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Vaccine response boost through immune enhancing microRNA delivery in ovo
Sub-Project No. 1.4.1

Researcher Contact Details
Mark Tizard
CSIRO Health & Biosecurity,
Australian Animal Health Laboratory
Private Bag 24
Geelong, VIC 3220

Email: mark.tizard@csiro.au
Ph: 03 5227 5753
Fx: 03 5227 5555

In submitting this report, the researcher has agreed to the Poultry CRC publishing this material in its edited form.

Poultry CRC Ltd Contact Details
PO Box U242
University of New England
ARMIDALE NSW 2351

Phone: 02 6773 3767
Fax: 02 6773 3050
Email: admin@poultrycrc.com.au
Website: http://www.poultrycrc.com.au

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Executive Summary

Efficient vaccination of broiler chickens using the Inovoject® system to prime the immune system of chicks prior to hatch has provided substantial benefits to the health, welfare and productivity of poultry. However it is known that the efficacy of the vaccination protocols have scope for significant enhancement, with collateral improvements in benefit to industry. The chicken embryo, in ovo, has an immune system that contains a full complement of immune cell types however many of these are not yet primed or stimulated for action. MicroRNAs (miRNAs) are a class of molecule found in action throughout the immune system, where they perform important roles in controlling the subtle changes in gene expression necessary for the development and activation of the most important classes of cell (Baltimore *et al*, 2008). Many microRNAs have now been characterised that are able to alter the balance of cell types, for example between B cells and T cells, and the activation state of those specific cell types, including monocytes and macrophages.

The objective of this project was to develop observations made in our laboratory, using virally vectored expression of miRNAs, in which immune cell populations were altered. For translation to industry practice it would be essential to move away from viral vectored delivery and onto a delivery vehicle that would not impose regulatory constraints due to the incorporation of genetic technology and that could be industrialized and scaled up to meet the potential demand from a large industry. CSIRO developed and patented polymer chemistry technology (**r**eversible **a**ddition and **f**ragmentation **t**echnology – RAFT) used to make nanoparticle delivery vehicles capable of getting small RNA molecules such as miRNAs into animal tissues and into cells by a means that maintained their function and had measurable biologically activity (USPTO WO2013003887-A1). This has the characteristics of being scalable to industrial level production.

Three key miRNA were selected (miR-146a, miR-181a, miR-155) which had suitable, valuable and measurable activities, and these were obtained from commercial manufacturers as synthetic miRNAs (smiRNAs). These were combined with appropriate RAFT polymers and assessed for their delivery to developing chicken embryos in ovo to determine if they would have a measurable impact on the immune cells of the developing embryo. The route of introduction of the smiRNA::RAFT complexes was assessed for efficiency of delivery and inocuity to the successful development and hatch of the chick. This was also assessed in relation to the timing of delivery from incubation day 2 to day 10. In addition to assessing the smiRNAs for their individual effects they were also assessed in combination to determine if there was synergy between their functions.

Result of these staged assessments showed that the smiRNA::RAFT formulations could be delivered in a way the retained normal levels of hatchability, a critical feature to retain the advantage that this might confer to industry. However this required reduction of dose levels. At these lower dose levels only very modest and subtle effects were seen in the levels of cell populations and the activation state of some of those populations. This was also reflected in combinations of smiRNAs. It could not be resolved if the need to reduce dose to maintain hatchability levels was due to the negative effects of the levels of smiRNA or the RAFT polymer delivery vehicle. The original observations based on viral vectored delivery would have provided a low to medium level of smiRNA dose but with a sustained release over the duration of development of the embryo. This could be the key issue, though at present the form of the RAFT polymer vehicle does not provide for a slow release system. However in certain experiments observations were made that revealed an

unexpected activity from certain RAFT polymers alone, in the absence of the smiRNAs. Further investigation revealed that the particular RAFT polymers were stimulating a “danger associated molecular pattern” (DAMP) response in the cells of the immune system. This was characterised first in the cell line HD11, which derives from a macrophage lineage, and then in splenocytes recovered from adult chickens.

The primary objective of the Sub-project was not attained and there is no strong evidence to support translational development of microRNAs for use as adjuvant in the Inovoject® process. However there were some indications that smiRNAs should not be completely dismissed as tools in the development of immunological protection measures for poultry in production, though this will require some focussed research and development. A useful finding emerging from this Sub-project is that some forms of the RAFT polymer delivery vehicles have demonstrated their own intrinsic action on immune cells. This unexpected finding reveals a generalised immune stimulation that could have value in the adjuvant field and is therefore of relevance to the initial objectives of the Sub-project.

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Introduction

The Embrex Inovoject® vaccination system has revolutionised the control of many diseases in poultry production, making the vaccination process simpler and more cost effective by automating the delivery of vaccines to the day 18 embryo while it is still in the egg. A number of products have been developed and tailored to this system to control a range of diseases including infectious bursal disease (IBV – vaccine, Bursaplex), Marek's disease (MDV – vaccine HVT) and coccidiosis (*Eimeria* sp – vaccine, Inovocox EM1). Although the immune system of the 18 day old embryo responds to these vaccines there is substantial room for improvement and there are other vaccines that are still lethal or ineffective if administered to the 18 day old embryo.

Advice from industry (Zoetis) makes note that there are issues with co-administration of some live attenuated poultry vaccines. In a specific example it is not possible to combine ILTV with either NDV or IBV for spray administration at day of hatch. Therefore ILTV has to be administered by drinking water from day 7 post hatch and onwards. Another issue of note is that the NDV vaccine is lethal if administered in ovo. Industry has expressed "a crying need for more effective vaccination regimens and the ability to protect hatchlings from greater range of diseases particularly during the critical first two weeks of life".

There is a significant opportunity to identify a system to boost the response to these current in ovo vaccines and provide more effective immunity to flocks against IBV, NDV, Marek's disease and coccidiosis. Rather than a traditional adjuvant approach the aim of this Sub-Project is to use miRNAs, small molecules which are master regulators of cell type development and differentiation, to accelerate the natural process of maturation of the cells of the embryonic immune system. Success with current vaccines could present the opportunity to develop more vaccines in the in ovo format, i.e. those that are currently embryo lethal and to develop new vaccination regimens with quantum improvements for producers.

This project presents the potential for economic value by helping to boost flock immunity to a range of disease thus reducing the associated production losses. In addition it will substantially improve the hatchlings ability to mount their own un-primed innate and adaptive immune responses to other pathogens encountered in the intensive production environment, including bacteria. The potential for this technology to improve natural immune responses and thus resilience to bacterial pathogens may help to contribute novel control measures for the possible emergence of drug-resistant strains of bacterial pathogens of medical significance as poultry production reduces and removes the use of antibiotics as growth promotants in line with European practices.

MicroRNAs have been recognised for many years as having a critical role in the haematopoietic process (Baltimore *et al*, 2008; Undi *et al*, 2013) and have been postulated as a means for immuno-regulation for cancer immunotherapy (Okada *et al*, 2010). MicroRNA-150 has been shown to induce myeloid cell differentiation (Morris *et al*, 2013), while miroRNA-181 has a critical role in natural killer (NKT) cell ontology and lymphocyte development (Henao-Mejia *et al*, 2013). These kinds of response could be harnessed to improve the responsive lymphocytic (T and B cell) populations and the myeloid (macrophage and antigen presenting) populations of the developing chicken embryo. MicroRNA-146a not only has a role in maturing T and B cells (Labbaye and Testa, 2012) but also plays an important role in regulating the Toll-like receptor 4 (TLR4) in a manner which helps to control overwhelming proinflammatory immune responses that can have a

pathological effect in neonatal immune response (Lederhuber *et al*, 2011).However some microRNAs are also know to negatively regulate immune responses for example microRNA-155 and microRNA-21 promote expansion of myeloid-derived suppressor cells (Li *et al*, 2014). It will therefore be critical to examine the responses to various proposed synthetic microRNAs (smi-RNAs) that will augment the numbers of particular immune cell populations and that will improve their activation state in readiness to response to the day 18 in ovo vaccinations. Our own results, with the delivery of microRNA-146a by a viral vector to early embryos, indicate that there is the potential to achieve this in the chicken. However the key to this is achieving this effect by a delivery system that is acceptable to industry. There is no indication in the literature that any groups are actively working on such an approach either to studying immune ontology in the chicken, or more practically in the immune enhancement of developing chicken embryos.

Patent searches were conducted (through the USPTO PatFT & AppFT, PGPUB, EPO Espacenet and Google Patents search systems) and revealed no obvious encumbrance of freedom to operate or restriction to the opportunity to establish new IP in this space. CSIRO holds three patents that cover applications of RAFT polymer chemistry for siRNA delivery and a patent for the Direct Injection technology.

There is a very strong commercial interest in production trait modulation in the poultry industry. A focus on an immediate issue for industry, the in ovo maturation of the immune system presents three clear benefits. First is the improvement of efficacy of current in ovo vaccines without the need for reformulation, second is the possibility that vaccines currently administered post-hatch will become viable as in ovo vaccinations and third is the possibility that chicks hatching with an already matured immune system will be more resilient to the pathogen loads (viral, bacterial and protozoan) that they can encounter in intensive production systems. This later point may help to address two additional issues that are looming for the industry. First is the increasing move to free range organic farming practices, which translates to increased exposure to pathogens in this production environment. Second is the move to withdraw antibiotics from animal feed in an effort to halt the spread of antibiotic resistance in bacterial pathogens of medical significance.

The strongest indication of commercial relevance of this project comes from Zoetis, who develop and manufacture the Inovoject® vaccines - more than 200 million doses are sold and administered every year. The dollar value of protection from production loses gained by industry enterprises is far greater. However as with any new technology there is room for improvement. The Inovoject® vaccines are widely used through Australia and across the global poultry industry and are used for both broilers and egg-layer birds.

Objectives

The overarching aim of the Sub-Project is to stimulate early maturation of the immune system of the developing embryonic chick, in ovo, such that at hatch it is primed to respond better to in ovo administered vaccines as well as to the immunological challenges of an intensive production environment. The aim was to achieve this through the delivery to the early embryo of miRNAs known to be involved in the maturation process through their regulation of key gene pathways in the process of haematopoiesis. Our hypothesis was that administration of these miRNAs would accelerate the maturation of the immune system and enhance the response of the late embryo to in ovo vaccinations that are widely used in the commercial setting (Embrex Inovoject®). Such an “adjuvant” effect could

enable more vaccines to be applied in ovo rather than having to wait until post hatch. In addition it is expected that the early maturation of the immune system will help to mitigate the removal of antibiotics as feed additives.

- Objective 1 Modulation of the developing embryonic immune system using synthetic microRNAs (smiRNAs) delivered with RAFT polymers
- Objective 2 Characterising the potential adjuvant effect of miRNA delivery to improve vaccine response
- Objective 3 Explore synergies between smiRNAs, how best to deliver them and define the first steps towards of commercial translation of the technology

Methodology

The Sub-Project followed a strategy to inject miRNAs (specifically miR-146a, miR-181a and miR-155), that are involved in maturation and activation of antigen presenting cells and responsive cells, into embryos at stages compatible with current in ovo vaccination strategies. There was an assessment of the biological impact of such injections into earlier stages of embryo development to assess any advantage or difference in these time points. Injected embryos were assessed for impacts on their immunological status (indicative of their ability to respond to vaccine doses).

The miRNA doses were formulated with both RAFT-delivery polymers and with the commercially available delivery material, Lipofectamine 2000 (L2000CD). This was injected into chicken embryos at day 2.5. Blood samples from the embryos were retrieved and the immune cell populations subjected to FACS analysis. This was used to assess the numbers of immune cells populations that perform of different functions; T cells, B cells and antigen presenting cells (dendritic cells). Using the capability of fluorescence activated cell sorting (FACS) to assess multiple cell surface markers, the cell populations were also assessed for their maturation and activation states.

The results of this analysis was used to assess the most suitable time to administer the miRNA-formulation to achieve the best activation state of the embryo by day 18 of development when vaccines are administered. Immune responses to the vaccine was compared with the response of control groups with no miRNA dosing. If there was an increase in detectable parameters such as antibody titre and T cell immune response then the general immune activation state was assessed using a challenge with a relevant infectious agent (influenza virus). This process of analysis was also applied following the injection of the miRNA-formulations into embryos at day 2.5, day 10 and day18 of development and assessing the various compartments for injection (allantosis, yolk sac, embryonic tissue). These data was used to evaluate the industry benefit of this novel adjuvant approach against the cost of dose, formulation and administration.

