Project periodic report
Publishable Summary

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Report against Grant Agreement – Amendment 2

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3.1 Publishable summary

Background

While vaccination is the cornerstone of influenza prophylaxis, current inactivated vaccines provide only moderate protection, requiring annual updating due to poor long-term immunity and antigenic drift of the virus. Efficacious, protective immunity requires humoral and cell-mediated defences. The UniVax goal is to develop the first multimeric and synthetic universal influenza vaccine based on self-replicating RNA replicons targeted to dendritic cell receptors by synthetic delivery vehicles, inducing humoral and cell-mediated immunity for broad, long-lasting protection.

Replicons are derived from defective virus genomes, from which at least one structural protein-encoding gene has been deleted – replicons replicate and translate but cannot produce virus. Inherent problems with many current replicons are their cytopathic nature (reducing the duration of immune response induction), the need for virus-like particles for delivery, and their derivation from human pathogens. Moreover, they are not targeted to dendritic cells – the critical cells for efficient immune response induction, and therefore efficacious vaccination.

UniVax employs pestivirus replicons (RepRNA) derived from a non-cytopathic porcine virus, which is non-infectious for humans. The RNA is delivered into dendritic cells by synthetic means, wherein the RepRNA efficiently translates and replicates. Our current RepRNA carries insertion sites that efficiently facilitate the accommodation of any gene of interest for prophylactic or therapeutic applications. RepRNA encoding influenza virus antigens offer the potential for broad universal protection against influenza. Co-formulation with novel mucosal adjuvants, such as c-di-AMP, potentiates robust humoral and cellular immune responses, including cytotoxic and multifunctional T cells – the latter related to robust protective T-lymphocyte immunity. Overall, this approach offers an effective alternative to pandemic and seasonal vaccinations, providing a means of reducing dependency on constant annual vaccine renewal.

Description of Work and main results until month 66

The active component of the multimeric influenza vaccine within the UniVax project is based on the patented pestivirus replicon RNA (RepRNA). Thus, an important advantage for the UniVax consortium comes from this intellectual Property for the nanoparticulate delivery of the RepRNA. This is owned by Partner 1:

1. Tratschin JD, Ruggli N, McCullough KC. US9670466B2. Pestivirus replicons providing an RNA-based viral vector system. Priority Date June 4th 2008.
2. Tratschin JD, Ruggli N, McCullough KC. US9249395B2. Pestivirus replicons providing an RNA-based viral vector system. Priority Date June 4th 2008.
3. Tratschin JD, Ruggli N, McCullough KC. WO 2009/146867 Al. Pestivirus replicons providing an RNA-based viral vector system. Priority Date June 4th 2008.
4. Tratschin JD, Ruggli N, McCullough KC. EP 2130912A1/ EP20090757250. Pestivirus replicons providing an RNA-based viral vector system. Priority Date June 4th 2008.

These patents claim delivery of said replicons by nanoparticulate delivery vehicles (“said delivery vehicle is selected from the group consisting of: liposomes, nanoparticles and nanocapsules.”), thus providing protection for the innovations arising from the UniVax Consortium.

Choice of the Pestivirus Replicon

Replicons are genetically modified viral genomes; they are self-amplifying (self-replicating) vaccines, incapable of generating infectious progeny. This is achieved by deleting genes encoding for essential viral structural proteins. Their translation capacity to promote this self-replication also produces the vaccine antigens from inserted genes encoding for these antigens.

The pestivirus replicon has major advantages over other replicons:
(i) Pestivirus RepRNA is non-cytopathogenic, unlike the majority of replicons in use today – including alphavirus- or flavivirus-derived replicons – which are cytopathogenic.
(ii) Pestivirus RepRNA efficiently translates and replicates in dendritic cells (DCs).
(iii) Importantly, pestivirus RepRNA will not kill DCs in which it is translating and replicating, rendering it more ideal as a vaccine than other replicons such as those derived from alphaviruses, which are cytopathogenic and thus reduce duration of immune induction.
(iv) The pestivirus genome and replicon do not carry 5'-triphosphates, in contrast with alphaviruses where the 5'-triphosphates can signal cell innate defence mechanisms to impede or even destroy the replicon, reducing their efficacy as vector vaccines.
(v) The pestivirus leader protein encoded by the genome (N^{pro}) interferes with cell-signalling pathways responding to the presence of the RNA that would otherwise lead to cell activation and interferon induction impairing replicon function.

These characteristics of the pestivirus replicon facilitate its retention in DCs for prolonged periods. As such, this fits well with the manner by which DCs slowly process antigen for presenting to the adaptive immune system over a prolonged period, thus promoting robust immune defences.

Application of the Pestivirus Replicon (RepRNA)

Existing constructs from the Replixcel, HCVAX and HCRus projects provided the starting pestivirus replicon material for UniVax – namely plasmids carrying the RepRNA encoding reporter genes (such as luciferase) and influenza virus haemagglutinin (HA (H5)) or nucleoprotein (NP). These plasmids were employed to produce the first RepRNA for testing with the different biodegradable, nanoparticulate delivery technologies. of the UniVax Consortium.
For more details, see

1. McCullough et al (2014) Self-replicating Replicon-RNA Delivery to Dendritic Cells by Chitosan-nanoparticles for Translation In Vitro and In Vivo. Mol Ther Nucleic Acids. 3:e173
2. McCullough et al (2012) Functional RNA delivery targeted to dendritic cells by synthetic nanoparticles. Ther Deliv.3:1077-99

Production of the RepRNA from the above plasmids requires linearisation and transcription. Thereby, an essential step involves a unique endonuclease site which does not recognise any sequence within that for the replicon. In order to assure continued production of the RepRNA as required, a second endonuclease site was sought to obviate dependence on the availability of a single endonuclease. However, no other unique site was found. Therefore, a second unique site was engineered into the construct, providing a choice for the endonuclease to be used in the production process for the RepRNA. The "backbone" construct was thus prepared, in which the "foreign gene of interest" (GoA) encoded for luciferase.

