mRNA Technology a Promising Strategy for Rapid Response Vaccine Applications against the Emerging Infectious Diseases

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Abstract

mRNA vaccine platform is potent and versatile could be used in prophylactic and therapeutic vaccines against cancer and emerging infectious diseases. They have the capacity to increase protein translation, and assist modulate the innate and adaptive immune responses with improved delivery. Naked/or Lipid encapsulated forms of mRNA could be optimized for mRNA therapeutics. Recently, mRNA vaccines have been used to elicit potent immune response targeted against many infectious diseases in the animals e.g. influenza, zika, rabies virus and others. COVID-19 mRNA vaccines, developed and completed the all trials during pandemic, are the proven examples of achieving success on this platform. The vaccine developers have already received the conditional and emergency authorization for mass vaccination in US, UK and other countries. The mass vaccination campaigns are going on amid pandemic. The two leading candidates, mRNA -1273 and BNT162b2, have achieved the highest efficacy ~ 95% boosting humoral and cell mediated immune responses equally well. Diverse approaches have been employed to mRNA cancer vaccines too. Dendritic cell vaccines and other direct mRNA injectables were used in many clinical trials in generating promising T-cell response with disease-free survival. Prophylactic and therapeutic considerations for mRNAs by regulatory bodies include good manufacturing practices cGMP production, safety and efficacy. It is imperative to elucidate the immune pathways activated by the various mRNA vaccine platforms.

Keywords: mRNA; Vaccines; Immune Response; Innate Immune Sensing; COVID-19

Abbreviations

APCs: Antigen Presenting Cells; BNT162b2: mRNA Vaccine Candidate; COVID-19: Coronavirus Disease-19; Cationic LNPs: Cationic Lipid Nanoparticles; cGMP: Current Good Manufacturing Practice; CTLs: Cytotoxic Lymphocytes; DCs: Dendritic Cells; DOTAP:1,2-Dioleoyl-3-Trimethylammonium-Propane Chloride Salt; DOPE: Dioleoylphosphatidylethanolamine; dsRNA: Double-Stranded RNA; FPLC: Fast Protein Liquid Chromatography; HIV:

Human Immunodeficiency virus; HPV16E7: Human Papillomavirus 16E7 Gene; IFNAR 1/2: IFN α Receptors 1/2; IRF9: Interferon Regulatory Factor9; ISG: Interferon Stimulated Gene; IVT mRNA: *In-Vitro* Transcribed mRNA; JAK1: Janus Kinase 1; MDA-5: Melanoma Differentiation Associated Protein 5; MERS: Middle East Respiratory Syndrome; MHC: Major Histocompatibility Complex; mRNA: Messenger Ribonucleic Acid; mRNA-1273: mRNA Vaccine Candidate; MyD88: Myeloid Differentiation Primary Response 88;

NF κ B: Major Transcription Factor; nsPs: Non-Structural Proteins; NTPs: Nucleoside Triphosphate; PAMPs: Pathogen-Associated Molecular Pattern; pDNA: Plasmid Deoxyribonucleic Acid; PEG-Lipid: Poly(Ethylene Glycol); PKR: Protein Kinase R; PRRs: Pattern Recognition Receptors; PTM: Post-Translational Modification; RNase L: Ribonucleases L; RNAi: Ribonucleic Acid Interference; RT PCR: Reverse Transcriptase Polymerase Chain Reaction; RIG-I: Retinoic Acid-Inducible Gene-I; SAM: Self-Amplifying mRNA; SARS: Severe Acute Respiratory Syndrome; ssRNA: Single -Stranded RNA; STAT: Signal Transducers and Activators of Transcription Protein; T7, T3 or Sp6: Bacteriophage; TBK1: TANK-Binding Kinase 1; TLR: Toll Like Receptors; TRIF: TIR-Domain-Containing Adaptor-Inducing Interferon- β ; TyK2: Tyrosine Kinase 2; UTRs: Untranslated Regions

Introduction

Vaccination is one of the most effective public health interventions to prevent/control the infectious diseases and save the lives of millions every year. Smallpox vaccine was used widely, to eradicate the disease from the world completely, is its outcome. The incidences in many childhood diseases have largely been reduced around the world. Conventional vaccine approach (live attenuated and inactivated) provides durable protection against various dangerous diseases like polio, measles, mumps, rubella, tuberculosis etc. However, there is still a major hurdle in vaccine development in case of those pathogens which evade the immune system e.g. Herpes, Hanta, HIV, SARS, MERS, Ebola and Zika virus [1]. Hence, there was an urgent need to develop more effective and versatile vaccine platform.

IVT mRNA (*in-vitro* transcribed) achieved success first when studied in animal models in 1990; to detect specific protein production in mice [2]. However, the initial results largely associated with mRNA instability, high innate immunogenicity and inefficient delivery.