Methods and materials

Synthetic siRNAs

The control siRNA used in this study was obtained from QIAGEN (USA). It is an anti-Green Fluorescence Protein siRNA sequence; the sense strand is

5’gcaagcugacccugaaguucau 3’ and the antisense strand is 5’gaacuucagggucagcuugccg 3’ and is referred to as si22.
All synthetic microRNAs (smiRNAs) were obtained from QIAGEN (USA) as “miScript miRNA mimics” (samples supplied as 20 nM in total).
Mir146a (Product name: Syn-hsa-miR-146a-5p, guide strand sequence: 5’-UGAGAACUGAAUUCCAUGGGUU-3’)
Mir155 (Product name: Syn-hsa-miR-155-5p, guide strand sequence: 5’-UUAAUGCUAAUCGUGAUAGGGGU-3’)
Mir181a (Product name: Syn-hsa-miR-181A-5P, guide strand sequence: 5’-AACAUUCAACGCUGUCGGUGAGU-3’)

Formulation of polymer/smiRNA complexes

A variety of different polymers were considered and tested for toxicity to the chick embryo. This included the polymers BC-28 (ABA tri-block), TL 39B (4-arm star), TL-112, TL-105 and TL-5D, S4-11 (Mikto star). Molar ratios of polymer to varying amounts of siRNA (si22) were also tested from 20 pmole up to and including 2 nmole to determine an optimal dose. Molar ratios of polymer to 50 pmole siRNA or siDNA were calculated. Complexes were formed by the addition of OPTIMEM media (Invitrogen, USA) to eppendorf tubes. The required amount of polymer resuspended in water was added to the tubes and the mixture vortexed. 50nM of smiRNA or di22 was then added to the tubes and the sample vortexed. Complexation was allowed to continue for 1 h at room temperature.

Delivery of miRNA to the embryo

Four different routes of inoculation were tested using the developing chick embryo, including intravenous (i.v.) delivery of the polymer:si22 or polymer:smiRNA complex at stage E2.5, into the yolk at stage E4, i.v. delivery at stage E10 and intra-allantoic delivery at E10. Delivery of the polymer:si22 or smiRNA complex to the E4 yolk or E10 allantois were selected as the best route of inoculation due to the ease in which these would be able to be scaled up if required.
E4 yolk inoculation: The pointy end of the egg was swabbed with 80% ethanol, and a small hole (<5 mm) opened to check egg viability. This was then re-sealed with 3M tape. The blunt end of the egg was swabbed with iodine solution and a small hole made in the shell using an egg-punch. The egg was positioned on its side and 100 µl of polymer:si22 or smiRNA complex was delivered to the yolk using a 25G (1”) needle inserted its full length into the egg and angled slightly downwards. The hole was then sealed with 3M tape. Eggs were then returned to the 37C incubator until stage E18.
E10 allantois inoculation: The lowest point of the egg air sac was marked avoiding areas with veins and on the opposite side to the embryo. Eggs were swabbed with iodine solution and a small hole made in the shell using an egg-punch approximately 2 mm above the marked line. 100 µl of polymer:si22 or smiRNA complex was delivered using a 26G (1/2”) needle inserted its full length vertically into the egg. The hole was then sealed with 3M tape. Eggs were then returned to the 37C incubator until stage E18.

Agarose gel electrophoresis

Samples at different molar ratios of polymer to 50 pmole siRNA were electrophoresed on a 2% agarose gel in TBE at 100V for 40 min. siRNA, smiRNA and siDNA was visualised by gel red (Jomar Bioscience) on a UV transilluminator with camera, the image was recorded by the GeneSnap program (Syngene, USA).

Cellular toxicity assay

CHO-GFP, A549 and Huh7 cells were seeded at $1x10^4$ or $2x10^4$cells per well respectively in 96-well tissue culture plates and grown overnight at 37 °C with 5% CO_2.
The polymer materials were serially diluted in water and added to 3 wells in the 96 well culture plates for each sample and incubated for 72 h at 37 °C in 200 µl standard media. Toxicity was measured using the Alamar Blue reagent (Invitrogen USA) according to manufacturer's instructions and described previously (Hinton *et al*. 2012). Results are presented as a percentage of untreated cells and the presented data are representative of three separate experiments in triplicate. Obtained data was analysed in Microsoft Excel. Results are presented as a percentage of untreated cells and the presented data are representative of three separate experiments in triplicate.

Cell Insight plate reader

A549, Hd11 and Huh7 cells were seeded at $1x10^4$ or $2x10^4$ cells per well respectively in 96-well tissue culture plates and grown overnight at 37 °C with 5% CO_2. Polymer/FAM siRNA complexes were prepared, cell media was removed and replaced with 200 µl OPTI-MEM. The siRNA: polymer complexes in a volume of 10µl was added to 3 wells of cells per sample and incubated for 5 h. Cells were then washed three times with 5mg/ml Heparin on ice followed by paraformaldehyde fixation. Cells were stained with DAPI and read on a Cell insight fluorescent plate imager. 1000 cells per well were counted and mean total FITC fluorescence was measured.

Flow Cytometry

The following antibodies were used for flow cytometry:
Mouse anti-chicken CD4-FITC (SouthernBiotech), Mouse anti-chicken CD28-PE (SouthernBiotech), Mouse anti-chicken CD8a-SPRD (SouthernBiotech, Human anti-chickenCD25:Alexa fluor647 (BIO-RAD), Mouse anti-chicken MHC class-II-FITC (SouthernBiotech), Mouse anti-chicken Bu1-Alexa fluor647 (SouthernBiotech), Mouse anti-chicken IgY-PE (SouthernBiotech), Mouse anti-chicken IgM-SPRD (SouthernBiotech). The following isotype control antibodies were also used for flow cytometry: Mouse IgG1-FITC (SouthernBiotech), Mouse IgG1-PE (SouthernBiotech), Mouse IgG1-PSPRD (SouthernBiotech), Mouse IgG2b-SPRD (SouthernBiotech), Mouse IgG1-APC (SouthernBiotech).

Confocal microscopy

A549 and Hd11 cells were seeded at $1x10^5$ cells on 13 mm round glass coverslips (Menzel, Germany) in 24 well plates (Nunc, USA) and grown overnight at 37ºC with 5% CO2. For positive controls [6FAM] labelled si22 was transfected into cells using Lipofectamine 2000 (Invitrogen, USA) as per manufacturer's instructions as described below. Polymer and labelled siRNA complexes were produced as described above and added to the cells for 5 h. To process cells for confocal microscopy, cells were washed in PBS and fixed in 4% paraformadehyde (Sigma, USA) in PBS for one hour. Coverslips with cells were mounted onto slides in Vectashield (Vector Laboratories, USA). Images were acquired on a Leica SP5 confocal microscope (Leica Microsystems, Germany). All samples were processed in parallel and laser and gain settings for [6FAM] labelled si22 were kept constant between samples.

DAMP molecule, Interferon and Interleukin activation assays in vitro

HD11 cells were seeded at approximately 2×10^5 in duplicates into 24 well plates and grown overnight at 37 ºC with 5 % CO_2. Poly I:C (Invivogen, San Diego, CA) a synthetic viral RNA homologue known to produce an interferon response was transfected into cells using Lipofectamine 2000 (Karpala et al. 2008; Stewart et al. 2011). Polymer complexes were added to cells and incubated for 3, 6 or 24 h. Supernatant was stored at -80 C for ELISA assay and RNA was harvested using an RNeasy miniprep kit as per manufacturer's instructions (QIAGEN, Hilden, Germany).

Preparation of splenocytes and splenocyte activation assay

Spleens were collected into cold PBSA from stage E18 embryos. The spleen was separated into a single cell suspension by passaging through a 70 µM cell strainer (BD). Splenocytes were separated by lymphoprep layered centrifugation. Cells were then washed twice in DMEM growth media before counting and plating in 96 well plates for assay. Alternatively splenocytes were prepared by harvesting a spleen from an adult white leghorn chicken in the AAHL small animal facility according to AEC instructions. Cells were prepared as above.

Supernatant from treated HD11 cells was added to 1×10^6 primary splenocytes in 96 well plates and incubated for 6, 24 or 48 hr. Supernatant from the culture was stored at -80 C for ELISA assay and RNA was harvested using an RNeasy miniprep kit as per manufacturer's instructions (QIAGEN, Hilden, Germany).

Reverse transcription and quantitative real-time PCR

RNA was harvested using the Trizol method (Chomczynski and Sacchi 1987), one microgram was treated with DNase (Promega, Madison, WI) according to manufacturer's instructions, quantitative real-time PCR (QRT-PCR) experiments were conducted using *Power SYBR green RNA to CT* kit (Applied Biosystems, Foster City, CA) according to manufacturer's instructions to measure cytokine expression levels. The sequences for all probes have been published previously (Karpala *et al*, 2008). Primers were obtained from Geneworks (South Australia). All quantification data was normalised against chicken GAPDH. QRT-PCR was performed on a StepOnePlus Real Time-PCR System, 96 well plate RT-PCR instrument (Applied Biosystems) under the following conditions: 1 × cycle 50 ˚C for 30 min followed by 95 ˚C for 10 min, 40 × cycles 95 ˚C for 15 sec followed by 60 ˚C for 1 min. The comparative threshold cycle ($\Delta\Delta$Ct) method was used to derive fold change gene expression. Data was normalised to chicken GAPDH.

In vivo influenza virus silencing

Polymer complexes were injected into the allantoic cavity of E10 eggs. The eggs were incubated at 37 °C for 24 h. PBS was injected as a control. After 24 h incubation with polymer influenza PR8 virus (H1N1) was diluted in 100 µL PBS to 500 pfu/egg and immediately injected into the allantoic cavity of the treated embryonated chicken eggs. The eggs were incubated at 37 °C for a further 48 h then allantoic fluid was harvested to measure virus titre.

$TCID_{50}$ Assays

$TCID_{50}$ assays were performed as described in (Liang et al. 1994). Briefly, tissue culture supernatants or allantoic fluid were assayed for virus infectivity on MDCK cells by endpoint dilution for cytopathic effect with a 10-fold dilution series. Titres were expressed as log_{10} $TCID_{50}$/ml ± SEM.

Histopathology and chorioallantoic membrane uptake

Embryonic chicken livers were obtained from the same embryos as the spleens studied for IFN- response at 24 h. Livers were fixed in 10 % buffered formalin for 24 h and analysed for H&E staining. Chorioallantoic membranes were removed and fixed in 4 % paraformaldehyde for 2 h. Membranes were permeabilized with 0.1 % Triton X-100 (Sigma, St. Louis, MO) in PBS for 30 min, and stained with 4',6-diamidino-2-phenylindole (DAPI, Invitrogen) for 20 min. Samples were then mounted onto slides in Vectashield (Vector Laboratories, Burlingame, CA). Images were acquired on a Leica SP5 confocal microscope (Leica, Wetzlar, Germany). PBS control and polymer injected samples were prepared in parallel, and laser and gain settings were kept constant between samples.

Statistics

The difference between virus infected groups was statistically analysed by one way repeated measures ANOVA, parametric, with Tukey post analysis. **$p<0.01$, *$p<0.05$, N.S. not significant. For FACS analysis to measure differences in cell populations the Kruskal-Wallis ANOVA was applied.

Results

Uptake and distribution of RAFT polymer and siRNA in embryo tissues

This task was approached using two labelling systems. This utilised a rhodamine fluorophore incorporated into the RAFT polymer delivery agent during its synthesis. Since many fluorophore molecules are incorporated into each polymer this gives a strong signal in fluorescence light microscopy. However it only shows the location of the delivery vehicle and not the location of the active agent, the smiRNA. So a second approach used an siRNA labelled with FITC fluorophore. The level of labelling per molecule is lower so the signal strength is not as great, however if localised to the same regions it provides a strong indication of delivery of the active ingredient (smiRNA) by the delivery vehicle (RAFT polymer). In order to keep the levels of (detectable) reagents high and the tissues in which they had to be screened relatively restricted this study was performed by intravenous injection into the aorta of embyros at day 2.5 of development (E2.5). This approach was used to assess a number of RAFT polymer delivery agents alongside Lipofectamine 2000 (L2000CD). Due to the higher doses and early embryo stage there was a high degree of mortality among the embryos treated (Table 1). The issue of dose level and toxicity but was addressed in later experiments.