For more details, see

Démoulins et al 2017 Self-Replicating RNA Delivery to Dendritic Cells", in "RNA Vaccines: Methods and Protocols", ed. T. Kramp & K Elbers, Chapter 5, Methods in Molecular Biology vol 1499, Springer Science+Business Media, New York.

RepRNA Replicon Constructs

From the luciferase-encoding construct, additional constructs have been generated. These encode for influenza virus HA (H1 Cal2009), neuraminidase (NA (N1)), M protein, and PB1 protein. A further construct encodes for ovalbumin sequence detected by OTI and OTII cells. This allowed for assessment of delivery systems in the OTI/OTII model, but also has applicability in future products as a cancer vaccine in certain animal models.

The above RepRNA constructs encoding a series of antigens translate to provide the means for inducing both humoral and cell-mediated arms of immune defence. New RepRNA prototypes

have been constructed to lack one, several or all the structural proteins of the original pestivirus sequence. The aim of UniVax was to formulate the RepRNA encoding influenza virus antigens for delivery, with particular emphasis on interaction with DCs.

The expression of the encoded vaccine antigens was assessed alongside expression of certain pestivirus proteins still encoded by the RepRNA. By such means the relative efficiency of translating the GoA encoding the influenza virus antigen was compared with the endogenous genes – the former are translated via the ribosomal entry site naturally occurring in the 5'-NTR of the pestivirus RNA, while the latter are translated via an additional ribosomal entry site inserted after the GoA. This allows determination of the efficiency with which the GoA near the 5' end of the RepRNA can be recognised and translated compared with the genes downstream of this, including those required for replicon replication.

The majority of the partners in UniVax would be involved with either the delivery of the RepRNA or the *in vivo* assessment of the delivery (in certain cases, also *in vitro* assessment). Thus, training of these partners in the methodologies for producing RepRNA was achieved via a number of workshops held by Partner 1, as well as follow-up visits for trouble-shooting. This aim was to ensure that the recipient partners became efficient at producing RepRNA in-house (Démoulins et al 2017 Methods in Molecular Biology vol 1499: Chapter 5). Partner 1 was in charge of this training, due to their ownership of the intellectual property for nanoparticulate delivery of RepRNA (see above) and their long-term experience in producing this material from the plasmid constructs they had generated.
See the aforementioned patents and references, as well as

Suter et al (2011) Immunogenic and replicative properties of classical swine fever virus replicon particles modified to induce IFN-α/β and carry foreign genes. Vaccine. 29:1491-503

Formulation for delivery to dendritic cells

The UniVax Consortium has assessed several different biodegradable delivery systems, and modifications therein, namely virus-like replicon particles (VRPs) developed by Partner 1 for pestivirus RepRNA, cationic lipids (lipoplexes) developed by Partner 2 to promote RNA delivery, lipopolyplexes developed by Partner 7 – Histidylated Lipid/Polymer/RNA particles (LPRs) combining advantages of lipidic and polymeric carriers, and controlled protonation (pKa) of histidines during endosomal acidification to promote early escape from endocytic vesicles (cytosolic translocation), neutral lipopolyplexes (LPP) developed by Partner 7, which have negligible protein adsorption, decreased complement activation and favour extracellular matrix and distribution to lymphatics upon interstitial administration, polyplexes using PEI of different molecule weight, with or without cell penetrating peptides, chitosan-based complexes developed by Partner 8/Partner 12, whereby particle cores can promote endosomal escape, while the outer shell accommodates targeting ligands.
new liposome delivery systems resembling virosomes – termed "HA-nanoparticle" – designed for Partner 1 using commercial liposome formulations, introduced by the UniVax Consortium due to the success observed by consortium partners with virosome vaccines
(see Ebensen et al 2017 Mucosal Administration of Cycle-Di-Nucleotide-Adjuvanted Virosomes Efficiently Induces Protection against Influenza H5N1 in Mice. Front Immunol. 28;8:1223)

All nanoparticulate delivery systems were physico-chemically characterized, with the exception of the VRPs. For each of the above delivery systems, a number of different components was analysed. The aim therein was to determine if modifications in the structure of the material or the formulations would impact on interaction with and delivery of the RepRNA. The exception was with the VRPs, which resemble the parent virus of the RepRNA and have been already well characterized. Due to their high efficiency at RepRNA delivery for translation and replication, they were also the reference point against which other delivery systems were compared.

Overall, the main criteria were that the delivery vehicles were
efficient at complexing RepRNA and protecting against RNase,
capable of interacting efficiently with DCs to promote cargo uptake,
readily tolerated by cells and host,
efficient at promoting RepRNA release within cells to facilitate translation of the encoded antigens.

Details of the assessments are shown below under sections on *in vitro* and *in vivo* evaluations. For further details, see also

1. Démoulins et al 2016 Polyethylenimine-based polyplex delivery of self-replicating RNA vaccines Nanomedicine 12:711-722;
2. Démoulins et al 2017 Self-Replicating RNA Delivery to Dendritic Cells", in "RNA Vaccines: Methods and Protocols", ed. T. Kramp & K Elbers, Chapter 5, Methods in Molecular Biology vol 1499, Springer Science+Business Media, New York;
3. Démoulins et al 2017 Self-replicating RNA vaccine functionality modulated by fine-tuning of polyplex delivery vehicle structure. J Control Release 28;266:256-271;
4. Englezou et al 2018, Self-amplifying Replicon RNA delivery to Dendritic Cells by Cationic Lipids, Molecular Therapy Nucleic Acids 12:118-134.
5. Perche et al., Versatility of neutral lipopolyplexes for in vivo delivery of conventional and replicative RNA. Molecular Therapy Nucleic Acids under revision

Targeting

The targeting approach sought to identify ligands reactive with receptors on DCs with the potential for enhancing RepRNA delivery. The UniVax consortium employed three approaches – hyaluronic surface decoration to target the CD44 on DCs and indeed many cell types; glycan probes targeting C-type lectin, SigLec or galectin receptors on DCs; RGD-containing peptides known for targeting lectins and related structures on DCs; functionalisation of selected glycans for conjugation to hyaluronic acid, PEI or other structures of the delivery vehicles employed within UniVax.