The outbreaks of Ebola, Zika and COVID-19 have demonstrated that how quickly the emerging infectious diseases can spread and accentuate the crucial need for rapid response vaccines or 'on-demand' technology platform [3,4].

mRNA based technology has been growing rapidly over the last two decades. Technological advancements in molecular biology and chemistry have guided to increase the stability of mRNA with

appropriate delivery system. This eventually accelerated the development of synthetic mRNA vaccines to acquire the extra features from potency and safety point of view. They have the ability to sustain long-lasting immune response in animals and humans during the clinical trials.

mRNA strategy is promising to have the generic and synthetic landscape with a potential for low-cost manufacturing. This technology has the capability to design and develop a vaccine placing in rapid response to the emerging infectious diseases and can be deployed for rapid and large-scale production.

mRNA, the most preferred nucleic acid-based vaccine

Nucleic acid-based vaccines are mRNA, plasmid DNA and viral vectors based and designed for their suitability for rapid response applications. Because they can be manufactured by rapid and flexible process approach. They have ability to induce broadly protective immunity. The main advantage of these vaccines is that they don't depend upon the coding antigens. Different vaccines are based on the same nucleic acid platform can afford to use the same production, purification methods, and manufacturing facilities requiring standardized and validated methods, therefore cutting the cost and time.

Nucleic acid vaccines mimic the infection by *in-situ* expression of antigen, inducing both humoral and cytotoxic T-cell response to eliminate the intracellular pathogen/or infection by acquiring the protective efficacy.

Nucleic acid vaccines with their special intrinsic adjuvant properties recognized by pattern recognition receptors (PRRs), key elements of innate immune response. With the help of these sensory receptors DCs are induced for adaptive immune response. Plasmid DNA and viral vectors have been evaluated as vaccine platforms performed safe and immunogenic in the human clinical trials. pDNA delivery into the nucleus of the target cells is less efficient. The viral vectors induce virus-specific immune responses against viral structural proteins especially on boosting.

mRNA vaccines don't generate infectious particles/or integrate to the genome of host cell. Antigen is expressed *in-situ* at increased translation level, without crossing the nuclear membrane. Complex antigens can be expressed without packaging constraints [4,5].

mRNA vaccines can be produced rapidly, possibly within days after obtaining gene sequence information, using completely synthetic manufacturing process. This platform is manageable considering multiple targets.

mRNA vaccines have several beneficial features over subunit, killed, live attenuated and even DNA vaccines:

- It is non-infectious.
- Non-integrated platform.
- No potential risks of infection/or mutagenesis.

mRNA is half degraded by the normal cellular processes; various modifications provide more stability and make them to increase the translation for high antigen expression. It also helps in inherent immunogenicity modulating to increase the protein safety profile. The delivery system also allows its rapid uptake and expression in cytoplasm [4,5]. mRNA have the potentials to fill-up the gaps of vaccine development for rapid, scalable and economical manufacturing in relation to *in-vitro* high yields transcription reactions.

Principle of mRNA vaccine

mRNA encode the specific genes or genes of interests to efficiently translate their genetic information through ribosomes in order to form required protein as vaccine to be used *in-situ*.

mRNA is constructed optimally from linear DNA template us-

ing T7, T3 or Sp6 phage RNA polymerase. The product contains an open reading frame (ORF) encoding the protein of interest (gene of interest), flanking UTRs, a 5' cap and 3'poly (A) tail. The five -prime cap (5' cap) is an altered nucleotide on 5', regarded as 7-methylguanylate cap (m7G).

Two major types of mRNAs are currently studied for vaccines: non-replicating mRNA and virally derived self-amplifying RNA. Conventional mRNA-based vaccines encode the antigen of interest and contain 5' and 3' UTRs (flanked untranslated regions), whereas self-amplifying RNAs encode not only the antigen but also the viral replication machinery that enables the intracellular RNA amplification for substantial amount of protein expression. The engineered mRNAs gravitate to simulate molecules naturally as fully processed matured mRNA occurred in the cytoplasm of eukaryotic cells. The transfection agent facilitates the mRNA uptake and protect it from degradation. Once mRNA penetrates the cytosol, the synthesis of proteins and peptides begins using ribosomes translating mRNA into polypeptide chains, which undergo post-translational modification (PTM) to form the mature product to unfold in a fully functional protein. This is the greatest advantage to use mRNA molecules in vaccines and protein production by facilitating their delivery to the target cellular compartments to warrant the precise function. Finally, mRNA is fully degraded by the physiological functions, therefore reducing the risk of metabolite toxicity [4,6].

Figure 1: Principles of conventional and self -amplifying mRNA vaccines and their mechanism to express targeted proteins to elicit the required immune response.

Various mRNA vaccines platforms have been developed and validated in recent years for immunogenicity and efficacy. Some novel advances made related to the formulations in mRNA vaccines using adjuvants and some still remained effective without using adjuvants. Geall has demonstrated the duration of *in-vivo* protein production from RNA replicons can be greatly improved by packaging them into lipid nanoparticles [7].