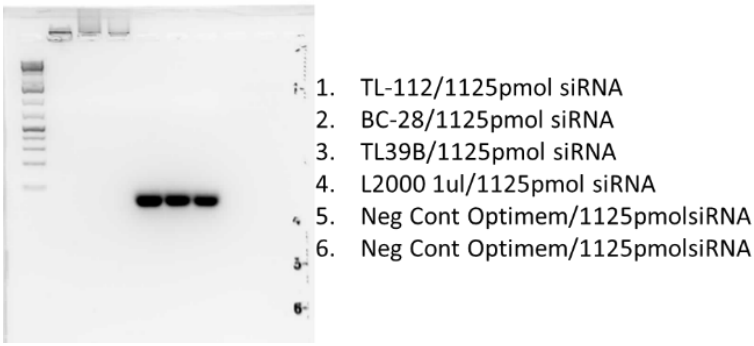
Table 1: Embryos survival post-injection

Complex	**24 hr PI**	**48 hr PI**
TL-112 *(Mikto star)*	4/5	1/5
BC 28 *(ABA tri-block)*	3/5	0/5
TL 39B *(4-arm star)*	0/5	0/5
L2000CD	5/5	5/5
si22-FAM alone (control)	3/3	3/3

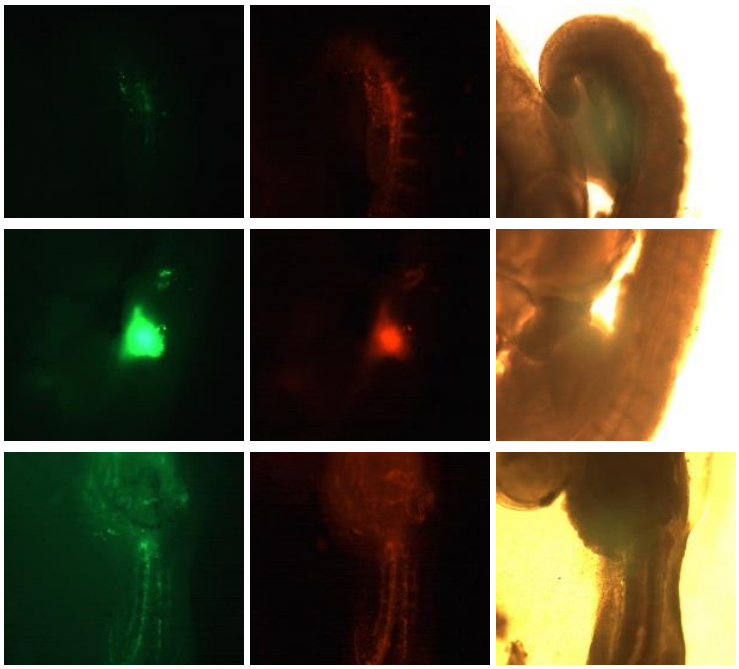
These results show that the labelled si22 did not intrinsically affect embryo survival and that the L2000CD reagent was also neutral in its effect on the embryos. Of the RAFT polymer reagents the TL-112 (Mikto star) had the least impact on embryo survival, at these higher doses. Fluorescence microscopy of the developing embryos showed good biodistribution of both this RAFT delivery agent and the siRNA in a wide range of tissues the (Fig. 1).

Figure 1: A) *Binding of the siRNA (si22-FAM) by the RAFT polymers, TL-112, BC-28 and TL39B and by the L2000 reagent.* B) *Biodistribution of siRNA (green label) and RAFT polymer TL-112 (red label) in embryo tissues 24 hrs post administration.*

A



B



Uptake and distribution of siRNA in embryo tissues using commercial transfection reagents.

Related to the availability of RAFT polymer the use of commercial delivery agent was assessed in parallel with RAFT as a control. There is no fluorophore version of Lipofectamine 2000 (L2000CD) available so it was therefore necessary to use the FITC labelled siRNA (method 2 above) which is known to be less sensitive. It is known from standard in vitro assessments of our batches of RAFT polymer that the L2000CD reagent does not appear to bind the siRNA (or smiRNA) as tightly as the RAFT polymer (Fig.2) however the L2000CD is still effective in cell culture analysis of gene silencing by RNAi.

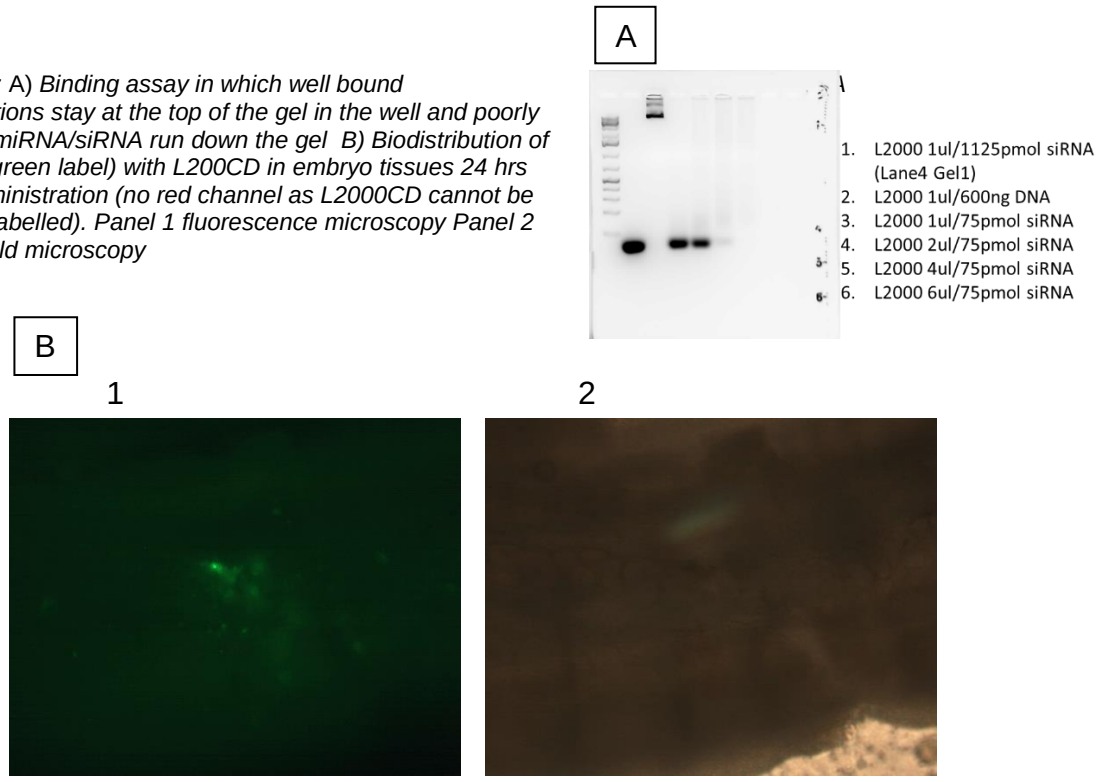


Figure 2: A) *Binding assay in which well bound formulations stay at the top of the gel in the well and poorly bound smiRNA/siRNA run down the gel B) Biodistribution of siRNA (green label) with L200CD in embryo tissues 24 hrs post administration (no red channel as L2000CD cannot be directly labelled). Panel 1 fluorescence microscopy Panel 2 bright field microscopy*

The standard gel shift assay used to assess siRNA binding to RAFT polymers shows that the interaction between siRNA and the commercial L2000CD reagent is much weaker, so the siRNA is not held in the wells as it is by RAFT polymers (Fig. 2 compared with Fig. 1A). However it is known that L2000CD can mediate delivery and gene silencing in cell lines in tissue culture. Fluorescence microscopy of the developing embryos (representative example in Fig. 2B) showed poor biodistribution of the siRNA when formulated with L2000CD. This reagent is widely used in cell culture experimentation and is effective in that environment, however it is not easy to formulate, not easy to scale up and is very expensive – thus not a viable tool for translation to industry practice.

Assays to measure effect of smiRNA delivery to embryos (Milestone 3)

Immunological assays for B and T cell populations were established. These were performed by fluorescence activated cell sorting analysis (FACS) of cell populations using the B cell markers IgY, IgM, Bu1 and the MHCII antigen, and for T cell markers CD4 and CD8 in combination with the activation markers CD28 and CD25. Splenocytes from E18 embryos and from adult birds were used to optimise the preparation of cells and to

determine the most effective concentration of antibodies. This formed the basis of from the analyses is shown in later in the assessment of embryos treated with smiRNAs.

Selection of most efficacious delivery vehicle (Milestone 4)

A range of RAFT polymer delivery vehicles were prepared by organic chemists at CSIRO Manufacturing. RAFT block copolymer structures; BC-28 linear tri-block, TL39B four armed star di-blocks, TL-112 mikto-arm star multi-block (structures shown in Fig. 3). The blocks create functional domains in the polymers which have a number of functions. First is the hydrophilic block which ensures solubility of the nanoparticle formed and provides a physical protection of the payload, the smiRNA or siRNA. Second is the cationic block, with a positive electrical charge which has two functions, to bind the negatively charged smiRNA locking it into the nanoparticle and helping to protect it, and also to provide a net-positive charge to the final nanoparticle, which is critical to the binding and entry of the nanoparticle into cells to release the active ingredient the smiRNA. The various batches of the RAFT polymer were first validated with the desired performance characteristics (low cellular cytotoxicity and high transfection delivery efficiency) through standardized *in vitro* assays. This was performed to ensure a single reliable batch of each RAFT polymer delivery agent was available to enable comparative analysis to select the most efficacious vehicle for the following milestones.

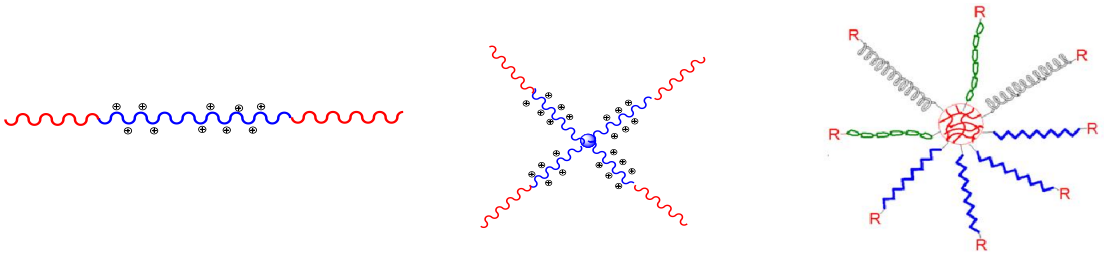


*Figure 3. RAFT polymer structures tested: ABA linear tri-block (BC-28), 4 arm star (TL39B) and the mikto star (TL-112) multi arm structure. Functional blocks are **red** – hydrophilic block; **blue** – cationic (positive charged) block; **green** – hydrophobic block.*

Comparison of data from RAFT polymer delivery and commercial transfection reagent delivery.

Assessments were performed to compare a commercial cationic lipid (chemically defined Lipofectamine 2000 - L2000CD) and three RAFT block copolymer structures; BC-28 linear tri-block, TL39B four armed star di-blocks, TL-112 mikto-arm star multi-block. The comparisons show that RAFT polymers and Lipofectamine delivery agents are effective in delivering smiRNA *in ovo*, as judged by bio-distribution assay and *in vitro* bio-activity. However it is important to note that from a commercial perspective Lipofectamine L2000CD would be cost prohibitive for the transfer of this technology to industry practice (at the time of this report) and its biodistribution was relatively poor compared to the RAFT polymers (cf Fig. 1B and 2B). Of the three RAFT polymer structures the mikto-arm star multi-block polymer TL-112 was the most active. It was also the best tolerated by embryos as judged by the two day survival rate post administration (Table 2 and 3). The focus of the next studies was with the polymer TL-112. This was manufactured in a sufficient quantity, with appropriate activity and toxicity analysis, to proceed with the study from a single comparable batch.

Table 2: Embryos survival post-injection – intravenous

Group	Complex	24 hr PI	48 hr PI	Total embryos
1 (e2.5)	TL-112 *(Mikto star)*	4/5	3/4	9
2 (e2.5)	L2000CD 4 (50 pmole)	4/5	3/4	9
3 (e2.5)	L2000CD 5 (75 pmole)	5/5	4/4	9
4 (e3.5)	TL-112 *(Mikto star)* e3.5	3/5	0/4	9

Impact of smiRNA and delivery vehicle on embryo survival.

Survivability analysis with some of the initial batches of RAFT polymer yielded relatively poor survival outcomes, in the range 20-60% (Table 1). The survival rates were also affected by the route of delivery of the RAFT polymer/siRNA formulation. Observations made during the injection procedure indicated that the overall cationic (positive charge) nature of the formulation caused clumping of red blood cells – a situation that could cause minor occlusions of capillary blood vessel with the potential for detrimental effects on embryo growth and development. This may be the reason that the intravenous route gave the lowest survivability scores. Lower doses of smiRNA/polymer formulation by the intravenous route gave higher survivability, from 60-100% (Table 2), but this did constrain the therapeutic ratio that could be applied in the studies that followed to assess the in ovo activity. It was clear that increasing the number or duration of clean up steps for the polymer batches did impact on survivability, suggesting that minor contaminants of the original monomers could be responsible, at least to some extent, for impacts on survivability. With the final (purer) batch of RAFT polymer TL-112 and a reduction in the overall dose administered survivability rates with smiRNA/TL-112 formulation via the intra-allantoic delivery were as high as 80-100%, which is indistinguishable from the natural hatch rate with the line of birds in use.

Optimizing point of delivery (Milestone 5)

It became clear during the study that the timing of delivery is intimately linked to the route of delivery in order to be able to access the right cells at the right time. Various routes of delivery were considered for the comparison of alternative points of entry the advantages and disadvantages of which were compared in Table 4.

Direct operator observations following intra-aortal injection of various formulations into the E2.5 embryo, of clumping of red blood cells, was interpreted as a result of the positively charged nanoparticles causing electrostatic cross-linking of the red blood cells. This would the lower 48 hour survivability of embryos treated with this route of administration. The alternative routes of the allantois, the amnion or the yolk presents the advantage that the dose does not immediately encounter high concentrations of cells. Of these three the yolk and the allantois are the most practical. The conclusion from the combined observations is that the dose of smiRNA/TL-112 via the allantois is the preferred route since it combines a

practical approach for future commercial development together with signs of efficacy of delivery of miRNA.

