11b (Macrophage/myeloid) cell marker in addition to muscle cells.
- **Platform B:** *Ex vivo* transfected macrophages with GFP encoding DNA carried and expressed GFP protein selectively to ischemic muscles and not to non ischemic muscles

of ischemic mice. Moreover, the ischemic muscles were found to express the GFP protein that implies the transfer of DNA/protein from macrophages to ischemic muscles.

- **Platform B:** Horizontal transfer of DNA from macrophages to muscle cells was studied by in vitro model of co-culture which proved transfer of DNA from macrophages to muscle cells only in presence of pluronics.
- **Platform C:** New cationic poly(2-Oxazolines) (POx) polymers HEZ008 formed polyplexes with pDNA in spherical morphology and 100nm size by TEM imaging.
- **Platform C:** HEZ008/DNA polyplexes, or BICs, were stable against thermal challenge and DNase I.
- **Platform C:** The HEZ008 polymers exhibited very low cytotoxicity to RAW macrophage cells as well as B16 melanoma cells comparing to PEG-PLL.
- **Platform C:** The HEZ008/pDNA polyplexes have very low serum protein binding and slow uptake by macrophage cells, allowing minimum non-specific cellular uptake and potential targeting delivery to selected cell population.

REPORTABLE OUTCOMES

Conference Presentations

1. Zhijian He*, Yingchao Han, Lei Miao, Devika S Manickam, Rainer Jordan, Robert Luxenhofer, Alexander V Kabanov, Poly(2-oxazolines) as Novel Drug Delivery Systems: Synergistic Combinations of Anticancer Drug Delivery and Polyplexes for Macrophage-targeted Gene Delivery. Gordon Research Conference-Cancer Nanotechnology, Mount Snow Resort, West Dover, VT, July 14-19, 2013.

CONCLUSIONS

- Mice receiving $sH5_3$ alone induced neutralizing antibody titers that were protective against a low pathogenic viral challenge, similar to mice receiving polyanhydride nanoparticles. These initial results display a proof-of-concept for this vaccine platform, emphasizing the strength and immunogenicity of the hemagglutinin trimer cloned and expressed.
- In response to administration of pVAX1-H5HA DNA and pVAX1-H5HA DNA/P85 or SP1017, Pluronic/pVAX1-H5HA DNA vaccine formulations have significant protective effect over PBS control treatment against Low pathogenicity influenza virus A/H5N1 (VNH5N1-PR8CDC-RG).
- Presence of distal inflammation and its effect on gene expression provides a novel track for pluronic gene therapy in disease models (that often involve inflammation).This would imply even higher expression of therapeutic genes in inflammation models when the therapeutic genes are driven by CMV promoters.

- We believe that Pluronic block copolymers selectively recruit antigen presenting cells (monocytes/macrophages) to muscle tissue upon DNA injection which phagocytose the plasmid DNA and then in turn transfect muscle tissue thus increasing the muscle transfection efficiency.
- New cationic POx polymer HEZ008 may be applied as a gene delivery platform that has targeting potential capable of transfer genes to macrophage cells.

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