(i) Hyaluronic acid decoration

Chitosan nanoparticulate delivery vehicles were modified to carry surface hyaluronic acid. Hyaluronic acid (HyA) functionalisation was developed, based on the introduction of boronic acids on the polymer structure for its decoration. The boronic esters formed are hydrolysed at acidic pH, facilitating detachment of the cargo (such as RepRNA) in the endosomal environment developing following endocytosis of the delivery vehicle formulation carrying the RepRNA. Work on this new class of synthetic and pH-reversible proteoglycans has been published:

> Montanari et al 2018 Tyrosinase-Mediated Bioconjugation. A Versatile Approach to Chimeric Macromolecules Bioconjugate Chem. 29, 8, 2550-2560.

There was no toxicity from these formulations, and delivery to cells proved efficient. Translation of delivered RepRNA was observed *in vitro*, and could lead to induced immune responses *in vivo*. More details are shown under the sections for *in vitro* and *in vivo* evaluation below.

(ii) Glycans

Over 300 glycoconjugates were assessed for their relative capacity at binding to human and porcine DCs compared with other mononuclear cells. This permitted definition of glycan binding capacity for the different human and porcine mononuclear cells, with particular emphasis on the DCs and DC subsets. The analyses also permitted identification of species-common and species-specific glycan-binding receptors.

In addition, the binding of probes interactive with DCs was assessed on cell lines, in particular the DC2.4 cell line of murine origin, which is supposed to resemble DCs, at least in their ability to present antigen to T-lymphocytes. However, the glycan binding profile for DC2.4 cells was distinctive from that for primary murine DCs and other mononuclear cells, as well as for human or porcine DCs (see under the sections for *in vitro* evaluation below). This work demonstrated that DC2.4 cells could not be used for assessment of nanoparticulate vehicle delivery to DCs, whereby the risk a false impression on binding or a lack thereof would be forthcoming. In contrast, activated THP-1 cells – of human origin – showed a closer relationship to human mononuclear cells; more with monocyte-derived cells, rather than blood DCs.

A selection of probes defined on their interaction with DC subsets and monocytes were further analysed by microscopy to define binding and internalisation, in particular the endocytic route employed by the cell. The aim was to determine which targeting glycans promoted endocytosis by the DCs, and which endocytic route was identifiable. Such endocytic routes are important due to the manner by which the cell will process material, in turn impacting on the release of the RepRNA for translation, or its degradation.

For more details see also

1 Rapoport et al 2018. Glycan recognition by human blood mononuclear cells with an emphasis on dendritic cells. Glycoconjugate Journal, doi.org/10.1007/s10719-017-9811
2 Rapoport et al 2019. Glycan-binding profile of DC-like cells. Submitted for publication

(iii) Mannosylated targeting

During the period 1, CNRS (P7) has first used their available mannosylated lipid to target lipopolyplexes (LPR). Those mannosylated LPR were efficiently targeted to splenic DC and induce conventional mRNA-based vaccination. Each type of formulation has been optimized according to their features to condense replicons RNA, size and zeta potential, decondensation ability and RNase protection as well as their gene expression efficiency *in vitro.* Similarly*,* OZB (P2) synthesized and formulated mannosylated lipids. For both delivery systems from CNRS and OZB, a selective targeting and uptake of dendritic cells were observed *in vitro*. Unfortunately*,* results demonstrated no gain in efficiency with mannosylatred vectots in comparison with classic untargeted ones for RepRNA expression albeit enhanced expression was observed for mRNA and pDNA. All vectors showed an interesting pattern of efficiency, with a reliable reproducibility. *In vivo* data were in line with the in cellulo experiments. These set of data indicated that positively charged (>30mV) made with LPR and OZB lipids were not suitable for CSFV RepRNA delivery.

Pichon C, Midoux P (2013). Mannosylated and Histidylated LPR Technology for Vaccination with Tumor Antigen mRNA. ***Methods Mol Biol****.;969:247-74.*

(iv) RGD-containing peptides

The RGD peptides employed are known for their high immunogenicity *in vivo*, which presents a risk of inducing an immune response against the RGD-containing peptides. This precludes their applicability as generic targeting ligands for the RepRNA delivery vehicles. Accordingly, they were used primarily as a means for defining endocytic processes employed by DCs, to facilitate identification of processes into which the glycan probes and delivery vehicles would target the RepRNA cargo. As such, particular processes were subsequently identified with the selected glycan probes. This allowed for a more informative conclusion on the applicability of the glycan probes for RepRNA delivery. Routes were defined of importance to protein antigen delivery; employ late endocytic pathways for processing and presentation, required for antigen processing prior to presentation, but likely to be more degradative for delivered RNA. The RepRNA requires cytosolic translocation to facilitate interaction with the ribosomal machinery for translation; this can be achieved with the early endocytic pathways in which the delivered cargo is retained for a more prolonged period in less acidic endocytic vesicles.

(iii) Evaluations
For more details of these targeting entities (hyaluronic acid, glycans, RGD peptides and hyaluronic acid with glycans), see under the section for *in vitro* evaluation, subsection (iv) Targeting RepRNA delivery, below. Modified delivery vehicles, selected from the *in vitro* evaluations, were employed to assess their influence on RepRNA cargo delivery and translation efficiency – for more details, see below under *in vitro* and *in vivo* evaluations.

Adjuvants

The characteristics of the pestivirus replicon (RepRNA) – non-cytopathogenic, slow translation, a lack of 5'-triphosphates to activate cellular innate defences, and a leader N^{pro} to regulate Type I interferon pathways – facilitate retention by and maintained translation/replication in DCs. This contrasts with other viruses and replicons, notably the commonly used alphavirus replicons with their 5'-triphosphates sensed by cellular helicases. Nonetheless, activation of innate immune mechanisms is required for maturation of DCs, essential for efficient induction of adaptive immune responses, as opposed to tolerance or anergy. Accordingly, the delivery vehicles employed with the RepRNA require a potent adjuvant.