Table 3: Embryos survival post-injection – intra-allantois

Group	Polymer	Delivery	siRNA	No. embryos injected	Harvest at E14 : embryos survival	Harvest at E19 : embryos survival
1	-	E10 allantoic/PBS control 100 uL	-	10	4/5	4/5
2	TL-112	E10 allantoic 0.5 nmol/ 100 uL	-	10	5/5	3/5
3	TL-112	E10 allantoic 1 nmol/ 100 uL	-	10	4/5	4/5
4	TL-112	E10 allantoic 0.5 nmol/ 100 uL	Si22	10	5/5	4/5
5	TL-112	E10 allantoic 1 nmol/ 100 uL	Si22	10	5/5	5/5
6	TL-112	E10 allantoic 0.5 nmol/ 100 uL	PB	10	5/5	5/5
7	TL-112	E10 allantoic 1 nmol/ 100 uL	PB	10	5/5	5/5

Table 4: Comparison of route of delivery for smiRNA formulations

Route	Advantage	Disadvantage
Blood stream (early embryo)	Embryo is small; therefore doses can be small (reduced cost).	Hard to access; immune cells may be too immature to respond to smiRNA treatment.
Blood stream (late embryo)	Larger vessel to access; immune cell progenitors in circulation.	Hard to access; larger doses required to maintain dose rates per tissue mass.
Yoke	Easy compartment to access; good for industry translation.	Complex liquid/nutrient content; may impact on functionality of smiRNA/polymer formulation (nanoparticles may become coated in protein or lipid, affecting activity)
Amnion	Small compartment; could be used for early embryo; small doses.	Hard to access the compartment; Not a direct route for formulations to access immune cell progenitors from this compartment.
Allantois	Easy compartment to access; good for industry translation; can be used early or late in embryo development; relatively low complexity liquid content.	Not a direct route for formulations to access immune cell progenitors from this compartment.

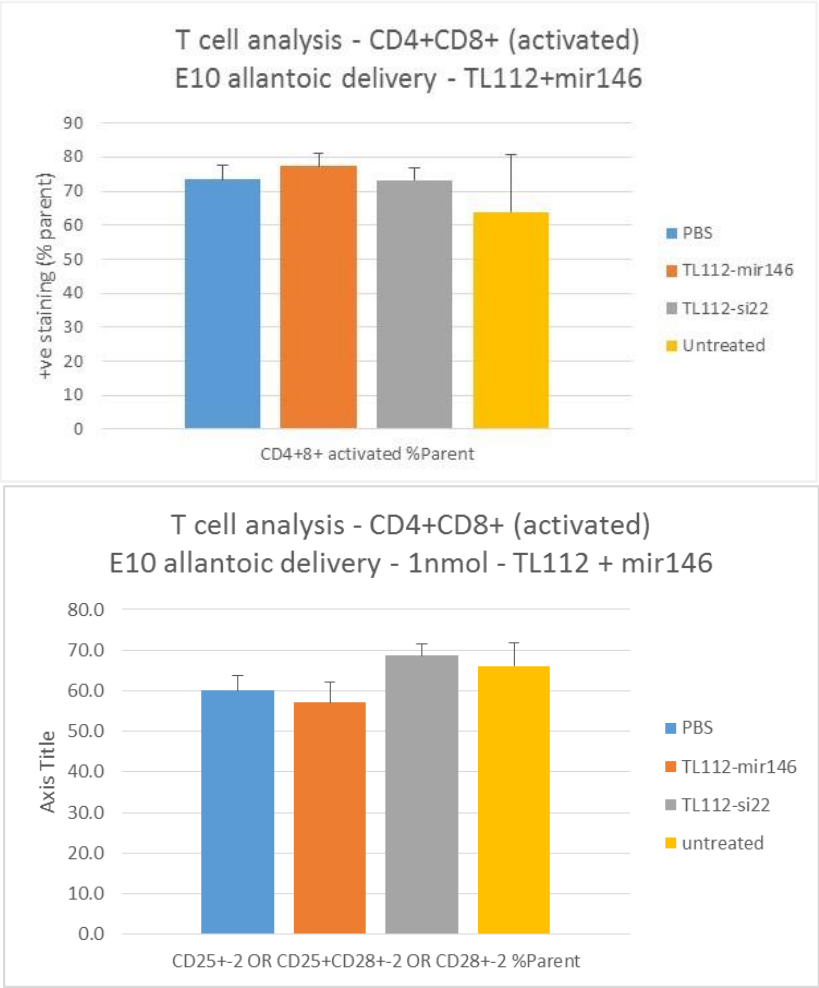
Measurement of effects of smiRNA on immune cell populations (Milestone 8)

Having assembled the necessary reagents, generated functional formulations and determined delivery route for the smiRNAs that yield acceptable levels of hatchability and that are practical for industry adoption, assays were established based on FACS analysis for immunological parameters of the key immune cell populations that could measure beneficial alterations in immune cell populations. These assays were applied to measure response to the smiRNAs selected, miR-146a, miR-181a and miR-155. These were formulated with the RAFT polymer TL-112, based on performance, and the intra-allantoic route of delivery at day 10 was selected as the optimal route for practicality of adoption by industry.

Assessment of smiRNA miR-146a

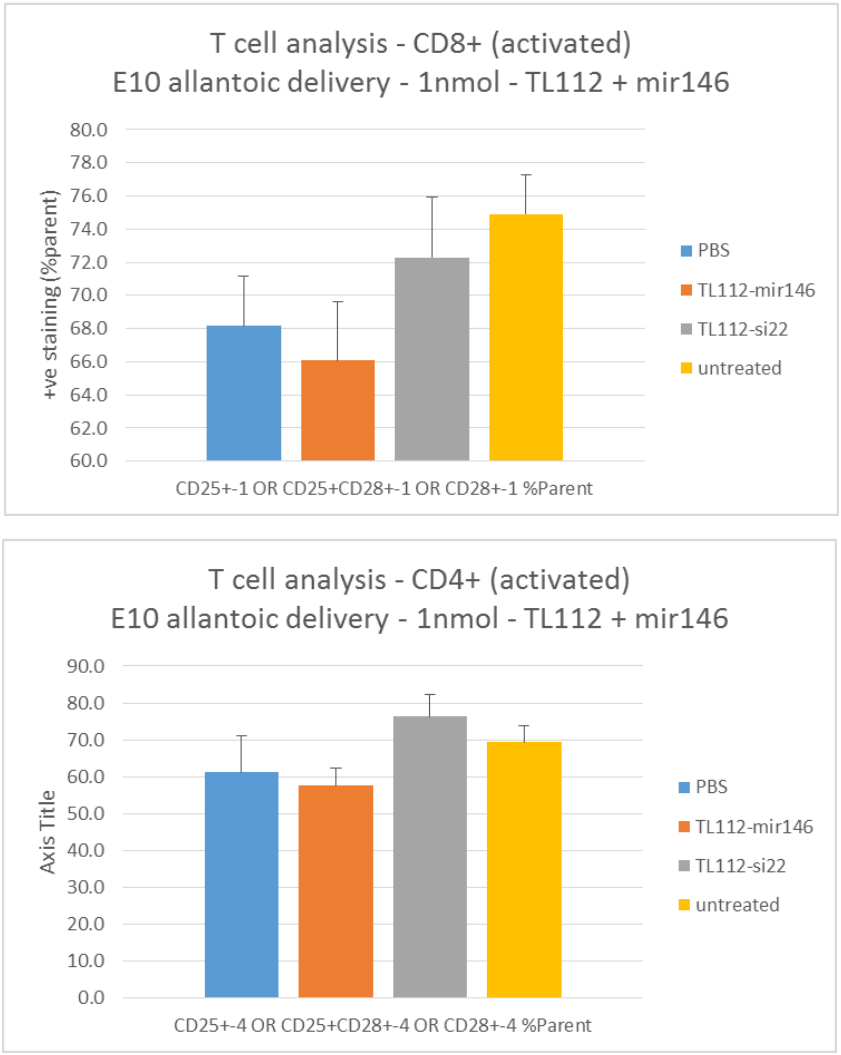
The smiRNA miR-146a was formulated at 200 pmol or 1 nmol doses with RAFT polymer Tl-112 and control preparations of the siRNA si22 were prepared at the same dose and RAFT polymer ratios. Assessment of smiRNA miR-146a showed no increase in the population of T cell precursors (CD4+ CD8+) displaying the CD25 and CD28 activation markers in comparison to controls, via allantoic delivery of smiR-146a with TL-112 at ED10 at doses of 200 pmol or 1 nmol per egg. In these cases assessments was conducted 8 days after dosing at ED18.

Figure 4: No effect of E10 allantoic delivery of 200 pmol or 1 nmol miR-146a + TL-112 on the CD4+ CD8+ precursor T cell populations



However there was a difference in the numbers of activated (CD25+ and CD28+) cells particularly the CD8+ and to a lesser extent the CD4+ T cell subtypes. This revealed a reduction in the numbers of these differentiated activated cells present in the spleen (site of immune cell storage and presentation). Upon activation the T cells (both CD4+ and CD8+) would be expected to migrate into circulation and the peripheral tissues to begin surveillance and activity and thus they would be expected to be depleted in the storage organ, as observed. The level of difference observed did not reach statistical significance by the Kruskal-Wallis one way analysis of variance (ANOVA) test. However there was an encouraging trend in the smiR-146a treatment group (orange bars), particularly in comparison to the direct control group of treatment with a formulation with an irrelevant siRNA (si22 – grey bars).

Figure 5: Reduction in spleen resident activated CD4+ and CD8+ T cells by E10 allantoic delivery of 1 nmol miR-146a + TL-112



There was also a small trend towards an increase in the population of B cells identified in the spleen of this same group of ED18 embryos, 8 days post treatment with 1 nmol of smiR-146a (Fig. 6). This could indicate an increased population of B cell precursors which have migrated to lymphoid follicles in the white pulp of the spleen to await activation and stimulation by specific antigens to generate plasma cells for antibody production. Once again this did not reach statistical significance by Kruskal-Wallis ANOVA but showed an encouraging trend.

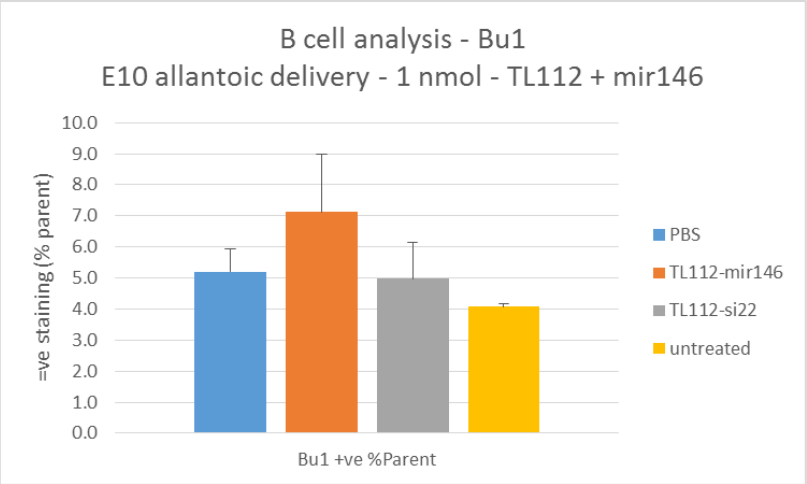


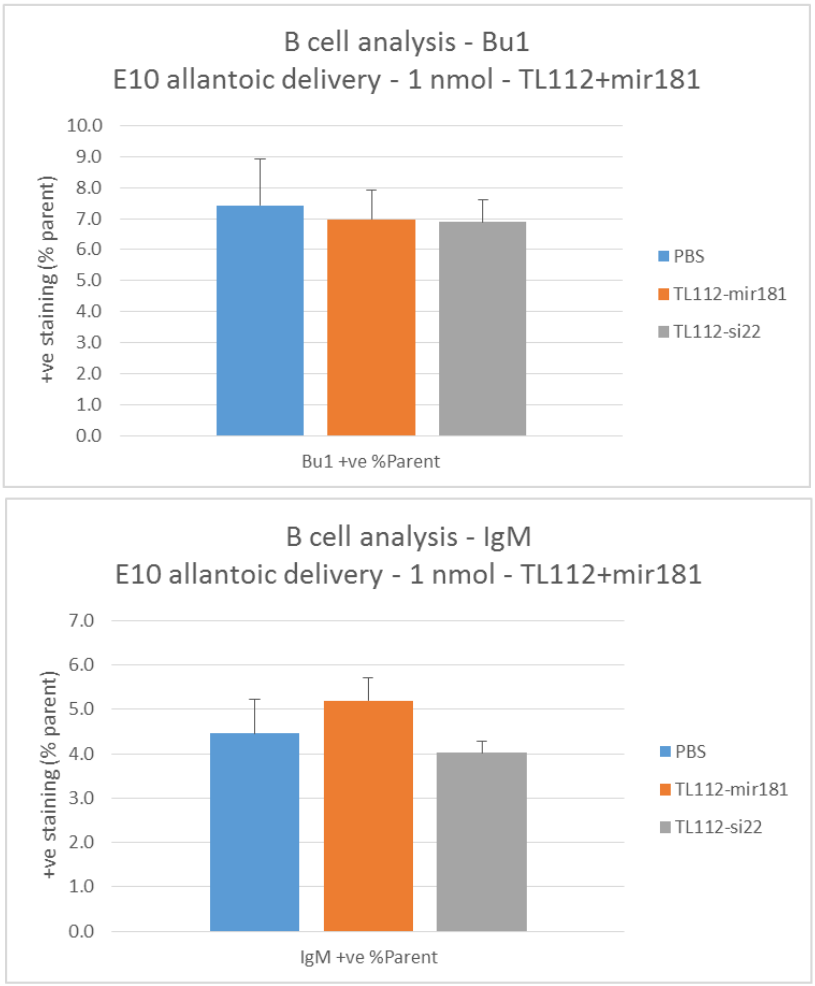
Figure 6: Increase in spleen resident B cell precursors by E10 allantoic delivery of 1 nmol miR-146a + TL-112

For the selected route of delivery via the allantois the optimised time of delivery was ED10. For the intravenous route the timing was originally set at ED2.5 but it was possible to adapt this to the ED10 time point to enable comparative data to be generated.

