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Towards RNA Nanoparticle Vaccines: Synergizing RNA and Inorganic Nanoparticles to Achieve Immuno-potentiation

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Abstract

Traditionally, vaccines have been composed of live attenuated or killed micro-organisms. Alternatively, individual protein subunits or other molecular components of the micro-organism can serve as the antigen and trigger an antibody response by the immune system. The immune system is a coordinated molecular and cellular response that works in concert to check the spread of infection. In the past decade, there has been much progress on DNA vaccines. DNA vaccination includes using the coding segments of a viral or bacterial genome to generate an immune response. However, the potential advantage of combining an RNA molecule with inorganic nanoparticle delivery should be considered, with the goal to achieve immuno-synergy between the two and to overcome some of the current limitations of DNA vaccines and traditional vaccines.

INTRODUCTION

Genetic medicines hold tremendous potential and there is a large literature already devoted to the topic of DNA vaccines. However considerably less work has been done on RNA-based vaccines despite several potential advantages, such as avoiding insertion, replication and transcription steps. Today there are literally thousands of papers in the literature also involving RNA nanoparticles. However few, if any, have considered whether the immunological profile of inorganic nanoparticles could synergize with the RNA to potentiate immune response. Here we summarize progress to date on RNA vaccines and address the molecular correlates and immunological responses desirable for potential RNA vaccine nanoparticles and their continued progression.

RNA VACCINES

While DNA vaccines have long been heralded, more recently an interest in RNA vaccines has emerged. RNA vaccines are being evaluated against a variety of diseases as summarized in Table 1.

As shown in Table 1, diseases for which RNA vaccines have been tested include; viruses such as Avian influenza virus, Cytomegalovirus (CMV), Flavivirus, Hepatitis C, HIV,

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influenza, and some cancers (e.g., melanoma) and measles. Delivery of the RNA vaccines has been accomplished via physical means such as electroporation or particle based methods such as gene gun and lipid nanoparticles. Routes of RNA vaccine administration include; subcutaneous, intradermal and intramuscular. Except for one case where RNAi was used, the overwhelming majority of RNA vaccines are constructed from either mRNA or self-amplifying RNA (saRNA), and these are discussed in the next section.

RNA VACCINE CONSTRUCTION

For RNA vaccines the delivery of a translatable RNA sequence into the cytoplasm circumvents many of the complications of DNA delivery, in that nuclear localization is not required and there are no integration, replication or transcription steps necessary. The structural elements an RNA vector must contain in order to be directly translated to protein once present in the cytosol is illustrated below in Figure 1.

Figure 1 illustrates basic structural features of RNA vaccine expression vectors. For example mRNA constructs contain a 5'-G cap, a 5'-untranslated region (UTR), the coding sequence for the antigenic protein, a 3'-UTR, and a 3'-poly(A) tail. The vaccine antigen to be expressed is encoded in the coding region. Within the 5'-UTR are sequences that promote the assembly of and stabilization of the ribosome on the mRNA. The 3'-UTR is generally thought to mediate mRNA decay rate. Self-amplifying RNA (saRNA) is a replicon-based vaccine that is longer than other RNA-based vaccines with typical molecular weights of approximately 10 kb.¹¹ The most studied saRNA vaccines have been derived from alphavirus genomes. Alphaviruses consist of a single copy of positive, single-stranded RNA that contains genomic RNA encoding its own replication machinery and sub genomic RNA that encodes viral proteins, such as capsid and glycoproteins.¹² When creating the vaccine, the sub genomic RNA encoding the viral structural proteins is replaced with RNA that encodes the antigen(s) of interest. First, the replicase complex is translated in the cytoplasm. The replicase then synthesizes a genomic anti-sense RNA that is used as a template for the genomic positive-strand RNA synthesis as well as for the sub genomic RNA encoding the structural antigen.¹³ No viral particles are produced because their genetic sequences have been replaced by those encoding the desired antigens and there is no cell-to-cell transmission. saRNA vaccines have the potential to be more potent than mRNA vaccines because of their ability to replicate and induce a stronger, broader, and more effective humoral and cellular immune response.¹⁴ Their delivery has advanced from being packaged into virus-like particles into synthetic formulations (i.e., lipid nanoparticles^{15,16}), which doesn't require a cell culture step. This provides simplicity and safety benefits that using a virus would impose.