Translation dependent stability of mRNA

Translation of mRNA is highly influenced by the flanking 5' and 3' UTR coding sequence elements, which is a critical concern for vaccine stability. These regulatory sequences greatly increase the half-life and expression of mRNA therapeutics. 5' cap is required for efficient protein production and poly (A) tail plays an important role in translation and stability (Figure 1). The codon usage has an impact on protein translation. Enrichment in G:C content constitutes another form of sequence optimization that has been shown to increase steady state mRNA levels *in-vitro* and in protein expression [4,6,8]. Codon alteration modulate the protein expression could affect the mRNA secondary structure, kinetics and translational accuracy with protein folding and the cryptic T-cell epitopes present in alternative reading frames.

A number of technologies have been used to improve the pharmacological aspect of mRNA. Exogenous mRNA is inherently immunostimulatory, as it is recognized by the various cell surfaces, endosomal and cytosolic innate immune receptors. Immunostimulatory profile of mRNA can be figured out by purification of IVT mRNA and introduction of modified nucleoside complexed with carrier molecules. Purification of IVT mRNA is critical to maximize the immunogen production in dendritic cells and to avoid unwanted innate immune response. FPLC- purified and nucleoside modified mRNA help understand the advance innate immune sensing to how to avoid the adverse effects, which substantially contribute to the current interest in mRNA-based vaccines and protein replacement therapies [4,9].

mRNAs complexed with carriers largely influence the physico-chemical properties in managing their distribution to the organs for precise delivery at cellular level. Direct injection of mRNA is comparatively rapid and cost effective, but it doesn't allow rigorous and efficient cell type delivery. The *ex-vivo* dendritic cell loading allows the accurate control of cellular targets and transfection efficiency. It elicits mainly cell-mediated immune response; which have been used primarily to treat cancer. DCs represent the effective target for transfection by mRNA vaccines, both *in-vivo* and *ex-vivo*. DCs *ex-vivo* loading method is heavily pursued in cancer treatment.

Unlike protein immunization, several formats of mRNA vaccines induce strong CD8+ T cell responses with efficient presentation of

MHC class-I molecules. In addition to that mRNA vaccines proved to generate potent neutralizing antibodies response unlike DNA immunization [10]. mRNA vaccines have shown the safety profile in animals and in human clinical trials in case of COVID-19 vaccine; and they are versatile and rapid to design for emerging infectious diseases like COVID-19 and also amenable to scalable GMP production.

Most currently used self-amplifying mRNA vaccines are based on an alphavirus genome. This platform enables the production of copious amount of antigen from extremely small dose of vaccine, owing intracellular replication of the antigen-encoding RNA. The development of RNA complexing agents brought remarkable improvement to the vaccine efficacy by producing T and B cell response [11]. The advantage of SAM (Self-amplifying mRNA) vaccines is that they can create their own adjuvants in the form of dsRNA structures, replication intermediates and other motifs that may contribute to their high potency. Immunogenicity of the replication protein may theoretically limit repeated use.

Non-replicating mRNA vaccines are an attractive format for their simple and economical administration, especially in less resourced settings. Although an early report demonstrated that immunization with liposome- complexed mRNA encoding influenza virus nucleoproteins elicited CTL response in mice. The noncomplexed mRNA encoded with pathogen sequence and complexed with protamine RNA adjuvants was immunogenic in multiple animal models [12].

Nucleoside -modified mRNA vaccine represents a new and highly efficacious category of mRNA vaccines. Immunogenicity level of LNP-complexed, non-FPLC-purified mRNA vaccines against influenza HA 10 neuraminidase, H10N8 and H7N9 influenza viruses, was detected in mice, ferrets, non-human primates and for the first time in humans. Clinical trials have also been initiated to evaluate the immunogenicity and safety of nucleoside-modified mRNA-LNP vaccines in humans for the first time [13,14].

mRNA vaccines with neo-antigens/or neoepitopes within the cancer cells, have been deployed as vaccine targets in humans. The cancer vaccines are more personalized therapeutics rather than prophylactic, in order to stimulate cell-mediated responses (CTLs), that are capable of clearing or reducing tumor burden. The first proof -of -concept of mRNA vaccines was published two decades ago. DCs are to play in initiating the antigen-specific immune response. DCs function is also modulated through mRNA encodes pro-inflammatory cytokines such as IL-2/or trafficking-associated molecules. The TriMix, a cocktail of mRNA-encoded adjuvants (CD70, CD40L) and constitutively active TLR4 can be electropor-

ated. This formulation has been endorsed efficacious in multiple pre-clinical studies by increasing the DCs activation and shifting to CD4⁺T cell phenotype from T-regulatory cells to T-helper 1 (TH 1) cells. Notable the stage III and stage IV melanoma treated with DCs loaded with melanoma associated antigens and TriMix adjuvant resulted in tumour regression by 27% [15,16].

The beneficial outcome of mRNA vaccination has increased when it combines with adjunctive therapies, such as traditional chemotherapy, radiotherapy and immune checkpoint inhibitors etc. Cisplatin treatment (Chemotherapy for multiple cancers) significantly increased the therapeutic effect of immunizing with mRNA encoding HPV16E7 oncoprotein and TriMix adjuvant leading to the complete rejection of female genital tract tumours in mouse model [17].