Assessment of smiRNA miR-181a

Having established that there was an observable, though not statistically significant, effect in response to delivery of miR-146a at E10 at 1 nmol via the allantois, this route, timing and dose was applied to miR-181a. In the analysis of effects on immune cell populations 8 days post treatment with miR-181a showed that there was a modest apparent effect on the level of IgM positive B cells in the spleen (Fig. 7), although this was not supported by the observed level of Bu1 positive cells (an alternate marker for B cells). It is possible that the Bu1 marker is showing both the B cell and B cell precursor population whereas the cells that are IgM positive population are in the matured or activated state. This will require more detailed analysis.

Figure 7: Increase in spleen resident activated B cells by E10 allantoic delivery of 1 nmol miR-181a + TL-112



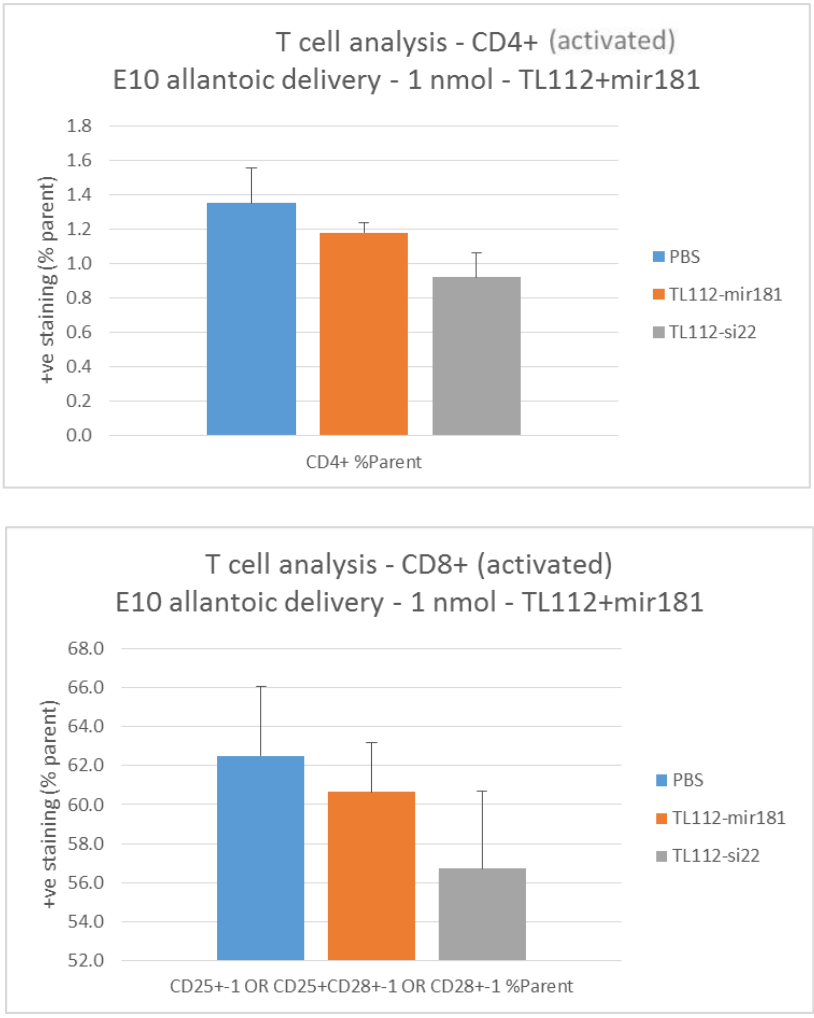


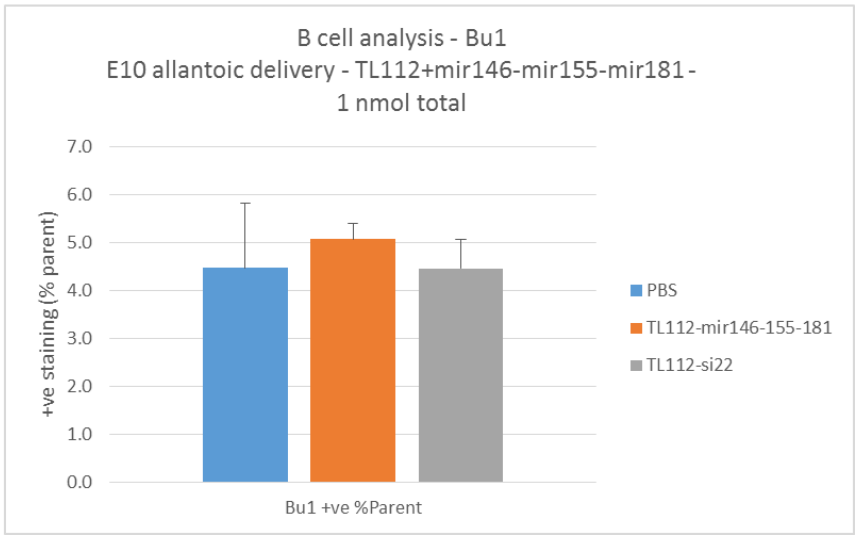
Figure 8: Increase in spleen resident activated CD4+ and CD8+ T cells by E10 allantoic delivery of 1 nmol miR-181a + TL-112

The T cell populations for CD8+ and CD4+ subtypes both show a modest (but not statistically significant) increase in the spleen in comparison to the spleen populations in embryos treated with the control siRNA, si22 (Fig. 8). However this was confounded by the fact that the untreated controls were equal to the miR-181a treatment group.

Combinatorial smiRNA (Milestone 10) and timing and point of injection (Milestone 11)

The effects of smiRNAs miR-146a and miR-181a although not statistically significant and somewhat subtle did indicate that the original objective of examining the combinatorial effect of each individual smiRNA was worth undertaking. Therefore the three smiRNAs produced for analysis, miR-146a, miR-181a and miR-155 were combined in a single formulation at the optimal dose of 1 nmol and was assessed by delivery through the optimal route of intra-allantois.

The observations showed that there was no effect of this combinatorial effect on the level of active or precursor B cell in the spleen of treated embryos (Fig. 9). This was also the case with respect to the levels of activated CD8+ and CD4+ T cells (Fig. 10) and for the levels of CD4+/CD8+ double positive T cells and the naïve precursor CD4-/CD8- lymphocytes (Fig. 10).



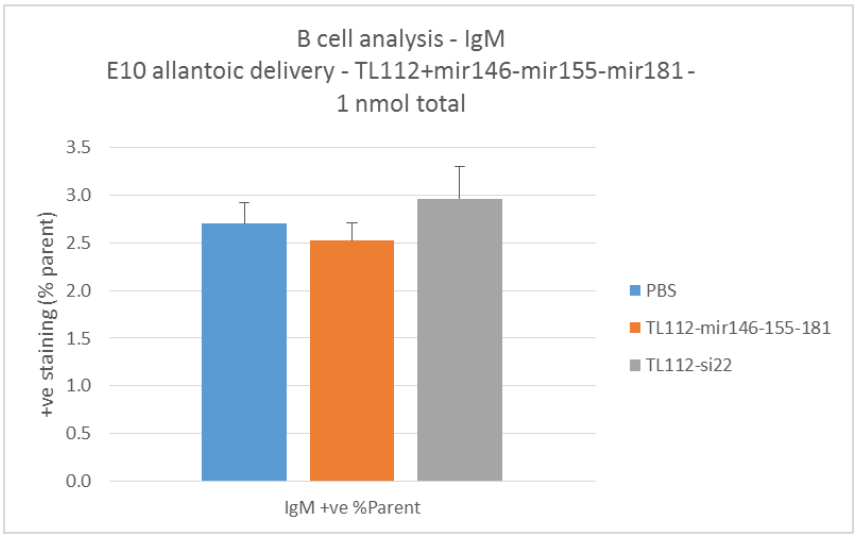
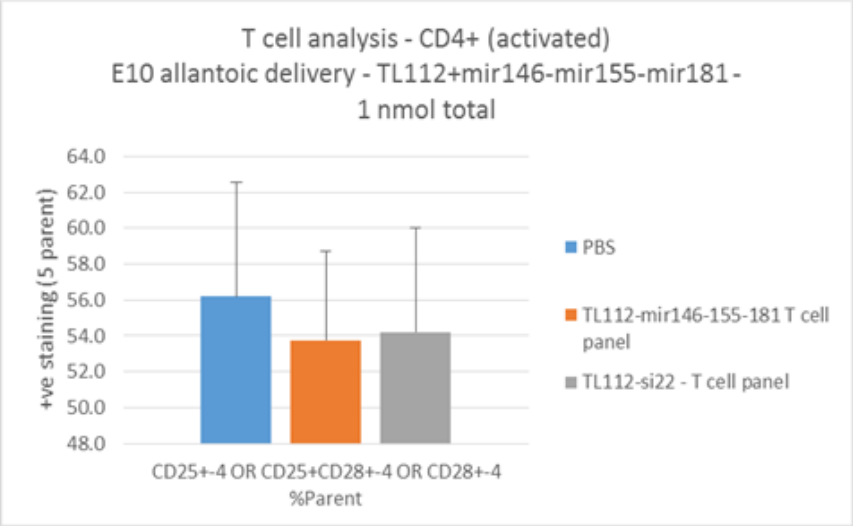


Figure 9: No change in spleen resident B cells and B cell precursors in response to E10 allantoic delivery of 1 nmol of combinatorial miR-146a-miR-155-miR-181a + TL-112



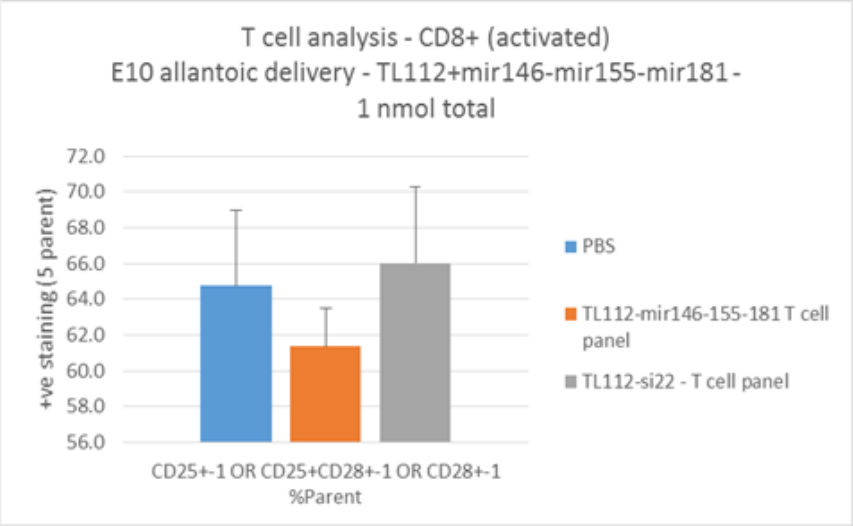
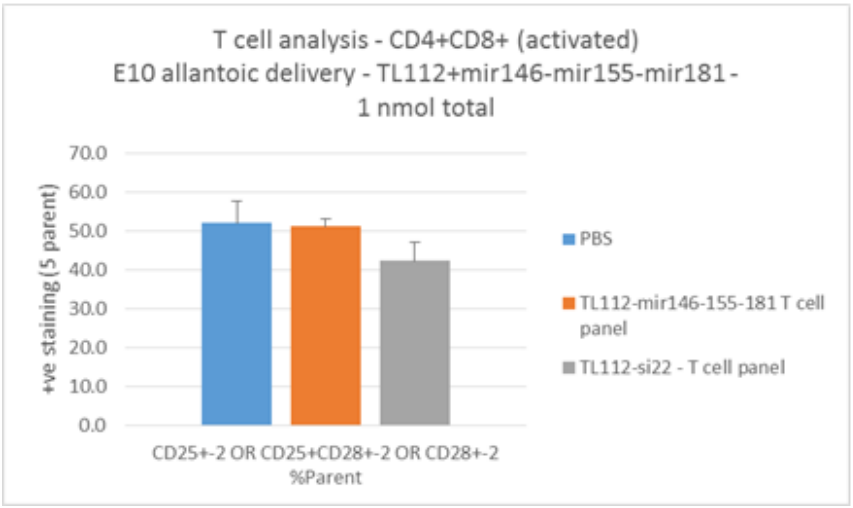