The adjuvants were formulated with delivery vehicles carrying RepRNA, selected in the most part from initial *in vitro* evaluations (see the section on *in vitro* evaluation below), as well as with the RepRNA alone. Several different adjuvants were compared for enhanced induction of humoral and cellular immune responses against the RepRNA-encoded vaccine antigen. Pulmonic and subcutaneous or intramuscular administrations were employed with readouts for humoral immunity, T-lymphocyte profiling, T-lymphocyte activity and cytokine profiling.

The most promising candidates were MALP-2 and cyclic-di-AMP (c-di-AMP), both having distinct modes of action. These offer additional advantages in being synthetical and applicable via both parenteral or mucosal routes. Importantly, the cyclic-di-AMP proved to be the most efficacious, by both parenteral and mucosal routes of immunisation. For more details, see below under the sections on *in vitro* and *in vivo* evaluations, and also

Ebensen et al 2019 The combination vaccine adjuvant system alum/c-di-AMP results in quantitative and qualitative enhanced immune responses post immunization, Front Cell Infect Microbiol. 19;9:31
Ebensen et al 2017 Mucosal Administration of Cycle-Di-Nucleotide-Adjuvanted Virosomes Efficiently Induces Protection against Influenza H5N1 in Mice. Front Immunol. 28;8:1223

In vitro Evaluation

(i) Efficiency of RepRNA delivery by different complexes.
For assessment of delivery efficiency, RepRNA was fluorescently labelled to facilitate its tracking; with VRPs, only translation was employed as an indicator of delivery. As expected, VRPs proved to be particularly efficient at delivering RepRNA leading to translation of the encoded antigens. As for the other delivery systems – lipoplexes, lipopolyplexes (LPRs), PEI polyplexes (with or without cell penetrating peptides), chitosan-based complexes, or the new liposomal HA-nanoparticles – delivery efficiency varied dependent on the components employed for each system. At least one form of each delivery system demonstrated efficient delivery of labelled RepRNA to DCs and to certain cell lines, particularly the porcine kidney cell lines (SK-6) in which replication of the RepRNA is readily assessed.

When DNA, conventional mRNA or siRNA molecules also employed, the pattern obtained for delivery of labelled RNA did not relate to that seen with labelled RepRNA. While many of the delivery formulations could deliver DNA or mRNA and siRNA molecules, this was not necessarily indicative of efficient RepRNA delivery. With some delivery formulations, RepRNA could be detected earlier than those RNA molecules, whereas with other formulations it was the converse. Consortium partners considered that the larger size of the RepRNA, and therefore its more

complex interaction with delivery vehicle components, likely had an impact on these observations. Moreover, delivery to cell lines did not guarantee delivery to DCs with the same efficiency. Nonetheless, the use of appropriate cell lines (such as SK-6 or activated THP-1) could prove of value for determining the potential for delivery to DCs.

(ii) Efficiency of RepRNA translation following delivery by different complexes.
From the above work, it became clear that certain formulations, or modifications to the components thereof, were more efficient than others at delivering the RepRNA. However, many of the formulations and components under test had been developed for delivery of conventional mRNA molecules or DNA. As witnessed from the above, this could not guarantee success with the much larger RepRNA and its more complex interactions with the delivery vehicle components.

Accordingly, it was critical to ascertain if the observed efficiency of delivery would in turn provide efficiency of translation for the RepRNA. This was particularly important when the delivery components had proven effective with siRNA or DNA, because the ultimate site for delivery of these nucleic acids has no relationship to the site for delivery of the RepRNA, namely the ribosomal machinery for translation. In contrast, delivery components successful with mRNA could identify candidates for delivery of RepRNA, due to their similar requirements for the ribosomal machinery. Nonetheless, one had to consider that major differences between mRNA delivery and RepRNA delivery could come from the greater size of the RepRNA and its more complex interaction with the delivery vehicle components or intracellular proteins. In other words, compaction of the RepRNA by the delivery vehicle might prove more problematic than with mRNA. How mRNA and RepRNA can be sense or attracted by immune sensors of other regulatory proteins is not known yet. Certainly, identification of efficient delivery would not distinguish this problem, although microscopic compartmentalisation of the delivered RNA within the cell could indicate the likelihood of compaction or decompaction, dependent on the image and site of intracellular localisation obtained. Ultimately, assessment of translation would bring to light any problems with release of efficiently delivered RNA.

Those delivery formulations and components found to provide efficient delivery of RepRNA to cells, particularly when the assessment had employed DCs, were further investigated for efficiency of delivery leading to translation of the encoded genes. This could not be assessed in combination with the delivery, because it was considered that the labelling of the RepRNA may have modified the RNA to impede ribosomal entry and/or translation of the RNA.

Irrespective of the delivery system employed, delivery leading to translation was seen to be dependent on both the formulation and the cells employed for assessment. Indeed, certain cell lines proved much less efficient for assessment of the translation. Moreover, the results demonstrated the importance for screening with primary DCs. Neither the efficiency of associating the RepRNA with the delivery vehicle, nor the efficiency of the RepRNA delivery into the cells could be related to this translation. Clear delivery of the RepRNA was essential, but only certain formulations facilitated the apparent release of the RepRNA for translation of the encoded antigens. By modifying the delivery vehicle component, one could modify this efficiency of delivery leading to translation, even when the delivery efficiency was apparently unaltered. Moreover, efficient delivery of mRNA molecules had no relevance to identifying formulations for efficient delivery of RepRNA that would ensure translation.

In the last period of Univax; Partner 7 decided to develop novel neutral lipopolyplexes (LPP) based on data obtained from cationic LPR. Those neutral LPP demonstrated a versatility for mRNA and RepRNA delivery both *in cellulo* and *in vivo*. They are able to both compact and protect RepRNA towards nuclease degradation and demonstrate a very good stability in presence of serum. LPP were amongst the 4 series of prototypes selected for the challenge experiments. Understanding the intramolecular interactions of those LPP with both mRNA and RepRNA and

the investigation of intracellular routing of those formulations compared to others that failed would be crucial for the future.

