mRNA vaccine manufacturing – challenges in plasmid DNA cloning vector design

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Abstract: In the post-COVID-19 era, there has been a notable surge in the development of mRNA vaccines. These vaccines are not only targeting various pathogens beyond SARS-CoV-2 but also hold promise in treating cancer and genetic disorders. These type of vaccines are revolutionizing vaccinology through their inherent possibility for rapid pandemic response, high efficacy, minimal side effects, and cost-effectiveness.

Achieving these benefits hinges on seamlessly integrating mRNA production steps, from plasmid DNA (pDNA) design and antigen cloning, *in vitro* transcription to lipid nanoparticle formulation.

A critical initial step in mRNA vaccine production is pDNA cloning vector design. The vector should be carefully constructed considering a copy number of plasmid, vector backbone with a promoter, the origin of replication, multiple cloning sites, polyadenylation signal, and markers for selection. However, despite careful design, challenges like poly-A tail deletion may arise, prompting the exploration of stable large-size and low-copy vectors, as well as linear and bacteriophage vectors. Additionally, for large-scale production and regulatory compliance, vector systems must be scalable and well-documented.

This overview aims to elaborate upon the intricacies in pDNA cloning vector design. The focus is on achieving accurate insert sequence, especially those encoding the complex antigens and gene expression, highlighting the critical role of pDNA design in ensuring vaccine effectiveness.

Although commercial vectors and automated synthesis facilitate gene construction, challenges still exist. This emphasizes that a multifaceted approach, combining molecular biology techniques, computational tools, and collaboration with experts in microbiology, molecular biology, and vaccine development, is required for successful mRNA vaccine development.

Keywords: mRNA vaccine; plasmid DNA cloning vector design

1. Introduction

A vaccine is a biological preparation that provides active acquired immunity to a particular infectious disease. It typically contains a weakened or inactivated form of the pathogen (such as a virus or bacterium) or its components or genetic material. Vaccines stimulate the body's immune system to recognize and remember a specific microorganism, without causing the disease itself (Larson et al., 2018). This plays a vital role in preventing, controlling, and eradicating infectious diseases, which saves millions of lives worldwide. Vaccination prevents approximately 2-3 million mortalities from measles, influenza, pertussis, and tetanus every year. The COVID-19 pandemic has emphasized the importance of safe and effective vaccines in global public health (Montero et al., 2023).

Traditional vaccines frequently contain pathogens or parts of them that have been attenuated or inactivated in order to elicit an immune response without causing illness. Many infectious diseases have been successfully prevented by these vaccines, which have been in use for decades. Examples include live attenuated vaccines (e.g., measles, mumps, rubella vaccines) and inactivated vaccines (e.g., polio vaccine, influenza vaccine). Traditional vaccines are made within standard manufacturing processes involving bacterial culture or cell culture systems for viruses, purifications, and formulation steps. However, manufacturing

processes often require time-consuming and labor-intensive procedures for development and production, particularly for live attenuated vaccines, which can lead to challenges in scaling up manufacturing capacity to meet global demand during pandemics or outbreaks (Larson et al., 2018).

Modern vaccines utilize advanced biotechnological techniques, including recombinant DNA technology and synthetic biology, to produce antigenic components of pathogens without the need for live or inactivated whole organisms. Examples include protein subunit vaccines (e.g., hepatitis B vaccine), virus-like particle vaccines (e.g., HPV vaccine), and nucleic acid vaccines (e.g., mRNA vaccines). This precision allows for the development of vaccines with improved safety profiles and reduced risk of adverse reactions. Moreover, modern vaccine production methods are often more scalable and adaptable than traditional methods. Techniques such as recombinant protein expression and mRNA synthesis can be rapidly scaled up to meet global demand, as demonstrated during the COVID-19 pandemic.

However, modern vaccines also have certain obstacles. For example, recombinant peptide vaccines encounter various problems of solubility, folding, glycosylation, immunogenicity, and significant danger of immunological escape, while DNA vaccines have long-term expression and genetic modification hazards, which could result in the development of anti-DNA autoantibodies (Gote et al., 2023; Zhang et al., 2023).