Target delivery of non-replicating mRNA

Non-viral delivery system is used to deliver mRNA to the target cells as described above. Plasma membrane is a major barrier for mRNA delivery. The hydrophilicity and strong net negative charge of RNA significantly impairs the cellular uptake. To counteract this, mRNA can be electrostatically complexed with cationic LNPs to improve cellular uptake as it wraps around acting like cargo and by masking the negative charge of the RNA. In many studies, it has been shown the non-replicating mRNA can mediate endocytosis through scavenger-receptor at caveolae, a lipid raft-rich membrane invagination [18]. The early endosomes normally fuse with late endosomes at pH~5 - 6 and become lysosome pH ~4.5 supplemented with RNA -degrading nucleases. mRNAs undergo endosomal escape at this time to relocate in the cytosol. The large mRNA molecules reach ribosomes and translation process starts. So, in the cytoplasm the mRNA interacts with different sets of cellular proteins that regulate siRNA movement and location [19].

Carrier molecules of nanoparticle system for mRNA vaccines make particular complexes with specific diameter for efficient delivery are commonly used as: naked mRNA with *in-vivo* electroporation; protamine complexed; oil-in-water cationic nano emulsion; complexed with PEG-lipid; polyethylenimine; polysaccharide (chitosan); cationic nanoparticles like 1,2-dioleoyl-3-trimethylammonium-propane (DOTAP) chloride salt or dioleoylphosphatidylethanolamine (DOPE) lipid; cationic lipids and cholesterol and PEG-lipid [4].

Nanoparticles influence the efficiency of expression through antigen presenting cells (APCs) having presence of pathogen-associated molecular pattern (PAMPs) in the dsRNA/or unmodified

nucleosides and the level of optimization of RNA sequence (codon, G:C content and 5' and 3' UTRs). Vaccine activates the T follicular helper (TFH) cellular germinal centre (GC) B cell responses.

Innate immune sensing

Innate immune sensing of mRNA is related to produce vaccine response to engage both innate and adaptive arms of the immune system (Figure 3). The exogenous mRNA interacts at multiple points to bring vaccine outcomes. RNA vaccines also have the ability to act as self-adjuvant through RNA sensing machinery, which presents in all cells of the body to provide an initial barrier against viral infections. TLR 3, TLR 7/8 (Toll like receptors), RIG-I receptors (retinoic acid-inducible gene-I like receptors) and MDA-5 (Melanoma differentiation protein 5) are the receptors of sensing machinery, which assist the exogenous RNA sensing process. TLR 3 recognizes double stranded (dsRNA > 45 bp) RNA, single stranded RNA (ssRNA) through double stranded structures and replication intermediates. TLR7 and TLR8 recognizes single stranded RNA (ssRNA) with some GU-rich sequence. Cytosolic RIG-I preferentially recognizes ssRNA and dsRNA bearing a 5C triphosphate below 1 kb in size with no sequence specificity and MDA-5 signals in response to chemically synthesized ds RNA such as poly I:C polyinosinic:polycytidylic, an immunostimulant which interacts with TLR3 [20].

TLRs are largely involved in the immune surveillance in DCs, macrophages and monocytes. RIG-I and MDA-5 are primary sensors in other tissues. When RNA vaccine is injected to muscle cells gets recognized by RIG-I/and MDA-5 sensors to acknowledge the delivered nucleic acid. TLRs and RLRs intensify the innate immune response involving in activation of type I IFN (IFN α and IFN β). The initial cytosolic signaling events occur through RNA sensors inducing IFN gene expression. IFN protein is secreted from the cell binds IFN α receptors 1/2 (IFNAR 1/2) in both autocrine and paracrine signaling ways that orchestrates the induction of potent ISGs (interferon stimulated genes) to amplify the antiviral response in the surrounding tissues. At this time IFN and ISGs locally induce transcriptional and posttranscriptional changes to augment the antiviral state. Viral replication is inhibited by changed mRNA translation rates, ribonucleases L (RNase L) upregulation by increasing the degradation of RNA and activation of pro-apoptotic pathways [20,21]. An active antiviral response is advantageous to train the adaptive immune cells and preventing the viral spread, it can become toxic to cells if uncontrolled. For example, activation of protein kinase (inhibitor of cellular translation) establishes the antiviral state. Hence, it is crucial to monitor the antiviral response in days after the delivery of mRNA.