See also

1. Démoulins et al 2016 Polyethylenimine-based polyplex delivery of self-replicating RNA vaccines Nanomedicine 12:711-722;
2. Démoulins et al 2017 Self-Replicating RNA Delivery to Dendritic Cells", in "RNA Vaccines: Methods and Protocols", ed. T. Kramp & K Elbers, Chapter 5, Methods in Molecular Biology vol 1499, Springer Science+Business Media, New York;
3. Démoulins et al 2017 Self-replicating RNA vaccine functionality modulated by fine-tuning of polyplex delivery vehicle structure. J Control Release 28;266:256-271;
4. Englezou et al 2018, Self-amplifying Replicon RNA delivery to Dendritic Cells by Cationic Lipids, Molecular Therapy Nucleic Acids 12:118-134.
5. Perche et al., 2019. Versatility of neutral lipopolyplexes for in vivo delivery of conventional and replicative RNA. Molecular Therapy and Nucleic acids (*under revision*)

(iii) Influence of delivery vehicle complexing with the RepRNA on translation efficiency.

A. RepRNA functionality and cell sensing

It was considered that the RepRNA may have been non-functional in certain complexes or compacted to a degree that would not be reversed adequately for ribosomal entry and translation of RepRNA-encoded antigens. Thus, the functionality of the RepRNA was assessed using virus replicon particles (VRP). These are constructed in complementing cell lines to create the original virus-like particles, but carrying the replicon in place of the virus genome. VRPs were efficiently delivered to cells – DCs and cell lines – promoting efficient translation of the encoded antigens, including the influenza virus antigens. These results clearly demonstrated that the RepRNA was indeed translation- and replication-competent.

Another consideration was the capacity of the cell to respond against the presence of this foreign RNA. It is well established that both pestiviruses and their replicons do not induce cellular innate responses (see above under the Section "Choice of the Pestivirus Replicon"). When another replicon to that employed within UniVax – an alphavirus replicon originating from a third party outside the consortium – was assessed, there was the expected cellular innate response, which may have influenced the alphavirus translation. However, this is irrelevant to the pestivirus replicon (RepRNA) employed within UniVax. Of critical importance to the UniVax aims and objectives, the pestivirus replicon of UniVax does not possess the 5'-triphosphate cap of alphaviruses – strong inducers of cellular innate defences – and also encodes a leader autoprotease N^{pro} that regulates cellular innate defence pathways. Moreover, attempted regulation of cellular innate activity, as induced by alphavirus replicons, would pose problems for vaccine application. Such cellular activity is required for the vaccine adjuvant to promote DC maturation and thus efficient induction of adaptive immune responses. Any interference with this would create problems for vaccine efficacy.

B. Delivery vehicle interaction with RepRNA

When the RepRNA of UniVax was compared with the above alphavirus replicon using particular delivery vehicles, the results for translation were similar for a particular delivery vehicle. Accordingly, it appeared that related events were occurring that impacted on both the RepRNA of UniVax and other replicons in terms of their efficiency for translation following delivery. It was therefore considered that the discrepancy between delivery and translation of the RepRNA with the different delivery systems may have related to the characteristics of interaction between the RNA and the delivery vehicle components.

Analyses with different PEI-based formulations added some clarity to the situation. While certain formulations were efficient at promoting translation of all encoded antigens, other formulations

provided for an imbalanced translation. The RepRNA "gene of interest" (GoI) – encoding influenza virus antigens or reporter genes such as luciferase – is under control of the ribosomal entry site within the 5'-NTR of the RepRNA. Endogenous RepRNA genes, such as that encoding the structural E2 protein, are situated after the GoI and therefore under the control of the second (inserted) internal ribosomal entry site. Certain formulations were efficient for delivery leading to translation, regardless of which gene was under scrutiny. In contrast, particular formulations promoted delivery for translation favouring either the GoI or the E2, despite showing similarly delivery efficiencies. This would impact on the efficiency of the formulation as a vaccine. Translation of genes downstream of the GoI with poor translation of the GoI would be inefficient at inducing the desired immune response against the GoI encoded antigens. If the translation favoured the GoI over the downstream genes, then the replicative complex for the RepRNA would not be generated, and thus replication would fail. Nonetheless, the problems concerned more a poor GoI translation with efficient downstream translation. This would imply a differential degree of compaction along the RepRNA, either preventing ribosomal entry or inefficiently protecting from RNase. Major influential parameters were
the delivery component molecular weight,
the ratio of the delivery component to the RepRNA,
and the presence of membrane perturbing entities (in the case of the PEI polyplexes, these were inclusion of cell penetrating peptides).

The above results demonstrated that different delivery formulations could differ in their interaction with the RepRNA such that only certain genes would be translated. That is, a major issue to be assessed with delivery formulations for RepRNA is the degree of compaction of the RepRNA. Even with efficient delivery, and apparent translocation to the cytosol for translation, it is necessary to define if all genes are being translated, or if there is interference with a portion of the replicon. Thus, delivery of these large self-replicating RNA molecules require definition with respect to translation of different genes, rather than just the GOI, for identifying optimal delivery for the desired immune activation *in vivo*.

See also

> Démoulins et al 2017 Self-replicating RNA vaccine functionality modulated by fine-tuning of polyplex delivery vehicle structure. J Control Release 28;266:256-271

Consequently, careful selection of the delivery vehicle components together with the RepRNA of UniVax has identified formulations capable of delivering to both DCs and cell lines, promoting translation of the delivered RepRNA. This was particularly notable with certain PEI-based polyplexes, newly developed LPPs (neutral lipopolyplexes), and modified chitosan-based delivery vehicles. In addition, the new liposomal HA-nanoparticle (virosome-like) delivery vehicle also showed *in vitro* delivery promoting translation. Nonetheless, overall the most efficient means of RepRNA delivery leading to translation of the RNA remained the VRP. These formulations were evaluation by mucosal and parenteral vaccination *in vivo* (see below under "*in vivo* evaluation").

(iv) Targeting RepRNA delivery.