DOUBLE-STRANDED RNA (dsRNA)

Double-stranded RNA (dsRNA) is an alternative approach to an encoding RNA strand, but which has also been shown to elicit an immune response. Because dsRNA is often associated with and produced by viruses, it is therefore recognized by the host's immune cells as an indicator of viral infection. In viral infections, host cells recognize viral components, such as dsRNA, via Toll-like receptors (TLRs), a family of innate immune

receptors that recognize pathogen-associated molecular patterns (PAMPs).^{17,18,19} One type of dsRNA, for which there has been a lot of recent interest, is polyinosinic:polycytidylic acid, poly I:C. Poly I:C (pIC) is a synthetic analog of viral dsRNA with one strand composed entirely of cytidine and the other strand of inosine. It has long been suggested as an adjuvant to potentiate vaccine immunogenic activity, but is more recently being considered for combination chemotherapy²⁰ or even as a stand-alone treatment.²¹ Diseases being tested for potential pIC treatment are summarized in Table 2.

As shown in Table 2, a variety of cancer and infectious diseases are being treated with pIC. In some cases, the pIC is used more as an adjuvant in conjunction with vaccine or in combination with cytokine (IL-2). However, in many cases the pIC or the related compound poly ICLC (polyinosinic-polycytidylic acid-polylysine-carboxymethylcellulose) is used as the active immunotherapeutic agent itself. Similar to RNA vaccines, in some cases, naked pIC is delivered locally such as subcutaneous or intramuscular injection or administered via intranasal, intraperitoneal or intravascular injection. Many of these have progressed beyond mouse studies into humans.

IMMUNOLOGICAL CORRELATES

One of the key factors for immunological activity of any potential RNA nanoparticle is the activation of innate and adaptive systems. A robust molecular response needs to be triggered for the cells of the immune system to communicate with each other and perform their critical functions. Table 3 presents an overview of these signaling systems.

As shown in Table 3, the immune system has a number of specialized cells including; neutrophils, eosinophils, basophils, mast cells, monocytes, dendritic cells, and macrophages. Producer cells, such as macrophages and dendritic cells, signal the receiver cells through expression of various cytokines. Major cytokines include, but are not limited to: Tumor necrosis factors (TNFs) such as TNF- α and TNF- β , and a variety of interleukins, such as IL-1, IL-6, IL-10, IL-12 and IL-15 and IL-18. Each elicits a different response on a variety of specific receiver cells or even on organs, such as the hypothalamus. Responses are overt and robust, and range from acute inflammation, the recruitment of a variety of cells to the site of infection, the release of killing agents, the production of chemokines, chemotaxis, and chemoattractant agents, as well as the signaling of a variety of other molecules involved. Receiver cells can be T cells, B lymphocytes, natural killer (NK) cells, neutrophils and other cells. The response of the receiver cell can be production of various classes of antibodies, MHC class I and II, cell differentiation and growth signals. Innate immune cells provide a quick, general response that typically leads to inflammation. These express genetically encoded receptors, called Toll-like receptors (TLRs), which recognize general danger- or microbe-associated patterns. These non-specific receptors cannot distinguish between strains of bacteria or viruses.^{17,18,19} Despite their lack of specificity, innate immune cells are essential in that they also activate adaptive immunity. Upon recognition of a microbe, dendritic cells and macrophages consume the invader and become antigen-presenting cells that activate lymphocytes.³⁷ Essentially, all cells involved in innate and adaptive immunity produce cytokines, but especially by T helper (T_H) lymphocytes, which are a part of adaptive immunity.^{38,39} IL-2 is considered an early responder to adaptive immunity and

activation of type II interferon is also considered indicative of adaptive immunity. In addition to antibody production other signaling molecules which get up-regulated in an adaptive immune response include VCAM, ICAM, CD and MHC molecules.