The main goal of mRNA technology is to instruct certain cells in our body to perform specific tasks, such as synthesis of new proteins to fight disease. mRNA vaccines are a groundbreaking technology in the field of vaccinology that offers significant advantages over traditional vaccine technologies. They can be rapidly developed, scaled, and adapted to address global health challenges. This makes them promise for mass vaccination campaigns, as they can be manufactured in large quantities to meet increasing demands in a short period of time. Additionally, the modular nature of mRNA vaccine technology allows for rapid adaptation to emerging viral/pathogen variants. The mRNA vaccine uses *in vitro* transcribed mRNA that encodes the antigen(s) of interest, avoiding the risk of mutagenesis in the host (M. Li et al., 2022; Pardi, 2021; Shin et al., 2020). When mRNA vaccine is introduced to a host cell, it is immediately translated into protein and presented to the immune system. This is one advantage of RNA over DNA: RNA cannot be incorporated into the genome and does not induce mutagenesis, whereas DNA can occasionally be absorbed into the target cell's genome and can then cause mutagenesis by gene insertion. The mRNA vaccines/therapeutics have the potential to be used not only for preventive vaccines but also for therapeutic applications. It can be employed in developing cancer vaccines, personalized medicine, and treatments for various diseases/genetic disorders (Ankrah et al., 2023; Ghattas et al., 2021; Nitika et al., 2021).

However, with the growing demand for mRNA vaccines, it has become clear that there are some challenges in the manufacturing process. This work aims to provide brief insights into the manufacturing process of mRNA vaccines, with a focus on the plasmid DNA (pDNA) vector design and selection.

Although plasmid DNA cloning vector design has become more automated, streamlined, and accessible through commercialization and outsourcing, there is still a need to develop a special strategy to ensure the stability, efficacy, and safety of mRNA vaccines.

2. mRNA vaccine manufacturing

The manufacturing of mRNA vaccines involves several steps, starting with the production of pDNA - the pDNA designing and cloning, the transformation of host cells, followed by high cell-density fermentation. The cells are then harvested, lysed, and obtained plasmids are purified using chromatography or precipitation techniques. After this, pDNA are sequenced to ensure accuracy and quality. The next steps are the linearization of purified plasmids and an *in vitro* transcription (IVT). Typically, IVT is performed using T7 RNA polymerase, and modified ribonucleotides are commonly incorporated into therapeutic IVT mRNAs to decrease their innate immunogenicity. The most commonly used modified nucleotide is N1-methyl pseudouridine (m1Ψ) (Ghattas et al., 2021; Nitika et al., 2021). Obtained mRNA molecules are subjected to enzymatic capping by adding a 7-methylguanosine cap (m7G cap) to the 5' end of the RNA molecules. This process is very important for evading detection by cellular innate immunity. After all steps, mRNA should contain five essential components, which are the 5' cap, 5' untranslated region (UTR), an open reading frame (ORF) of the gene of interest, 3' UTR, and a poly(A) tail.

To obtain functional RNA for various applications, several processes may be involved, depending on the specific requirements of the RNA molecule and its intended use. Some of these processes include IVT, enzymatic capping, 2'-O-Methylation (2'-O-Me), and polyadenylation. Those enzymatic reactions are often carried out in a sequential, or one-by-one, manner and are subject to specific conditions. The necessity of purifying RNA after each specific reaction depends on the nature of the reaction, the quality requirements of the RNA, and the downstream applications. Contaminants present in the RNA sample, including residual DNA, small RNAs, nucleotides, proteins, and other

reaction components can affect the accuracy, quality, and integrity of the mRNA molecules. To meet clinical quality standards, the mRNA generated upstream must undergo multiple purification steps. The purification method chosen for mRNA vaccines depends on various factors, including the desired purity level, scalability, cost, and downstream applications. Purification protocols can vary depending on the specific requirements of the experiment or application, and various purification methods, such as column-based purification (diverse types of chromatography, such as HPLC - high-performance liquid chromatography), or tangential flow filtration TFF techniques phenol-chloroform extraction, or magnetic bead-based purification, may be employed (Whitley et al., 2022).

The delivery of mRNA vaccines into cells is not without challenges. RNA is inherently unstable and must be protected from degradation in the extracellular environment. In recent years, lipid-based nanoparticles (LNPs) and polyplexes/polymeric nanoparticles have been two of the most used mRNA vaccine delivery systems (X. Li et al., 2024; Zeng et al., 2022). LNPs are widely used as delivery systems for mRNA vaccines. The lipid formulations in mRNA vaccines encapsulate and protect the mRNA molecules, facilitating their delivery into cells.

The composition and properties of the lipid formulation can be optimized to modulate the immune response elicited by the mRNA vaccine. Ionizable lipids are effective at encapsulating and protecting mRNA, promoting endosomal release and cytoplasmic transport of the mRNA cargo, which is important for efficient protein expression. PEG-ylated lipids have a hydrophilic coating of PEG on their surface, which enhances biocompatibility and reduces cytotoxicity. Additionally, polymeric nanoparticles (e.g. polyplexes) can be engineered to enhance their stability and targeting specificity. In general, the choice of delivery system depends on several factors, including the specific characteristics of the mRNA vaccine and the desired transfection efficiency, safety, stability, and target specificity (Bouazzaoui et al., 2021; X. Li et al., 2024; Zeng et al., 2020).