A. Selection of targeting ligands.

Over 300 glycoconjugates were assessed for their relative capacity at binding to DCs compared with other mononuclear cells obtained from porcine and human donors. This collaborative effort involving Partner 3 (SYN) with Partner 1 (IVI) allowed clustering of the probes into different groups dependent on their relative binding to different cell populations. This facilitated a definition of glycan binding capacity for different mononuclear cells, with particular emphasis on the DCs and DC subsets.

As described above, it became evident that assessment of glycan binding on cell lines, in particular the DC2.4 cell line, was unusable, often providing a false impression on lack of binding.

Accordingly, the selection of glycan probes for further analysis was on the basis of binding to human and porcine DCs, in particular efficient interaction with different human blood cell populations as well as differentiating the degree of binding to DC subsets and monocytes.

B. Assessment of endocytosis

The selected glycan probes were further analysed by microscopy to define binding capacity and internalisation efficiency, as well as the endocytic route employed by the cell. The aim was to ascertain that the glycans were indeed capable of promoting endocytosis by the DCs, and not just binding to the cell surface – some cell surface receptors can bind ligands, but rapidly eject them either before or shortly after endocytosis.

The RGD-containing peptides allowed refined definition of the DC endocytic pathways, combining with markers for different compartments and structures within these. This high level of definition was also applied to the glycan probes, which had been extensively pre-screened for their binding profiles for DCs. Only the glycans capable of promoting enduring endocytic processes would prove applicable for targeting RepRNA delivery. Such endocytosis is important due to the manner by which the cell will process the material, in turn impacting on the release of the RepRNA for translation.

Certain of the glycan probes were found to promote efficient forms of endocytosis, but not all probes tested gave clear indication of efficient endocytosis. When endocytosis was clearly detectable with a particular glycan, the process into which the probe would target its delivery was found to be dominated by slow endosomal involvement typical of macropinocytosis or caveolar endocytosis. Both processes are important for RepRNA delivery – being slowly maturing (acidifying) processes, they would create an environment more readily accommodating cytosolic translocation. Of course, for the latter this would further depend on the characteristics of the delivery vehicle and to what degree compaction of the RepRNA would impact on cytosolic release the RNA.

C. Glycan probes for evaluation

On the basis of the above analyses, selected probes were modified to interact with certain of the delivery vehicles. Partner 3 (SYN) synthesised probes in their biotinylated form and then coupled with the respective biotin-functionalized carriers through streptavidin-mediated conjugation. This allowed for conjugation with low molecular weight chitosan/hyaluronic acid nanoparticles – with a strategy for functionalisation of the surface hyaluronic acid on chitosan nanoparticles assessed by Partner 12 (IIT) – and PEI-based polyplexes – with functionalised PEI assessed by Partner 1 (IVI). The selected probes were based on GalNAcα1-3Galβ and Neu5Acα2-8Neu5Acα2-8Neu5Ac, either in monomeric or polymeric form. Negative controls were included as well. The different glycan probes were:

A_{di}-biot (GalNAcα1-3Galβ), **"**Adi Mono**"**

$(Sia)_3$-biot (Neu5Acα2-8Neu5Acα2-8Neu5Ac) "Sia Mono"

αLacNAc-biot (Gala1-4GlcNAcb) "Neg Mono"

Biot1-pHAA-Sug(20) cat #87 Sug = GalNAcα1-3Galβ (A_{di}) "Adi Poly"

Biot1-pHEAA-Sug(20) cat #73 Sug = Neu5Acα2-8Neu5Acα2-8Neu5Ac (Sia_3) "Sia Poly"

Biot1-pHAA, no Sug, as negative control (no Sug ligand) "Neg Poly".

How the conjugation with streptavidin affected the carriers properties was studied. An increase both in size and dispersity was observed, but the characteristics of low molecular weight carriers were only slightly affected. The screening allowed for the provision of the conjugated delivery vehicles for assessment *in vitro* and *in vivo*, namely their influence on RepRNA cargo delivery and translation efficiency.

For lipopolyplexes formulations, it seems that mannosylated targeting is the best one amongst all targeting motives tested. Therefore, the last neutral formulation LPP has been produced with mannosylated lipids and gave the best results obtained so far with an effective Luciferase RepRNA expression both in vitro and in vivo (intra-muscular injection).

In vivo Evaluation

(i) In vivo evaluation models

Following the *in vitro* identification of the most potentially efficacious delivery formulations for promoting translation of the UniVax RepRNA, prototypes were assessed by immunogenicity studies in mice and pigs. This extensive work employed a number of *in vivo* evaluations by conventional immunogenicity studies on RepRNA encoding influenza virus antigens in mice and pigs. In addition, pre-immune mice were also employed. In order to enhance the capacity of the *in vivo* assessment, the TCR Ova model (OTI and II) together with RepRNA encoding the Ova antigen was employed.

With both the conventional vaccination models in mice and pigs, and the TCR Ova model, in-depth analysis assessed most aspects of immune responsiveness. This included antibody induction, T-lymphocyte subset induction, cytokine profiling and assessment of individual lymphocyte activities (see below under the different subheadings).

(ii) RepRNA requires particular delivery vehicle formulations

The initial lipoplex, polyplex and lipopolyplex formulations did not promote the clear specific immune response anticipated from the *in vitro* evaluations and the published work that initiated UniVax (see McCullough et al, 2014, Mol Ther Nucleic Acids. 3:e173). However, modifications of the polyplex (PEI), lipopolyplex (LPR) and chitosan/hyaluronic acid formulations showed increased efficiency *in vitro* for delivery to DCs and cell lines leading to translation of the encoded gene of interest. To this group were added the new liposomal HA nanoparticles (virosome-like), again showing high promise from the *in vitro* evaluations in DCs and cell lines. New *in vivo* assessments employed both murine and porcine models, with the murine experiments comparing a mucosal and parenteral route of injection.

These experiments confirmed the capacity of the new modified formulations to induce immune responses against the influenza virus antigens (or Ova) encoded by the RepRNA. Overall, combination of the observations from all *in vivo* evaluations showed that not all formulations of any particular type of delivery vehicle (lipoplex, polyplex, lipopolyplex, chitosan-based particles and lipid/HA-nanoparticles) would prove effective. While this related to the situation observed *in vitro* whereby only certain formulations of each type of vehicle would deliver RepRNA leading to translation, the selectivity and restraint of delivery vehicle efficiency was more acute *in vivo*.