Figure 2: A downstream signaling pathway to induce I IFN production in response to exogenous mRNA with help of activation of RNA sensing machinery. TLR 3, TLR 7/8 sense the dsRNA and ssRNA respectively. dsRNA and 5 C triphosphorylated forms of ssRNA and dsRNA are recognized by RIG-I. Kinase TBK-I is activated by RIG-I, MDA-5 and TLR-3 signaling,

Adaptive immunity involvement

APCs are involved in this process as they take vaccine antigen which is processed internally in the cytosol and the resulting peptides are presented to other T-cells through MHC-II receptors. APCs are recruited to the vaccine site via innate immune response, generated by secreted cytokines, triggered in response to adjuvating/or mRNA sensing process. They primarily upregulate the co-stimulatory molecules bearing CD80 and CD86 by APCs; mainly expressed in dendritic cells, monocytes and macrophages. This creates a microenvironment not only requires receptors of MHC-peptide complexes to engage, but also align the co-stimulatory molecules on their surface. They all signals the pathways to amplify the expression of inflammatory cytokines to increase the production of Th 1 T helper cells [22].

mRNA vaccines moderate the anti-viral response

mRNA vaccines have demonstrated that lipid-complexed mRNA vaccines could help achieve adequate adaptive immunity, most likely by its self-adjuvating effect of activating type IFN-I [23]. If RNA vaccine is complexed with protamine, develops stronger humoral immune responses that are dependent on TLR7 signaling. DCs like other immune surveillance cells express a high level of all TLRs to mediate the rapid induction of cytokines and increase presentation of microbial peptides to T-cells, which activates antigen

-specific immune response (Figure 3). This vaccination approach enhances the antigen presentation and cultivating the stronger adaptive response with ‘super antigen presenting cells’ [24]. In some disease models, the immune sensing of mRNA reduces the translation of mRNA-encoded antigen. Antigen-specific T-cell responses to mRNA vaccine were enhanced in IFNAR 1/2 - mice [21,25]. Induced pluripotent stem cell have shown to go through the RNA-sensing evasion tactics to efficiently reprogram cells with mRNA. Evading strategies of RNA sensing are attained through incorporation of modified nucleotides into mRNA during their *in-vitro* transcription reaction. Chemically synthesized and modified nucleosides have revealed moderate RIG-I and TLR-mediated signaling, resulting in a significantly reduced anti-viral response to exogenous mRNA. IFNAR 1/2 imitation derived from vaccinia virus(B18R) is also used as a method to limit the downstream anti-viral signaling following the production of type I IFNs. In addition, the recombinant B18R protein to cell cultures and delivering mRNA encoding B18R to cells have been successfully utilized to improve both conventional mRNA and self-amplifying mRNA-based cell engineering outcomes. These findings have shown that it is possible to evade an antiviral response to an actively replicating self-amplifying mRNA, while eliciting an effective vaccine immune response. However, it might be advantageous to temporarily alleviate the immune response to the delivered RNA assisting mRNA encoded antigens to be translated and presented [21].

Figure 3: mRNA activates the adaptive immune system along with RNA innate sensing.

Alphavirus replication pathway promote the mechanical delivery of mRNA to the target cell, without affecting the cellular viability or function. They have sophisticated mechanisms that circumvent the cellular signaling and transport to traffic within cells delivering specific genome in subcellular compartment. It was previously known that the alpha viruses enter cells via receptor mediated endocytosis, followed by membrane and cell membrane fusion event in endolysosomes to release the RNA and nucleocapsid into the cell cytosol. Another work challenges this model as alphavirus enters independent of endocytosis, acid pH and membrane fusion. However, the cell entry is still initiated by the viral engagement of specific cell receptor, but this would quickly trigger a viral membrane conformational change resulting in formation of a transmembrane pore. The virus then injects its RNA genome directly into the cytosol of the cell. Many non-enveloped viruses follow this model for infection. Once an alphavirus enters the cytosol, it immediately embraces the host environment to use its machinery for the translation and replicase activity from encoded nsPs [6,21,26].

The simplified model is that the alphavirus replication machinery and mRNA are to be relocated in a membrane invagination as 'replication complexes' for immune opportunities. This replication complex is transferred to the cytosolic surface of endolysosomes [27]. Here, a complementary negative strand RNA is transcribed from the positive strand of RNA, which in turn synthesize the new positive strand, leading to the exponential increase in alphavirus RNA synthesis. It is still needed to discover whether lipid-complexed self-amplifying RNA apply the same model and how long the replication complexes would remain immune privileged.

Furthermore, the studies for replicon life-cycle and *in-vivo* tracking using RNAi should be performed to route the activities of virus-derived replicons. Some studies on RNAi have been started to track the siRNA delivery by LNPs have revealed that complex RNA travel through receptor mediated endocytosis and settled in endosomal vesicles. It is highly likely that muscle cells play a predominant role in the antigen production, after having intramuscular injection. DCs internalize the mRNA through micropinocytosis as a part of immune surveillance [28,29].

Self-amplification of RNA replicon presents challenges and opportunities for RNA vaccines. It takes 1 - 2 weeks minimum to express high level of antigen, consequently producing antigen specific antibody repertoire. However, the mechanisms of RNA entry, cytosolic replication, and host interactions are still being developed. Immune evasion strategies could be utilized experimentally, provided the addition of a modified nucleotide to prohibit self-amplifying RNA activity, since the replicon polymerase can't properly transcribe these bulkier residues.

mRNA an excellent tool for active and passive immunotherapy

In the past years the mRNA vaccines are experiencing a burst in basic and clinical research. Majority of the mRNA vaccines focused on cancer research/applications and wide variety of infectious disease pathogens, including influenza virus, Ebola, Zika, *Streptococcus spp.* and *T. gondii* etc. Advances to understand and reduce the innate immune sensing of mRNA have aided efforts not only in active vaccination, but also in several applications of passive immunization or passive immunotherapy for infectious diseases and cancer.