Figure 10: Decrease in spleen resident activated CD8+ T cells in response to E10 allantoic delivery of 1 nmol of combinatorial miR-146a-miR-155-miR-181a + TL112



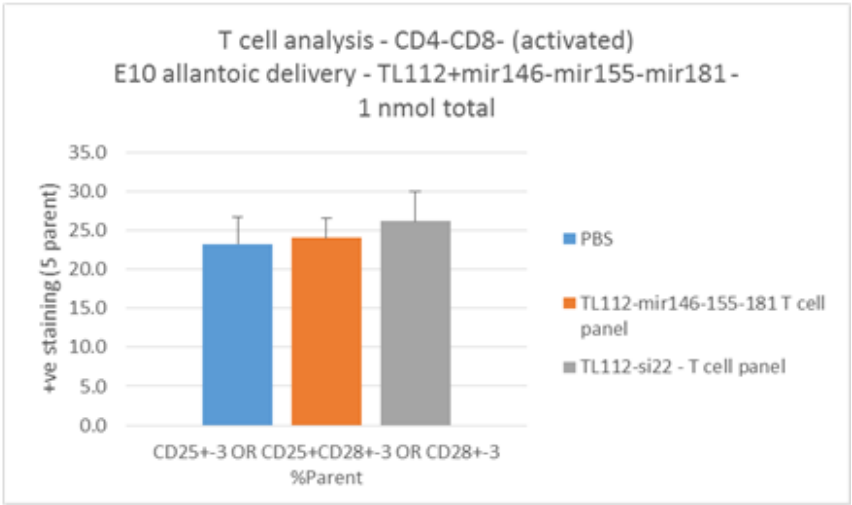


Figure 11: No change in spleen resident CD4/CD8 double positive or double negative T cells in response to E10 allantoic delivery of 1 nmol of combinatorial miR-146a-miR-155-miR-181a + TL112

Induction of a "danger associated molecular pattern" (DAMP) response to RAFT polymers

Initial investigation with the ABA tri-block indicated that when used to deliver anti-viral siRNA in chicken embryos delivery of the polymer with a negative control siRNA was also able to induce a low level of virus silencing. Subsequent analysis indicated that this was due to a interferon alpha response (Hinton *et al*. 2013). Previous data (Hinton, unpublished) using enhanced nanoparticle systems has shown that some can induce a DAMP response. Therefore the RAFT polymer siRNA formulations were investigated for their potential to activate this response. The chicken macrophage cell line HD11 was selected as a robust cell type that would be in the front line of interaction with nanoparticles injected into a chicken (embryo or adult). First the toxicity and uptake of the polymer nanoparticles in HD11 cells was assessed. Both the linear AB tri-block and the mikto star polymer were shown to be non-toxic at 3 and 24 h at up to 200nM. (Fig. 12A). Uptake of both polymer fluorescent siRNA complexes by HD11 cells was also observed at 24 h. This indicated that the nanoparticles had entered the cell (Fig. 12B).

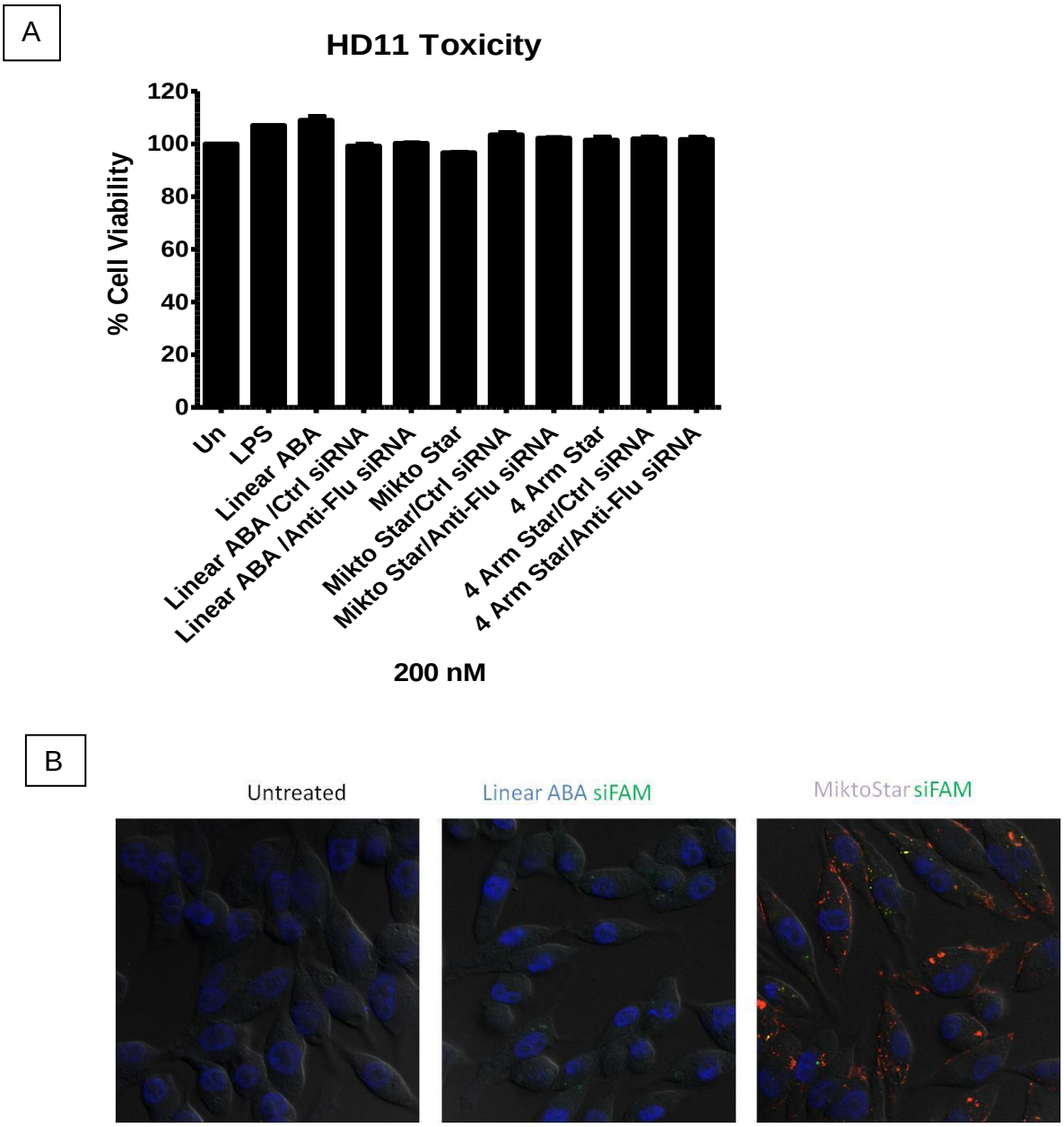


Figure 12: A) Toxicity of siRNA RAFT polymer formulations in HD11 cells in vitro; B) Uptake of siRNA RAFT polymer formulations in HD11 cells in vitro.

The first response expected is the activation of NALP3, which triggers cathespins which then activates Caspase 1. To determine if both NALP3 and Caspase 1 were being activated HD11 cells were incubated with RAFT siRNA nanoparticles for 6 and 24 h (Fig. 13). Both linear ABA tri-block complexes and the Mikto star complexes resulted in a 1.5-3 fold increase in NALP3 by 6 h which continued for the remaining 18 h of the assay (Fig. 13A). Caspase activation was also observed, as expected, following with a 1.5 – 2 fold increase observed at 6h with the mikto star and at 24 h with the ABA tri-block (Fig. 13B). The LPS control resulted in a 3 fold activation of NALP3 and 2 fold activation of Caspase 1 (Fig. 13) as would be predicted, whilst the viral RNA mimic PIC did not activate either NALP3 or Caspase 1.The siRNA control resulted in NALP3 activation at 6 h which had resolved by 24 h with minimal activation of Caspase 1(Fig. 13), demonstrating that the sustained NALP3 and Caspase 1 response was trigger by the structure of the RAFT siRNA nanoparticle.

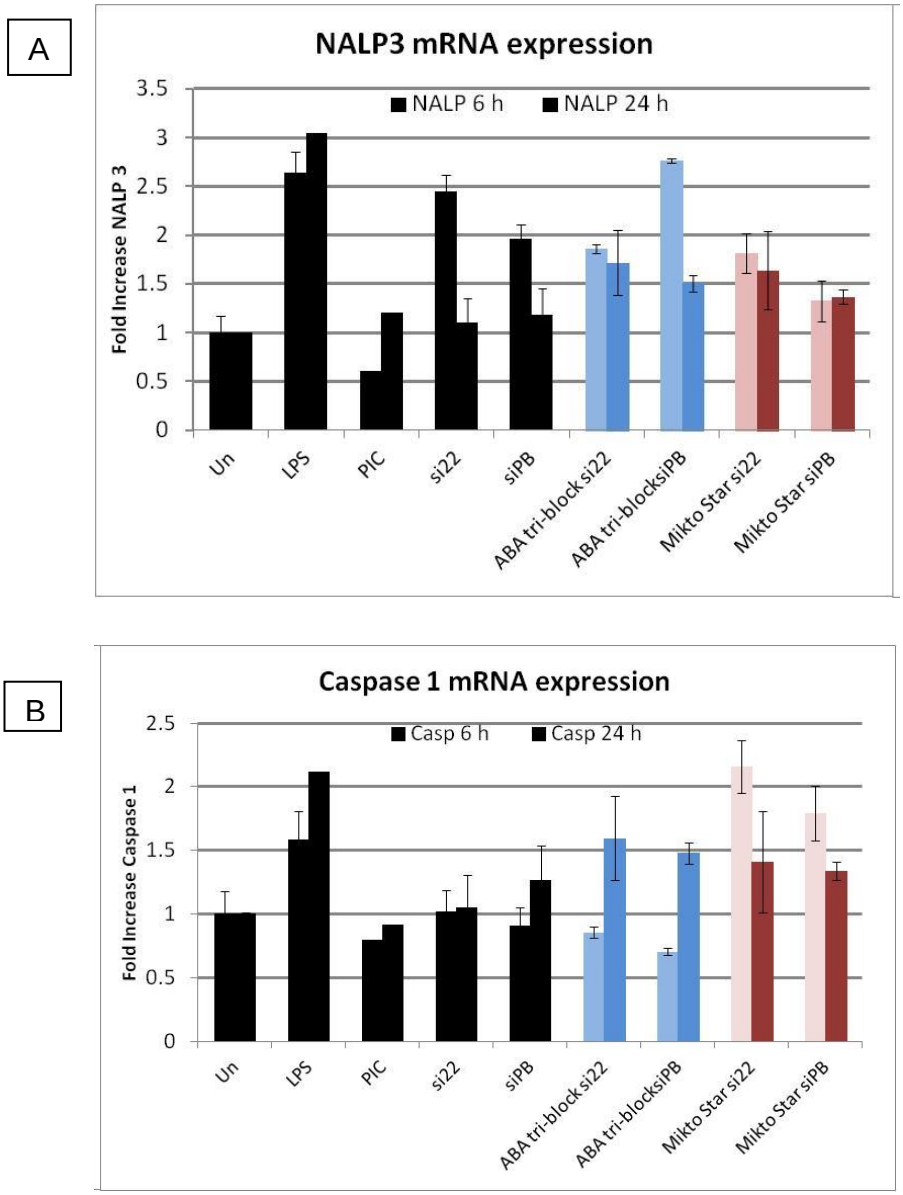
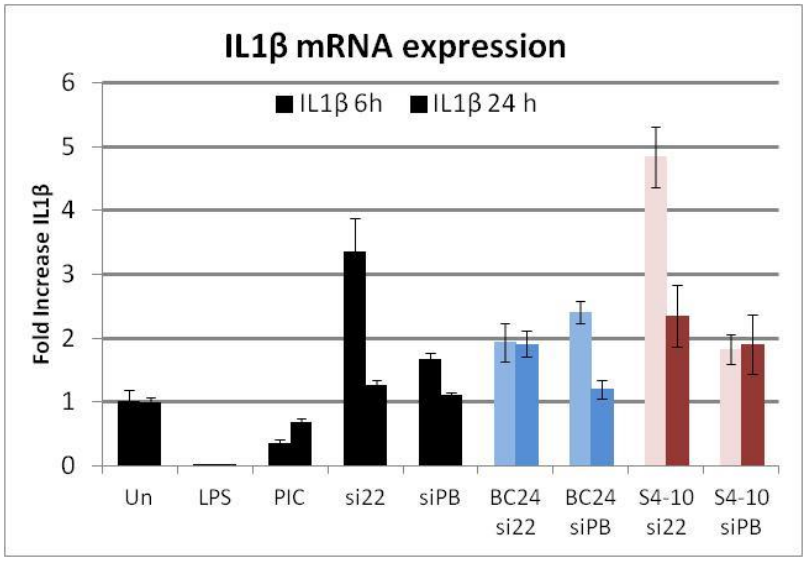