Moreover, when forms of each type of delivery vehicle that were effective *in vitro* were employed *in vivo*, certain formulations were more efficient at delivering RepRNA inducing one compartment of immune responses – humoral or cell-mediated. Nonetheless, particular formulations were identified that delivered RepRNA leading to induction of both humoral and cell-mediated immune responses.

(iii) Importance of the adjuvant in RepRNA/delivery-vehicle formulations

Importantly, the power of the cyclic-di-AMP has been clearly demonstrated, and that it is more potent with the RepRNA than any of the other synthetic adjuvants investigated. Cyclic-di-AMP adjuvant plays an essential role with the RepRNA for ensuring efficient induction of immune defences. In particular, cyclic-di-AMP proved to be a powerful mucosal as well as parenteral adjuvant. This was observed with all delivery vehicle types assessed, including the VRPs. Of particular interest was the observation that cyclic-di-AMP with RepRNA alone (no other delivery

vehicle) could prove effective at inducing immune responses against the RepRNA encoded Influenza virus antigen.

(iv) Evaluation of the final selected RepRNA/delivery-vehicle formulations

Using the data obtained from all previous *in vitro* and *in vivo* evaluations, final experiments were performed in mice and ferrets using the RepRNA/delivery-vehicle formulations showing the greatest efficacy following both mucosal and parenteral administration. These were one selected lipopolyplex, one selected chitosan/hyaluronic acid nanoparticle and one selected liposomal HA-nanoparticle. That latter was chosen without the surface HA, due to *in vitro* evaluations showing that this did not apparently influence the efficiency of delivery leading to translation of the delivered RepRNA.

The vaccines were composed of nanoparticulate delivery vehicles carrying RepRNA encoding for influenza virus HA (H1), NA (N1), NP and M1. This was an advance on previous experiments in which the delivered RepRNA encoded for either HA or NP. It is important to note that the RepRNA molecules encoded for a single Influenza virus antigen. This was designed to avoid the problems associated with multiple ribosomal entry site insertions, which are required for multiple foreign genes, in turn causing dramatic reduction in translation efficiency of the foreign genes.

Following vaccination (prime or boosts), there were no observed indicators for toxicity or signs of other adverse reactions with any of the formulations under test. This related to the previous observations with RepRNA encoding for HA or NP. Toxicity assessment of these forumulations, including histopathology, confirmed this absence of toxic signs. It was concluded that for all formulations assessed, the highest dose is well tolerated and safe.

Specific immune responses were detected in the animals vaccinated with the different delivery formulations. Interestingly, antigen-specific lymphocyte proliferation and cytokine responses were also observed with the RepRNA plus cyclic-di-AMP adjuvant, without delivery vehicle, implying that the adjuvant could also offer delivery potential. This had been observed in previous experiments using mice, but was less clear with the immunization of pigs (although the RepRNA/cyclic-di-AMP vaccination was not without immunogenic effect); in this context, it should be stressed that the immune response induction was dependent on the amount of RepRNA delivered to the mice, whereas only a single dose was tested in the pigs.

For the successfully efficient delivery formulations for RepRNA, each formulation showed a more pronounced efficacy for particular immune response components. Certain formulations favoured more an IL-2 and/or IL-12 response, while other formulations favoured IFN-γ-producing CD8+ T lymphocytes. While the lipopolyplex and chitosan/hyaluronic acid nanoparticles favoured antigen-specific T-lymphocyte responses, the HA-nanoparticle delivery was particularly efficient – for both $CD4^+$ and $CD8^+$ lymphocytes. Importantly, all delivery systems required the presence of the cyclic-di-AMP adjuvant. Intriguingly, when the combination of RepRNAs (encoding HA, NA, NP and M1) were employed with just the cyclic-di-AMP, an efficient induction of both antigen-specific $CD4^+$ and $CD8^+$ lymphocytes was observed.

The quality of immune effector functions has been related to the induction of multifunctional T cells, as a correlate of protection against influenza virus (Darrah et al. 2007). The above delivery formulations – lipopolyplex, chitosan-based, HA-nanoparticle and the cyclic-di-AMP adjuvant without delivery vehicles – all delivered RepRNA leading to antigen-specific induction of at least one cytokine – IL-2, IL-4, IL-6, IL-17, IFN-γ or TNF-α. Dominance of a particular cytokine was dependent on which delivery system was employed. Nonetheless, antigen-specific induction of IFN-γ and IL-17 dominated the profile regardless of the delivery. HA-nanoparticle delivery of RepRNA proved to be particularly efficient in this respect, while the lipopolyplex delivery of RepRNA was particularly effective for $CD8^+$ lymphocyte IL-17 and TNF. The multifunctional T-

lymphocytes were also induced by RepRNA delivery with the different formulations. This was most notable for the HA-nanoparticle, lipopolyplex, or cyclic-di-AMP without any other vehicle.

Current status on assessment of RepRNA/delivery-vehicle formulations

These results clearly demonstrated that a delivery formulation could not be chosen on the basis of success with DNA or small RNA molecules. Such characteristics could not guarantee success for delivery of RepRNA leading to translation and induction of immune responses *in vivo*. The effort of the UniVax consortium demonstrated that the delivery vehicle must be created specifically for RepRNA, taking into consideration the size and complexity of this molecule, the very particular requirements for its delivery, and the peculiarities associated with RepRNA release and translation for inducing immune responses.