NANOPARTICLE IMMUNOLOGY

While a great deal of work has already been described for the immunology of organic nanoparticles, carbon nanotubes, liposomes, etc, there is a growing body of literature which suggests that inorganic nanoparticles are also immunologically active as highlighted in Figure 2.

Figure 2 reflects the significant immuno-activity of nanoparticles. Here literature published the last 5–6 years for inorganic nanoparticles of general interest in biomedical nanotechnology including; silicon or mesoporous silicate nanoparticles (MSN), calcium phosphate (CaP), iron oxide (Fe_2O_3), zinc oxide (ZnO) and cobalt oxide (Co_3O_4) nanoparticles were compared. As can be clearly seen, all these types of nanoparticles have been reported to elicit some type of immunological response. Common molecular markers investigated include various interleukins, TNF- α , IFN- γ , chemokines and others. For example silicon oxide (SiO_2), titanium dioxide (TiO_2) and Fe_3O_4 have been shown to activate the transcription factor NF- κB leading to increased secretion of TNF- α , IL-1 β , and interleukin-6 (IL-6) in cultured brain microglia and neuronal cells.⁵⁴ In human vascular endothelial cells or human keratinocytes IL-6 induction has been reported to be mediated via potassium channel or P2Y11 receptor.^{55,56} While our work and others suggests IFN- γ , TNF- α , IL-1 β may be indicative of nanoparticle pro-inflammatory activity,^{58–62} IL-6 is recently considered as the keystone cytokine in disease.⁶² Therefore the above results should be interpreted with some caution and may be cell type and route of administration dependent. These need to be balanced by modulation of CD, increases in serum IgG, IgM, chemokines, MHC-I, and MHC-II reported for MSN and other nanoparticles which could otherwise contribute a desirable immunological component to an RNA vaccine or double stranded RNA (dsRNA) as discussed next.^{66–68}

ACHIEVING SYNERGY

Nanovaccines incorporating RNA may have several advantages. First the core nanoparticles themselves are capable of augmenting immunogenic activity as shown Fig. 2. Further more recent evidence for this is seen for example when MSN and CaP nanoparticles have been tested in conjunction with vaccine and have been shown to enhance and sustain T-cell responses and in the case of CaP to protect against influenza challenge.^{66–69} The RNA molecule does not have to encode an antigen to potentiate an immune response either, as shown previously for example with pIC where its signaling through Toll-like receptors (TLRs) and pathogen-associated molecular patterns (PAMP) are immuno-stimulatory. Thus another means of achieving synergy has been shown for example with pIC containing CaP nanoparticles which in addition to molecular signaling as above have the additional advantage of T cell activation and the induction of dendritic cell maturation.^{66,67}

Lipid coated CaP co-loaded with pIC, STAT3 siRNA and ovalbumin (Ova) antigen have been referred to as nanovaccines and have been shown to induce an effective anti-tumor immunological response.⁷⁰ Therefore similarly, another attractive approach for achieving synergy against cancer is to co-load the nanoparticle with pIC and another anti-tumor agent such as Zoledronic acid.⁷¹ As mentioned previously, pIC is a much needed double edged sword against cancer capable of stimulating an immunogenic and anticancer approach. Bob Langers group showed that surface functionalization is required for iron oxide to bind RNA,⁷² and this is true for pIC and consistent with our results for another magnetic nanocarrier, manganese oxide.^{73,74} There is however a great deal of current interest in targeted combination chemotherapy with pIC nanoparticles,^{75–77} especially as a novel approach for intratumoral immunotherapy against melanoma and potentially other cancers.^{78–80} For additional synergy it would be desirable to be able to load the RNA onto for example zinc oxide or cobalt oxide nanoparticles which have been shown to possess inherent anti-cancer activity themselves.^{81–84} A schematic of the synergistic advantage of such an approach is illustrated in Figure 3.