Figure 13: A) NALP3 and B) Caspase activation 6, 24 hr post treatment

The next step in the pathway is the activation and secretion of IL1β. The pro-form of IL1β is already present in the immune cells. This is cleaved to become active IL1β. However upon stimulation more IL1β mRNA will also be produced. Therefore qRT-PCR was performed to determine if IL1β mRNA was upregulated and IL1β ELISAs were performed on cell culture supernatant to determine if active IL1β was being secreted (Fig. 14)

A



B

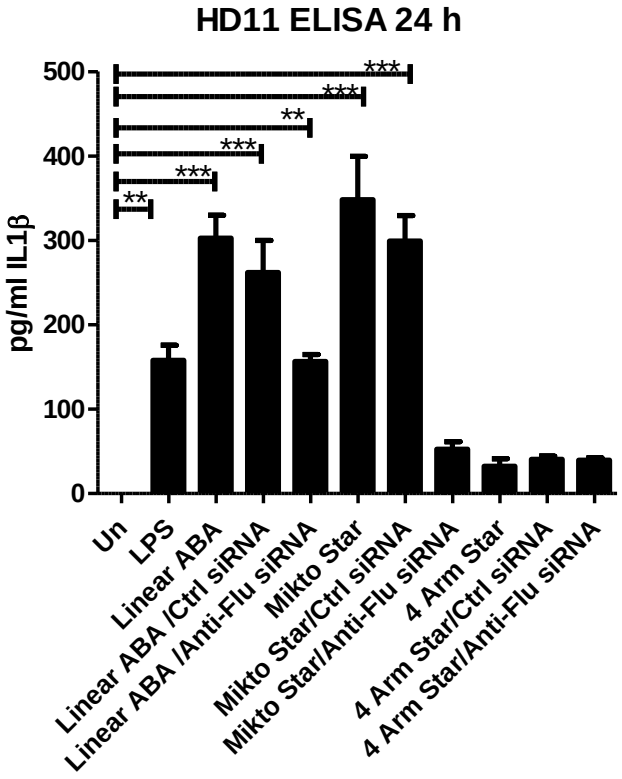


Figure 14: A) IL1B mRNA activation and B) protein secretion from HD11 cells 6, 24,

From these result it is clear both RAFT polymer siRNA complexes are inducing a DAMP response in HD11 cells. To ensure this was inducing a T helper response primary chicken splenocytes were exposed to HD11 supernatant for 24 and 48 h and T cell activation was determined by upregulation of CD25 on the cell surface. Co-staining with CD8 and CD4 staining antibodies allowed identification of the different T cell subtypes. Both the linear tri-block and the mikto star RAFT polymers caused specific up-regulation of T helper cells (Fig. 15).

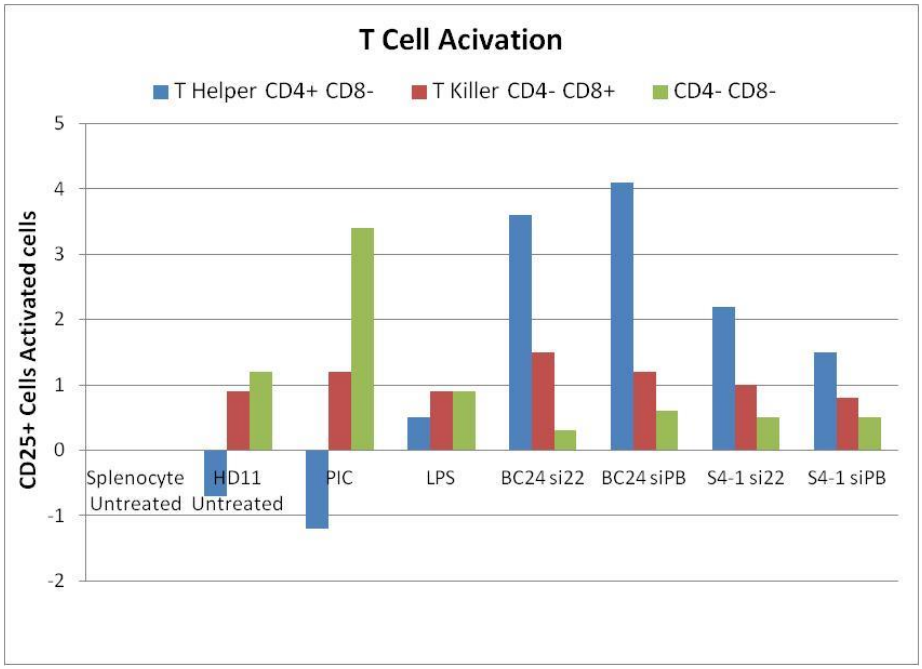


Figure 15: Activation of T cell subsets in splenocytes stimulated with supernatants HD11 treated with RAFT:siRNA nanoparticles

Finally to determine if the T helper response was driving an antiviral response interferon α and γ induction was assessed in these primary splenocytes (Fig. 16). Again both polymers resulted in 5-10 fold increase in IFN α and γ after 48 h. This response is slower than would be expected for a viral infection, however this is most likely due to the fact there is only 1 initial dose of polymer, whilst viral infection will resulting in increasing amounts of stimulant.

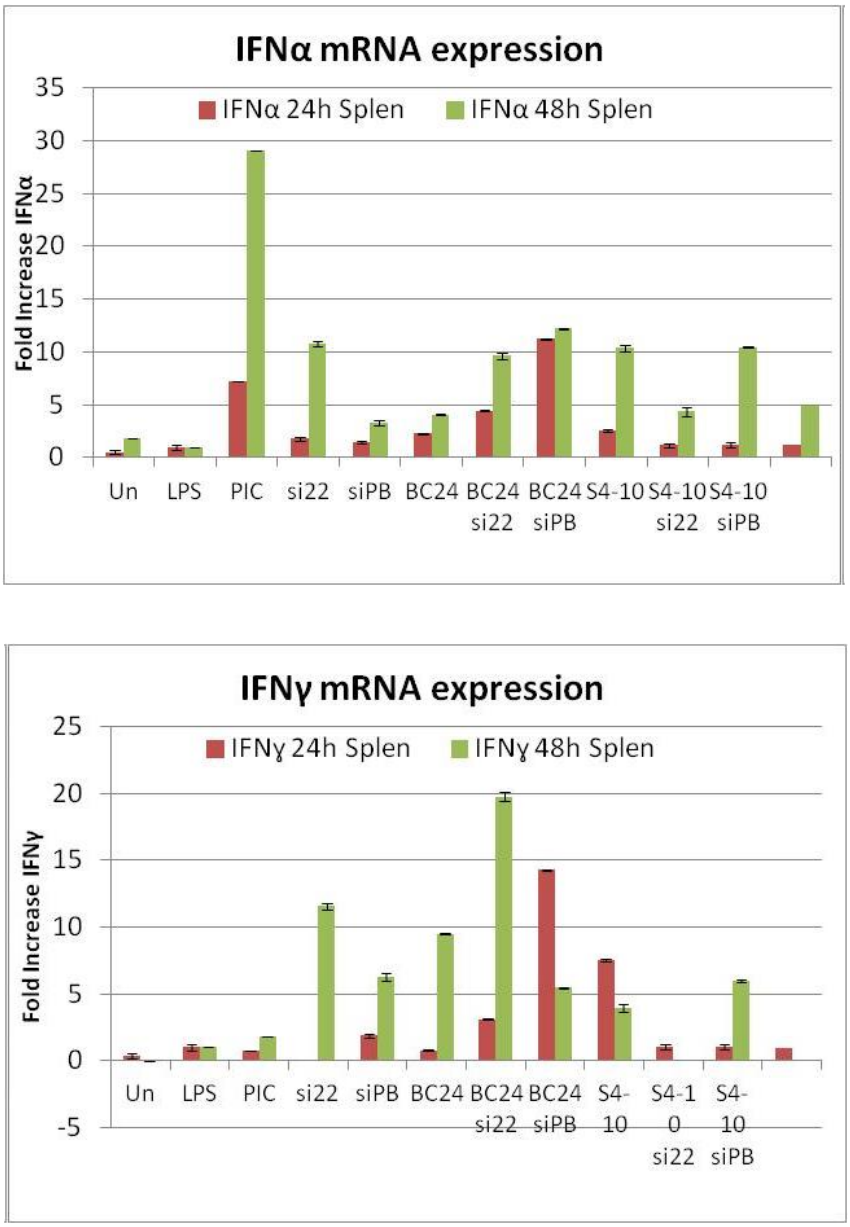
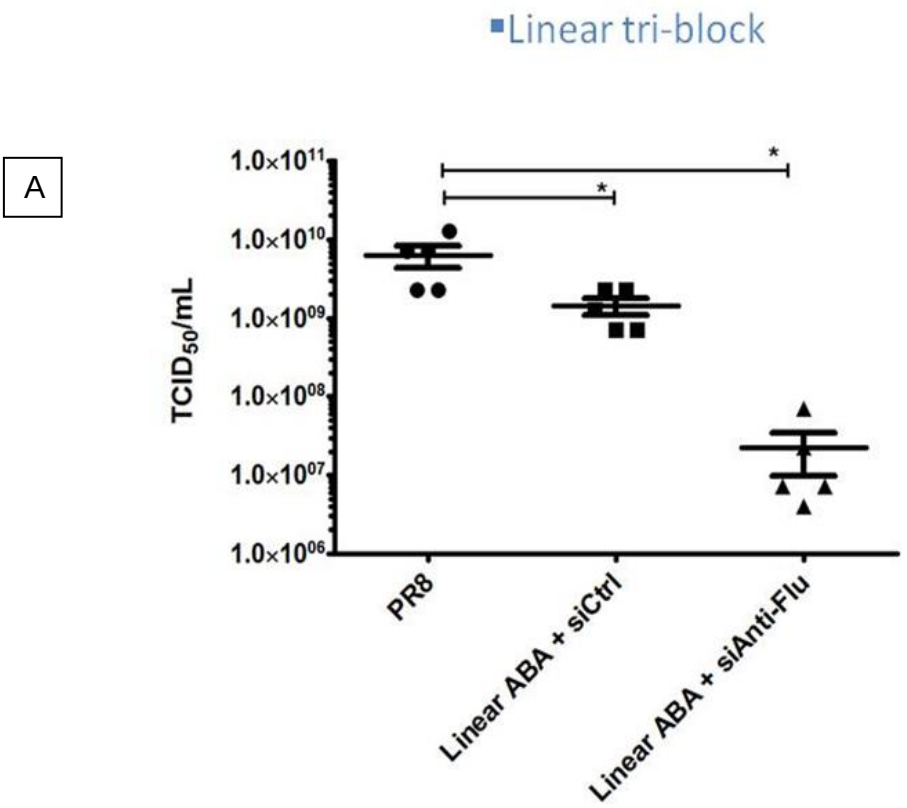


Figure 16: Splenocyte activation assessed by the increase in IFN α and γ mRNA 24 h and 48 h after stimulation with supernatants HD11 treated with RAFT:siRNA nanoparticles

The final functional assessment of the DAMP response generated by the RAFT-siRNA nanoparticles was conducted by the treatment of ED18 followed by challenge with influenza virus. The control siRNA (siCtrl = si22) targets the GFP gene (which is not present in the system) in comparison with an siRNA targeting the influenza virus polymerase basic protein (siPB also labelled siAnti-Flu). The response with both the linear tri-block RAFT and the mikto star RAFT showed that there was a significant reduction (~ 1 log = 10 fold) in replication of the virus without the siRNA targeting its polymerase PB gene, which is therefore presumed to be attributable to the DAMP response (Fig. 17 A & B). As expected the strongest reduction in influenza virus titre was in response to the treatment with RAFT-siPB (also labelled saint-Flu).