A number of *in vivo* immunisations, using either mucosal delivery or parenteral delivery, identified formulations with clear efficacy for delivery, resulting in clear induction of specific immune responses. For all, the inclusion of the cyclic-di-AMP adjuvant was essential. The efficacy was especially notable after intra-pulmonary injection. One particular HA-nanoparticle formulation with RepRNA proved effective for leading to induction of both humoral and cell-mediated responses, as did a particular lipopolyplex, a particular PEI polyplex and a particular chitosan/hyaluronic acid nanoparticle. These formulations were effective by both mucosal and parenteral routes of administration. In contrast, a cationic lipid lipoplex formulation chosen for mucosal delivery was different from that chosen for parenteral administration. The particular HA-nanoparticle formulation, particular lipopolyplex formulation, and particular chitosan/hyaluronic acid formulation delivery of RepRNA also provided evidence for levels of protection against H1N1 virus challenge in mice and ferrets.

These *in vivo* experiments provided clear evidence of immunogenicity for nanoparticulate delivery of RepRNA. An important discovery was the particularly powerful immunomodulatory capacity of cyclic-di-AMP, both parenterally and mucosally. Moreover, these results have allowed the selection of the delivery formulations showing the greatest promise for efficacy at inducing influenza virus-specific immune responses. The most efficient delivery system remains that based on VRPs, with which the added value of including the cyclic-di-AMP adjuvant was observed.

For more details see

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Immunological Evaluation of Biobank material

(i) Biobank evaluation in relation to Clinical Trial

A clinical trial at Haukeland University Hospital in Bergen, Norway was used to evaluate local and systemic immune responses after LAIV in children and adults. Clinical trial samples have been stored in a biobank of blood and saliva samples collected at different time points.

Overall, the biobank samples provide material from influenza H1, H5 and H7 infections or vaccinations, including seasonal vaccine studies. These have been employed to dissect the immune responses, in terms of human B and T cell epitopes, particularly potential universal epitopes. The HA stalk is highly conserved allowing the influenza A viruses to be divided into two groups. HA head and stalk specific antibody binding has been assessed, together with the avidity of binding and the functionality of stalk-specific antibodies using virus neutralization and antibody-dependent cellular cytotoxicity (ADCC). HA stalk-specific antibodies may have an important role in protection through neutralization and ADCC in people who respond poorly to traditional inactivated vaccines.

The data obtained to date shows that particularly young children respond well to LAIV with serological responses to H3N2 and B strains, along with local and systemic antibody secreting and memory B cell responses. The H1N1 strain did not elicit antibody responses, although T cell responses were detected in blood and tonsils. LAIV induces systemic and local T cellular responses including protection associated cross reactive T cells which may provide heterologous protection in children. This information is especially valuable to the RepRNA vaccine of UniVax, which possesses at least similar characteristics of immunogenicity to an LAIV. In particular, this concerns the ability of RepRNA to self-replicate and more closely mimic the situation with an Influenza virus infection, and therefore the type of immune responses induced.

(ii) Biobank evaluation with respect to immune responses of the elderly

Immune responses following influenza vaccination in elderly were assessed employing biobank material from 234 volunteers aged ≥ 65 years. Vaccine recipients were stratified in responder and non-responder by their increase of the hemagglutination inhibition (HAI) titres. PBMCs drived from responders and non-responders were used for in-depth multi-parametric flow cytometry analysis. While responders showed enhanced functionality upon vaccination, non-responders displayed a more suppressive immune phenotype. The acquired data is still being processed, together with correlation studies to gain a broad picture of vaccine-induced processes relative to age and responder status.

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Expected final results and potential impact and use

Influenza is a serious public health problem affecting more than 100,000,000 people per year. Most recover from the symptoms within a week without requiring attention, but there is a high risk for severe illness (3-5 million cases) or death (250,000 to 500,000) (WHO); most deaths occur among the elderly. The most effective way to prevent the disease is vaccination, although the vaccine among the elderly reduces severe illness and complication by only up to 60%. Annual vaccination is currently recommended for pregnant women, children 6 to 59 months of age, healthcare workers with patient contact, the elderly and people with underlying chronic health conditions such as respiratory, cardiac, metabolic, neurological and immunosuppressive diseases.

The outcome of the UniVax project will be the first synthetic replicating RNA vaccine against influenza. UniVax has generated essential data on integrating innovative technologies for RepRNA, synthetic delivery to dendritic cells, glycoconjugate-based targeting of dendritic cells, and mucosal adjuvants. The first ever prototype synthetic RepRNA vaccines with innovative new generation mucosal adjuvants have been evaluated pre-clinically, providing data on enhancing efficacy of vaccine delivery for inducing both humoral and cell-mediated immune defences.

UniVax has a direct outcome in terms of a novel clinical technology, with considerable benefits for health of the human population. The project also provides tools in basic research (RNA delivery systems to DCs, new adjuvants, replicative RNA technologies). It can now enable the widespread use of nucleic acid delivery to dendritic cells and other “immune cells” such as monocytes, macrophages, natural killer cells or even lymphocytes with the purpose of investigating cellular functions and overexpressing or silencing genes in therapeutic strategies.

Most importantly, UniVax provides a generic platform for creating innovative, biodegradable, biosafe vaccines. As biosafe, replicative entities, they have potential to replace less efficacious inactivated (non-replicating) vaccines. When vaccinations have to be repeated on a regular or even annual basis, the advantages of a self-replicating vaccine offer a much-reduced vaccination programme, or even single shot vaccination. This is both more efficient and more agreeable to individuals being vaccinated. Importantly, the nature of the RepRNA with the UniVax innovative vaccine formulation provides particularly high generic potential. The plasmids carrying the RepRNA constructs can be easily modified to encode any antigen, therapeutic agent or gene of

choice. The insertion sites within the RepRNA sequence allow for replacement of the so-called “gene of interest” in a rapid and efficient manner. This allows for rapid modifications of the vaccine in case of antigenic change, as occurs during pandemic Influenza, or replacement with a new sequence for whatever vaccine or therapeutic use is sought. The only question now remaining is that surrounding the compaction of the rather large RepRNA molecule. UniVax partners are continuing research along these lines to enhance cytosolic release of the delivered RepRNA in a decompacted manner that will enhance the translation of tboth the gen of interest and the downstream genes facilitating replication of the RepRNA and therefore enhanced immune defence induction for robust and long-lasting immunity form a small number or even single shot administration.

Contact and further information: https://univax-fp7.eu