Passive therapeutics, the recombinant monoclonal antibodies are rapidly transforming the pharmaceutical market and have

become one of the most successful therapeutic classes in treating autoimmune disorders, infectious diseases, osteoporosis, hypercholesterolemia and cancer. High-cost protein production pose the limitation to wide spread accessibility. Antibody gene transfer technology could potentially overcome these difficulties, as they administer nucleotide sequence to the patients enabling *in-vivo* production properly folded and modified protein therapeutics [21,30,31].

Advancements in mRNA vaccines

In the last decade, the technological advancements in mRNA molecular biology, chemistry, along with effective delivery systems enabled the efficient and stable manufacturing of mRNA products, despite achieving the promising results earlier.

A crucial need for a rapid response vaccine was prompted during 2013 - 2016 outbreaks of Ebola and Zika. mRNA vaccine platform attains all the relevant features to fulfil the demand and has confirmed during the COVID-19 pandemic.

The COVID-19 ongoing pandemic exploded in December 2019 in Wuhan, China. It is over a year now, the virus has propagated all over the world creating havoc claimed deaths over 2.2 million reported by Feb 2021 (worldometers.info/coronavirus), by shutting down the businesses, making people jobless, and countries are still imposing the lockdown due to emergence of various virus variants despite the fact that some vaccine candidates have already given the approval for mass vaccination.

Immune responses generated against the virus are adaptive and innate. Viral pathogenic stages in the host are asymptomatic, symptomatic and severe symptomatic. Virus can easily evade the innate and adaptive immune responses during the symptomatic stage, consequently the patients lead to be in the hyperinflammatory state of "Cytokine storm" due to release of excessive cytokines. Due to the early sequencing of the virus provided the insight to design RT PCR molecular test for the virus detection in suspected hosts and to develop serological tests to detect the past infection [32,33].

Vaccine development is normally a longer and expensive process in case of conventional vaccines. Attrition is higher, it takes multiple candidates and many years to produce the licensed one, which follow the linear sequence of steps with multiple pauses for data analysis and manufacturing process checks. But, in opposite

to the conventional vaccines, the COVID-19 mRNA vaccines are prepared on the basis of novel platform. The virus genome was sequenced in January 2020 and the first human trials began in March 2020. Vaccines were tested in cells and animals and passed through the regulatory criteria along with conformation in safety and efficacy testing.

These are:

1. Pre-clinical testing: To check the immune response generated by the new active candidate in cell culture and animals (Monkeys and mice).
2. Phase I safety trials: Vaccine is given to small number of people to test safety and confirm dosage to stimulate immune response.
3. Phase II expanded trials: Hundreds of people are given vaccines in various groups as children, elderly groups for further safety and efficacy.
4. Phase III trials: Thousands of people are given the vaccine to see any adverse effect against the placebos.
5. Approval: In pandemics vaccines received emergency use authorization before getting formal approval. Even licensed vaccines are continuously being monitored for safety and efficacy.

Using this novel platform, the recent advancement in COVID-19 mRNA vaccines is validated to be successful by attaining 95% efficacies in preclinical/clinical trials in animals and humans. Two mRNA vaccine candidates have already given approval for mass vaccination.

Companies named Pfizer and BioNTech and Moderna successfully developed the COVID-19 vaccine candidates BNT162b2 and mRNA-1273 respectively, amid pandemic. The encoding antigen is S-2P consisting of SARSCoV-2 glycoprotein with a transmembrane anchor and an intact S1-S2 cleavage site. The lipid nanoparticle capsule was formulated in a fixed ratio of mRNA. This efficacy achieved is immense i.e. 30% more than the set criteria limit of the vaccine [34,35]. The Pfizer's vaccine candidate has given the conditional approval by MHRA regulators (UK) first time in the world for mass immunization.

Vaccines tend to generate the protective immunity while eliciting potentially both the T and B-cell response.

Figure 4: COVID-19 mRNA vaccine to train the protective immune system. Lipid nanoparticles carry the specific viral RNA sequence and it bumps on to the vaccinated cells. The particles invaginate into the cell releasing mRNA into the cytosol. RNA gets associated with ribosome and translated into spike proteins individually. Consequently, these spikes presented on the antigen presenting cell. T-cells detect these fragments and alarm the other T and B immune cells for further action. B-cells attach to the spike protein produced by Helper T-cells to manufacture antibodies against spike proteins and they commence their proliferation. After infection antibodies attached to coronavirus spikes and destroy the virus before it gets into the cell. The APC also activates the killer T-cells which destroy the infected cells. The vaccine with dose regimen uses to prime the immune cells and 2nd dose is given 21 days apart. The active immunity remains for months and later it gets lowered down. Memory B and T-cells retain the information for many years/or whole life to combat the same pathogenic attack.