As shown in Figure 3, the delivery of pIC by zinc oxide nanoparticle would be expected to trigger an appropriate immune response as discussed above, and in addition activation of TLR3 causing translocation of interferon regulatory factor-3 (IRF-3) and nuclear factor (NF)- κ B across the nuclear membrane into the nucleus where type I interferons and proinflammatory cytokines, including TNF α , IL-6 and IL-8, would be expected to induced. This would essentially activate the immune system against the tumor. Further through TLR3 signaling, as well as RIG-1 and MDA-5, poly IC would be expected to trigger apoptosis, and essentially cause a molecular chemotherapeutic effect. Further, mechanisms of apoptosis caused via nanoparticles, ROS induction, etc. are not expected to be the same as poly IC, and thus the molecular signaling pathways may be complementary leading to such immunochemotherapeutic potentiation.

CONCLUSION AND FUTURE DIRECTIONS

In summary, RNA vaccines are in development against a variety of cancers, viral infection and type I allergy. RNA vaccine vectors thus far have been either mRNA or self-amplifying RNA (saRNA) constructs. Double-stranded RNA (dsRNA) such as pIC is also immunogenic and in addition to anti-viral activity is being evaluated as an immunotherapy or combination immunochemotherapy against melanoma and several other solid tumors, as well as ischemia, severe acute respiratory syndrome, Hepatitis B, herpes zoster in children with cancer and others. While liposome delivery of either RNA vaccines or pIC has been evaluated, the immunogenicity reported for inorganic nanoparticles has gone surprisingly underexploited. As clearly in the literature the last few years ample evidence has now been reported, particularly for calcium phosphate (CaP) and mesoporous silicate nanoparticles (MSN), but also for iron oxide, zinc oxide and cobalt oxide, of pronounced immunogenic activity. In the last year CaP and MSN have shown profound application as nanovaccines accordingly.^{69, 70} RNA nanovaccines may therefore have promise and application against cancer, where in this case exploring the RNA nanobio interface of zinc or cobalt iron containing nanoparticles which could combine indigenous anti-cancer and immunogenic activity with direct RNA binding may therefore achieve the synergy long sought after. Particularly given recent

reports of the antimetastatic activity of pIC^{85–87}, it is imperative now to evaluate its activity in conjunction with nanomaterials.

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FIGURE 1.
Illustration of the functional sequences in an mRNA or saRNA construct

FIGURE 2.
Molecular immunology of inorganic nanoparticles^{40–64}

FIGURE 3.
Synergy expected between zinc oxide nanoparticle and p1C.

Table 1

Diseases with potential RNA vaccines

Disease	RNA type	Delivery	Model	Reference
Cancer	mRNA	Naked	N/A	1
HIV	RNAi	Lipid Nanoparticles; Subcutaneous	Mouse	2
Melanoma Cancer	mRNA	Gene Gun	Mouse	3
Melanoma Cancer	mRNA	Naked; Intradermal injection	Human	4
Type I Allergy	mRNA	Naked	Mouse	5
Avian influenza virus	Self-amplifying RNA	Naked; intramuscular	Chicken	6
Cytomegalovirus	Self-amplifying RNA	Naked; intramuscular or subcutaneous	Human	7
Flavivirus	Self-replicating viral RNA	Gene gun	Mouse	8
H7N9 Influenza	Self-amplifying RNA	Lipid Nanoparticles	Mouse	9
Measles	Self-amplifying RNA	Naked; intradermal	Infant Macaques	10