A



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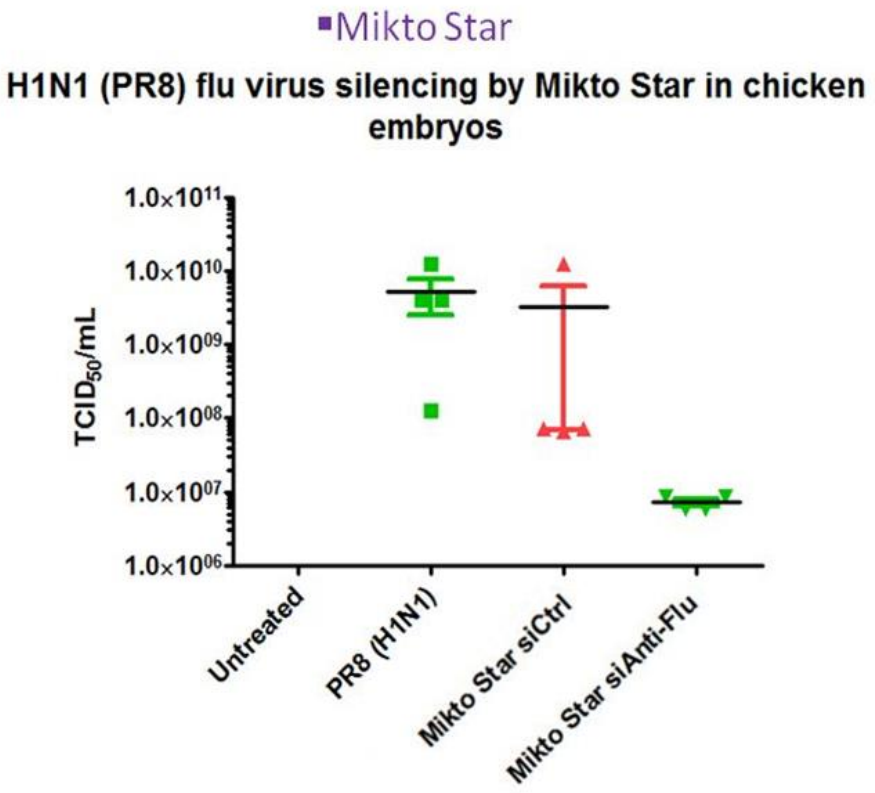

Figure 17: DAMP response following RAFT:siRNA nanoparticles treatment of ED18 embryos assessed by inhibition of replication of influenza virus PR8 (H1N1) ($TCID_{50}$ measured).

Discussion of Results

Functional studies of three different RAFT polymers designed to bind, protect and deliver small therapeutic RNAs, such as siRNAs and smiRNAs, indicated that the linear tri-block (BC-28), the four arm star (TL39B) and the mikto arm star (TL-112) had the best performance characteristics suited to the application of smiRNA to the developing chicken embryo. This included showing a low level of toxicity in tissue culture, a high capacity and protection of the small RNA payload in vitro and high efficacy in cell culture gene knock-down assays. These were paired with commercially sourced synthetic miRNAs, miR-146a and miR-181a which are involved in the development and maturation of lymphocytic cell of the B and T lineage (Baltimore *et al*, 2008) and also miR-155, which plays an important role in activating macrophages and is effective during viral disease response (Izzard *et al*, 2015).

Formulations of RAFT polymer and control siRNA material was used to investigate the bio-distribution and also the potential toxicity of formulations in the developing chicken embryo, in comparison with a commercially available small RNA delivery vehicle, Lipofectamine 2000. Dose rates were determined that were suitable to the route of delivery and the timing. For early embryos with lower tissue mass 75 pmole was found to be the highest tolerable level of smiRNA without reducing the hatchability of the treated chicks below an acceptable level (i.e. ≥80%). Previous work from our laboratory has shown that a dose of 50-200 nM of an siRNA is sufficient to mediate effective knockdown of target gene expression. 50 pmole of siRNA delivered to an E2.5 embryo (at ~0.5 g of tissue) is equivalent to 100 nM dose concentration. Since the material delivered to the E2.5 embryo was intravenous it was considered to be direct to the tissue mass of that embryo. Dose rates to the allantois and to the yolk were considered to be directed to large reservoir that could persist and yield a slower delivery to the growing embryo from the time of delivery (E10 or E4 respectively) for the duration of growth until hatch of the chick at a final mass of ~ 10-15 g. For these two route the dose rate of 1 nmole was used, this representing an overall dose concentration of 75-100 nM. Thus the dose rates of via each route are in an experimental range that is known to be effective and in each case shown by the experiment here to be tolerated by the developing embryos (based on hatchablity).

The biodistribution studies showed that the RAFT polymers and the siRNA could be found taken up by tissues of the embryo at E2.5 in a manner consistent with functional uptake in cell culture model systems. For intra-allantois injection we have evidence from previous studies that siRNA delivery by this route is found in the cells of the chorio-allantoic membrane, particularly around the blood vessels through which there is material exchange to the developing embryo (Hinton *et al*, 2014). The yolk is a difficult material to handle and analyse due to its complex composition (particularly lipids and proteins) and thus it was not possible to track the fate of smiRNA formulations directly in this material. Use of this route was built on the supposition that it is clearly connected with embryonic tissues acting as a slow release nutrient reservoir during the full course of development. The allantoic and yolk routes were designed with a final tissue dose rate set for the hatchling such that a steady supply of smiRNA formulation would be steadily delivered as the embryonic immune system developed. The mikto arm RAFT polymer TL-112 was better tolerated than either the linear tri-block (BC-28) or the 4 arm star (TL39B) in terms of chick hatchabilty. This may be due to the inclusion of a certain proportion of hydrophobic arms in the multi arm architecture of this RAFT polymer, a characteristic lacking in the linear tri-block and 4 arm star polymers. Though we have not been able to devise a methodology to assess the physiochemical characteristics imparted by these hydrophobic arms it is

interesting to note that a mikto arm RAFT polymer constructed without inclusion of that hydrophobic component had much lower efficacy in an *in vitro* cell culture gene silencing assay. The TL-112 polymer was therefore selected for the remaining assessments of smiRNA delivery.

There was less supporting evidence for formulation of smiRNAs with the commercial delivery reagent L2000CD. Although it was well tolerated by embryos in terms of hatchability (~100%) the biodistribution data was not as strong. In addition it was clear that the pricing of L2000CD (at the time of this report) would be prohibitive to industry adoption.

The established tolerable doses and the optimal route of administration were used to formulate the smiRNAs miR-146a, miR-181a and miR-155 and treat embryos. After treatment key immune cells were harvested from E18 embryos (the time point at which Inovoject® vaccination would take place) to assess any changes in the relative levels of these cells and their activation state, using FACS analysis for well characterised cell markers. The observations, made across an number of iteration of the experiments and a full range of B and T cell markers, although indicating modest trends in population changes did not establish any statistically significant effects stimulated by any of the three smiRNAs being used. Since these data did not reach statistical significance it was not possible to justify (to the satisfaction of animal ethics conduct) the planned experimental vaccination and possible challenge that might have followed from a stronger indication of beneficial immunological changes. However in the natural circumstance of a general immune activation it is likely that multiple microRNAs would act in concert with one another. The individual smiRNA data were sufficient to encourage attempts at a combinatorial approach.

The outcome of combining all three smiRNAs in a single dose administered intra-allantois or intra-yolk was not successful in terms of increasing any of the non-statistical differences observed with single smiRNA administration. The data therefore did not justify more extensive *in vivo* assessments of vaccination and/or challenge. However observations made in parallel to the pursuit of this Sub-Project, developing the RAFT polymer systems for anti-viral siRNA delivery indicated that some forms of the RAFT polymers had intrinsic immune patenting properties that appeared to be generating “danger associated molecular pattern” (DAMP) responses. Experiments to assess this in greater detail showed that RAFT polymers of the structure equivalent to TL-112 would trigger a DAMP response in the chicken macrophage cell line HD11. This was determined by the demonstration of specific stimulation of interferon (IFN α and γ) and interleukin (IL1β) responses in embryonic and adult splenocytes incubated with the activated HD11 supernatants – demonstrating that they containing specific signalling molecules released in response to RAFT polymer exposure.

The in vitro data were tested in a viral challenge experiment conducted in ovo (prior to hatch) which utilized the RAFT polymers formulated with anti-viral siRNA (as a positive control) and with a control siRNA (si22) as an assessment of the intrinsic immune stimulation generated by the RAFT polymer. The outcome was a demonstration that a DAMP response generated by both the linear tri-block and the mikto arm RAFT polymers was sufficient to reduce influenza virus replication to by between 5 – 10 fold. These observations are encouraging in relation to a potential adjuvant role for the RAFT polymers per se. This is something that will require further studies particularly since the initial screen of these RAFT polymers was for the distinct biological function of siRNA delivery. It is possible that the synthesis of RAFT polymers with function blocks of different physicochemical property and composition may have enhanced performance in the

context of DAMP response stimulation. Such molecules if they can be identified could provide an alternative to the use of smiRNA to enhance in ovo vaccination and could be useful in vaccination strategies for adult birds in the poultry production system.

Implications

MicroRNAs play a critical role in gene expression and thus the regulation of important processes including cell-type differentiation and development. It is no surprise that they therefore play critical roles in the development, maturation and activation of all key cell types in the immune system.

The Subproject has made significant steps forward towards the goal of assessing the potential smiRNAs (particularly smiR-146a and smiR-181a) to alter the maturation state of immune cells in the young embryo to enhance its ability to respond to *in ovo* vaccination. Although none of the changes in B and T cell populations has reached clear statistical significance by ANOVA there is still a trend which indicates that there is some biological impact.

The findings of this Sub-Project indicate that the technology for delivery of synthetic microRNAs as adjuvants to enhance Inovoject® vaccine responses is not sufficiently developed to consider industry transfer. Though there are some encouraging signs that smiRNA delivery to the developing chicken embryo in ovo can influence immune cell population development the level of change does not meet statistical significance. Increasing the dose to increase the effect was counter-productive since the higher concentration of the smiRNA::RAFT polymer formulation causes defects in development and failure of chicks to hatch at an unacceptably high percentage. The implications are that with the currently available technology for delivery of small RNA such as smiRNAs it is not possible to influence the development of the immune system of the early embryo to a level that is of value in the desired industrial application. Since siRNAs and smiRNAs have significant therapeutic potential in human medicine the search continues for the optimum vehicle for their delivery (Shajari N *et al*, 2016). Should such a material be found it would be of value to reassess this strategy, however in its current form this technology is not ready for industry translation. An unexpected observation made during the course of the work was that certain forms of the RAFT polymer delivery vehicle, particularly the “mikto-arm star polymers” (TL112 and S4-10), were able to stimulate a DAMP, that is to say an immune activation in a chicken macrophage cell line and in primary splenocytes from adult chickens. Though smiRNAs may not have been validated for in ovo adjuvant effect it appears that the RAFT polymers hold some promise as adjuvants for use in adult birds.

Recommendations

There is still great potential in the use of synthetic microRNAs for the immune potentiation of chicken embryos and a role in creating step improvements in the *in ovo* vaccination strategy. However improved small RNA delivery reagents will need to come to the market place in order to justify re-initiating further research. It is possible, even likely that such reagents could emerge in the mid-term since there is a great deal of research being performed in the medical field as siRNAs and smiRNAs still have great promise as therapeutics in human medicine.

The current format of RNA polymer delivery reagents is not sufficiently active to proceed any further for applications for the Australian poultry industry. However the unexpected observation that RAFT polymers can stimulate DAMP responses that could be useful in general adjuvant strategies is a benefit of the outcomes from the Sub-Project. Further developments of these observations awaits the identification of broader industry partners for whom this might generate a valuable tool to improve infectious disease impacts.

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POULTRY CRC

Plain English Compendium Summary

Sub-Project Title:	Vaccine response boost through immune enhancing microRNA delivery in ovo
Poultry CRC Sub-Project No.:	1.4.1
Researcher:	Dr Mark Tizard
Organisation:	CSIRO Health & Biosecurity
Phone:	03 5227 5753
Fax:	03 5227 5555
Email:	mark.tizard@csiro.au
Sub-Project Overview	The Sub-Project aimed to use microRNAs to stimulate the immune system of the chick while still in the egg to enhance the performance of a range of vaccines that are administered by the automated Inovoject® system. Direct injection of synthetic microRNAs (smiRNAs) formulated with RAFT polymer was assessed in relation to the development, maturation and activation state of immune cell populations in developing emmbryos.
Background	The Inovoject® system for broiler vaccination has provided substantial improvements in the health, welfare and productivity of poultry. However there is still significant room for improvement if the immune system of the pre-hatch chick could be boosted. MicroRNAs are small RNAs that are involved the development and activation of many types of cell including the key cells that make up the immune system. To exploit the potential of microRNAs to reprogram and enhance the immune system of a developing chick they must be delivered effectively in the egg.
Research	CSIRO has patents on the use of RNA interference (which microRNAs are involved in) and on the production of biomaterials called RAFT polymers that are able to deliver small RNAs such as microRNAs. The objective of the research was to test the ability of RAFT polymers to deliver microRNAs into the tissues of a developing chick in the egg and for this have a beneficial effect on measurable immune parameters.
Sub-Project Progress	RAFT polymers were found to be able to deliver small RNAs into the tissues of the developing chicken embryo in the egg. The dose level of smiRNA and RAFT polymer complex was found to be limiting to maintain normal levels of hatchability of the eggs. Some small changes to the immune cell populations and their activation were achieved but they were not dramatic. By completion of the Sub-Project it was clear that the technology was not sufficiently advance to be considered for translation to industry. An unexpected observation showed that certain forms of the RAFT polymer were able to stimulate and activate chicken immune cells. This may be useful for developments of adjuvants, immune stimulants, for standard vaccination strategies.
Implications	The Inovoject® system for broiler vaccination is a robust and valuable tool to improve health, welfare and productivity in production. Improvement of its performance through the application of synthetic microRNAs as adjuvants although promising is not sufficiently advanced to be translated to industry practice at this time.
Publications	None.