The interim guidance has been developed by WHO based on data emerged during clinical trials and the advice issued by the

Strategic Advisory Group of Experts on immunization on January 2021 for both the leading mRNA vaccine candidates from Pfizer and Moderna. If a person with history of anaphylaxis and allergy to polyethylene glycol (PEG) or related substance should not be administered the vaccine [36,37].

Considerations for mRNA vaccines

mRNA vaccines represent a promising platform because they are effective, safe and have capacity for rapid and economic development/manufacturing. The direction for consideration of this platform for widespread use is described below.

Recognition for cGMP

It is possible to manufacture mRNA in small facility footprint, because of its *in-vitro* reaction with recombinant enzymes, ribonucleotide triphosphates, simple and rapid production of DNA templates than the traditional vaccines. The sequence profile can be made primarily followed by purification making the manufacturing process sequence independent. This process is mainly suitable to standardize any encoded protein immunogen, making it applicable for rapid response to the emerging diseases. All enzymes and reaction components are obtainable of cGMP grade including animal components free products to avoid the occurred problems of adventitious agents unlike cell-culture based manufacturing of biologics. All source materials for example plasmid DNA, phage polymerase, capping enzymes and NTPs are available of GMP grade, could be an economical option.

The production process starts with a template plasmid DNA produced in *E. coli* linearized by restriction enzymes to begin the synthesis of runoff transcripts with polyA at 3'. mRNA is synthesized by DNA-dependent RNA-polymerase (from bacteriophage T7, SP6 or T3) using NTPs. Finally, mRNA is enzymatically/chemically capped to enable efficient translation *in-vivo*. 2 g/l yield of full-length mRNA can be obtained under optimized conditions. After synthesis, it is processed through several purification steps to eliminate remanent reaction components, residual DNA, free nucleotides, enzymes and truncated fragments. LiCl precipitation is used in large scale for removal of dsRNA and other contaminants are critical for final efficacy of the product. Final formulation must be free from RNase as RNA is susceptible to degradation and the formulation buffer components have chelators and anti-oxidants. Chelators and anti-oxidants reduce the effects of reactive oxygen

and metal ions which contribute to the instability of mRNA [38]. Pharmaceutical formulation is an active area of development.

Most of mRNA products are stored at -70°C, but the standard formulations can make RNA stable at higher temp ranges. Even the product can remain active after lyophilization to be stored at 5° - 25°C for 3 years and at 40°C for 6 months [39]. Naked mRNA is stable at refrigerated conditions at least 10 months. Stability of the product is improved by packaging with nanoparticles/or formulations made with RNase inhibitors [21].

Regulatory facet

Initially there was no specific guidance available from FDA (US Food and Drug Administration) and European Medicines Agency (EMA) for mRNA products. Although many guiding principles were defined for DNA vaccines and gene therapy vectors [40,41]. These are likely to be applied to mRNA vaccines with some adaptations to reflect the unique feature of mRNA. As of now mRNA vaccines are being used for mass and the guidance are available by 2020, that will draft the requirements to produce and evaluate new mRNA vaccines.

Safety aspect

Safety is the extremely stringent aspect of mRNA vaccines. They don't require toxic chemicals for manufacturing and pose no risk of adventitious viruses which are mainly associated with conventional vaccines. Theoretical risks of infection and integration of vectors into host cell DNA are not the concern for mRNA. mRNA vaccine platform is safe comparably.

Potential safety concerns are likely to be evaluated in preclinical and clinical studies. These include the local and systemic inflammation, stimulation of autoreactive antibodies, bio-distribution and persistence in expressed immunogen, and potential toxic effects of any non-native nucleotides and delivery system components. mRNA based vaccine platform induce potent type I interferon responses, which have been associated not only with inflammation, but also potentially with autoimmunity [42]. The extracellular mRNA increases the permeation to endothelial cells thus contribute to the formation of oedema. It can also promote the blood coagulation/thrombus formation.

mRNA therapeutics combine the safety with impeccable dose control and potential for multiple administrations, with no pre-

existing or anti-vector immunity. Earlier studies showed great immune response in mice to secrete functional antibodies. Lipid nanoparticles encapsulated mRNA encoding the light and heavy chains of anti-HIV antibodies VRC01 rapidly produced high level of functional antibody in serum and protected the humanized mice from HIV infection. LNP system with specific mRNA in cancer, rabies virus, botulinum toxin and B-cell lymphoma has given protection from the challenge doses and no toxic effects observed. This observation suggests that mRNA offers a safe, simple and efficient alternative to therapeutic monoclonal antibody protein delivery, with potential application to any therapeutic protein [4,21,43-45].

Vaccine efficacy perspective

Vaccine efficacy is the percentage of reduction of disease in vaccinated groups as compared to the unvaccinated group under the favorable conditions.

$$\text{Vaccine Efficacy} = (1 - \text{RR}) \times 100\%$$

RR= Relative risk to develop the disease in vaccinated people as compared to the unvaccinated people.

The main benefit of vaccine efficacy is to have the control on all biases found with randomization, prospective, active monitoring for the disease attack rates and tracking of vaccination status for a study population. The vaccine efficacy is the primary endpoint estimate for a placebo-controlled efficacy trial should be at least 50% with alpha adjusted confidence interval in case of COVID-19 vaccine [51].