Table 2

Diseases With Potential Poly I:C dsRNA Treatment

Disease	Delivery Method	Model	Reference
Brain Tumors	Poly-ICLC; intramuscular	Human	22
Cancer	Vaccine containing poly I:C	Human	23
Cancers	Poly ICLC + IL-2 injections	Human	24
Cerebral Ischaemia/reperfusion injury	Naked; Intraperitoneal and intravascular injection	Mouse	25
Gastric Adenocarcinoma	Liposomes; Subcutaneous	Mouse	26
Glioblastoma	Poly-ICLC; intramuscular	Human	27
Hepatitis B	Naked; hydrodynamic injection	Mouse	28
Herpes Zoster in children with cancer	Topical Poly I:C	Human	29
Influenza A Virus	Naked; Intranasal	Mouse	30
Influenza A/Puerto Rico/8/34 virus	Naked; intramuscular and intranasal	Mouse	31
Laser induced Skin Wounds	Topical Poly I:C	Human and mouse	32
Leukemia (solid tumors)	Poly ICLC injection	Human	33
Severe Acute Respiratory Syndrome	Naked; Intranasal	Mouse	34

Table 3

Molecular and cellular correlates of immunity [adapted from references 35 and 36]

Immunity	Cytokine	Producer Cells	Receiver Cell	Response
Innate	Tumor necrosis factor-alpha (TNF- α)	Monocytes, macrophages dendritic cells, T _H 1 cells and others	Endothelial, Macrophages Neutrophils, Hepatocytes, Fibroblasts, Muscle and Fat Cells, Hypothalamus	Production of: chemokine (to induce diapedesis, chemotaxis, and leukocyte recruitment), acute phase protein, interleukin-1 (IL-1), extracellular killing, stimulation of catabolism for energy conversion, fever and sleep
/*Innate	Type I/Interferons	Any virus infected cell IFN- α is produced by T-lymphocytes, B-lymphocytes, NK cells, monocytes, macrophages. IFN- β is produced by virus-infected cells, fibroblasts, macrophages, epithelial cells, and endothelial cells	All uninfected cells	Production of enzymes capable of degrading mRNA. Induction of MHC-I antigen expression needed for recognition of antigens by cytotoxic T-lymphocytes
Adaptive	Type II Interferons	Interferon-gamma (IFN- γ) is produced by activated T _H 1 cells, CD8 _T cells and NK cells	Cytotoxic T-lymphocytes, NK cells, macrophages, monocytes, neutrophils, antigen-presenting cells	Induction of: proinflammatory responses, MHC-I and MHC-II and co-stimulatory molecules by APCs in order to promote cell-mediated immunity, promotes differentiation of T4-lymphocytes into T _H 1 cells and inhibits T _H 2 cell proliferation, activates macrophages and increases antimicrobial and tumoricidal activity
Adaptive	Interleukin-2 (IL-2)	T4 and T8-lymphocytes	NK, T and B cells	Acts as a growth factor, increases NK cell killing ability, stimulates antibody synthesis
Innate	Interleukin-6 (IL-6)	T-lymphocytes, macrophages, monocytes, endothelial cells, fibroblasts	Liver cells, B-lymphocytes, Neutrophils	Proliferation of neutrophils and acute phase protein production
Innate	Interleukin-10 (IL-10)	Macrophages and T _H 2 cells	Activated macrophages and dendritic cells	Inhibits production of IL-12, co-stimulator and MHC-II molecules, Promotes an anti-inflammatory response
Innate	Interleukin-12 (IL-12)	Macrophages and dendritic cells	T-lymphocytes and natural killer cells	Production of interferon-gamma and induction of killing activity
Innate	Chemokines: IL-8, CCL CXCL, MIP, MCP, GRO, and other molecules	Leukocytes, endothelial cells, epithelial cells, and fibroblasts	Leukocytes, white blood cells (WBCs), B and T-lymphocytes, dendritic cells	Leukocyte chemoattraction and regulation of cell movement through the lymph nodes and spleen. Release of killing agents for extracellular killing, ingestion of damaged tissue, increase leukocyte integrin affinity for vascular wall, ligands during diapedesis, regulation of actin polymerization
Adaptive	VCAM/ICAM, CD, MHC	Dendritic cell, antigen presenting cell	Effector T cell	T cell activation, cellular and humoral immunity, tolerance. ICAM/VCAM expressed by endothelial cells and promote leukocyte adhesion to vessel walls during extravasation.