Future Prospects

The development of RNA vaccines has confirmed by the initial proof of concept in cancer [2]. The COVID-19 vaccine development with its successful trials and conditional authorization for mass immunization, amid pandemic, are the emerging trends in the field of vaccines against the infectious diseases.

At present, the RNA vaccines both non-amplifying and self-amplifying are progressing the clinical testing for prophylactic/therapeutic applications. The COVID-19 mRNA portfolio is being used. It has shown robust T and B-cell responses benchmarked against the state-of-the-art vaccine technologies such as adjuvant-based subunit and live-attenuated vaccines. RNA platform is versatile and being able to provide a wide range of opportunities for future im-

provements. Working on the RNA interaction with host cell could assist limiting the IFN-based immune response. RNA element has the property to act as self-adjuvant leading towards more potent vaccine [46,47].

Advanced technologies in synthetic biology and chemical modifications will generate more stable and uniform RNA vaccines.

By producing other viral replicons, and their co-expression would provide better understanding of their activities in new generation vaccines. Nevertheless, the emerging RNAi field provides siRNA imaging technology to work on their delivery by LNPs have shown that the complexed RNA enters the immune cells and help suppress the IFN response and manipulate to increase the RNA polymerase fidelity is a fully new concept could be applied to new generation RNA vaccines [48].

Formulations and delivery technologies are broadly researched, a critical area in context of DNA vaccines. However, the preliminary research in this area, in case of RNA vaccines, indicated that significant modifications and alternative strategies will be required for the efficient delivery of RNA [49].

Significant progress has been made in cell-specific targeted nanoparticles, could be useful in the emerging field of prophylactic and therapeutic applications of large RNA molecules. RNA vaccines provide important knowledge in basic and clinical research for vaccinology. *Ex-vivo* manipulations of the DCs could be a powerful tool to treat cancer in personalized medicines [21].

The current published data and development made on the COVID-19 vaccine has provided the insights for commercialization of this novel technology in the field of cancer vaccines, universal flu vaccine, or rapid response vaccines. Self-amplifying vaccines even require lower dose would help the rapid response platform in pandemic could provide benefits:

1. It is completely synthetic process, don't require cell culture and manageable to automation.
2. Based on generic process for robust manufacturing of vaccine against any changing viral strain/or variant.
3. Could be feasible in small manufacturing foot print utilizing standard disposable equipment.

4. Convenience in stocking the raw material in single facility, accelerating the progress from sequencing to the production.

RNA vaccines easily bypass the inherent potential safety limitations unlike live attenuated vaccines. This technology has the potential to enable new products because of its synthetic nature, cell free manufacturing approach.

mRNA will continue as future prophylactic and therapeutic for various ailments and disorders, with the emphasis on the delivery systems to increase the efficiency in targeting the infection. The better understanding of the mechanism of action would provide a rational design for the next generation RNA vaccines.

Conclusion

mRNA approach has all the attributes of vaccine 'on-demand' technology. It has been approved with the successful development of COVID-19 mRNA vaccine candidates, amid pandemic, including all the clinical trials for mass vaccination. Medicines and Healthcare products Regulatory Agency (MHRA), UK has granted the approval for first COVID-19 mRNA vaccine in the western world, developed by Pfizer and BioNTech in December 2020 [50]. FDA and EU have also recommended the grants for commercial marketing of this vaccine.

The novel platform of mRNA is scalable and cold-chain free, offers the agility in drug discovery and target development with cost effective production against novel threats. These therapeutics would fill the gaps by rapid developments with increased safety and efficacy. More exploration is needed to be carried out in the front of immune response generation and its impact including effective and safe delivery system.

More research is required on the prospects of nucleoside modified vs. unmodified mRNA, self-amplifying mRNA vs. conventional mRNAs, efficient and safest delivery system and best route of administration, proinflammatory innate immune responses and dose formulation of contributing components.

The modern vaccine design is based on genetic engineering, immunology, structural biology, and system biology [1,2,3,6]. The need of conventional vaccines seems unmet because in some chronic infections the pathogen lapses the adaptive immune response or require active cellular immune response against the

emerging diseases like Zika, Ebola, Nipah and pandemic influenza. The unpredictable emerging and re-emerging outbreaks almost every year with high morbidity and exponential spread, could create substantial social impact. In those circumstances the vaccine 'on-demand' approach is highly desirable. Nonetheless, it may not be adaptable to the conventional vaccine manufacturing approach which requires complex, expensive, lengthy research and development process.

Understanding of the sequence events of antigen expression, innate and adaptive activation responses could further guide developing mRNA portfolios with efficient delivery systems. This will undertake different applications, such as delivery of vaccine antigen vs. therapeutic molecules (nano antibodies), prophylactic vs. personalized therapeutics, infectious disease vs. host disease etc.

The detailed prospects of this technology are still under development.

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Conflict of Interest

There is no conflict of interest exists.

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