The formulation process is followed by additional purification, buffer exchange, and sterile filtering before filling and finishing. Numerous quality assessments at the LNP, mRNA, and LNP-mRNA levels tightly control the process (Gote et al., 2023; Pardi et al., 2018; Pollard & Bijker, 2021; You et al., 2023; Zhang et al., 2023).

3. Challenges in pDNA vector design

Although manufacturing mRNA vaccines involves several key steps, selecting an appropriate pDNA vector is a crucial aspect of this process. Precise vector design can help optimize gene expression and ultimately contribute to the efficacy of the vaccine manufacturing process.

In the molecular cloning, there are seven essential steps involved to clone any DNA fragment. These steps include selecting the appropriate host and cloning vector, creating recombinant DNA, producing DNA clones, choosing organisms for multiplication of recombinant DNA, screening desired DNA insert clones, and analyzing their biological characteristics.

3.1. Selection of Antigens

Identifying antigens or epitopes for a vaccine, especially for a multivalent vaccine targeting multiple pathogens or strains, requires understanding of the pathogens, their virulence factors, and the immune response they trigger. This could be based on the disease burden and the prevalence of the pathogens, as well as their ability to cause severe illness. To identify potential antigens, virulence factors or epitopes, various screening methods such as genomics, proteomics and bioinformatics can be utilized. These methods can help identify proteins or peptides that are highly conserved among different strains of the pathogen or that are unique to certain strains. It is important to ensure that selected antigens or epitopes are sufficiently conserved among different strains or variants of the pathogen. Conserved antigens are less likely to undergo antigenic variation and are more likely to provide broad protection against diverse strains.

For multivalent vaccines targeting different strains or related pathogens, the degree of cross-reactivity among the selected antigens must be assessed.

Finally, epitope mapping studies can be conducted to identify specific regions within the antigens that are recognized by the immune system. This allows for the selection of immunodominant epitopes that elicit strong antibody or T cell responses.

3.2. The sequence accuracy and size of DNA encoding the selected antigens

After antigens or epitopes are selected, designing compact and efficient genetic constructs is the next step. Accurate sequence and the production of adequate antigens by host cells are critical for the intended immune response and therapeutic outcome. To ensure correct gene expression, it is essential to confirm the presence of the gene in the host organism and verify its sequence. All additional signal sequences should be included in the plasmid vector (e.g., leader sequences or secretion signals) if needed for proper protein expression, secretion, and folding.

The original sequence of the potential antigen is often optimized for improved expression or other

desired effects. Multiple strategies can be used to optimize sequence. A strategy that adjusts the original codon sequence to match the natural distribution of the host codons has been recognized as the best way to optimize codons. Codon optimization can significantly improve protein expression levels, enhance translational efficiency, and optimize the performance of the mRNA construct in the host organism. Also, optimal codon usage can be used in order to avoid unwanted rearrangements, deletions, or mutations that can occur, and can affect the functionality of the cloned gene (Gote et al., 2023). Other parameters like GC content, RNA secondary structure, and restriction endonuclease sites also affect protein expression. Deep learning methods are increasingly being used to optimize codons and have shown impressive applicability in biological and medical research. In this study, the concept of a codon box is used to recode DNA sequences, and the BiLSTM-CRF sequence annotation method is adopted to annotate amino acid sequences with codon boxes or codons directly (Fu et al., 123 C.E.).

Validation of the gene of interest accuracy can be accomplished through the implementation of quality control measures, utilizing sequencing and analytical techniques.

An important consideration about cloning is the limited carrying capacity of pDNA vectors. The insertion of additional genetic material may lead to increased size, and it may affect the efficiency of cloning, or lead to unstable constructs. Most general plasmids (i.e. pUC or pBluescript, and pGEM-T) can cope with inserts up to 15 kb. If a large DNA should be inserted, there are special types of vectors suitable for such applications. However, large plasmids may be challenging to manipulate and propagate, and they may have lower transformation efficiencies.

3.3 The main components of pDNA vector backbone

pDNA cloning vectors usually have a backbone that is well-characterized, stable, and suitable for large-scale manufacturing. Besides gene(s) of interest pDNA cloning vectors have an origin of replication that allows for semi-independent or autonomous replication of the plasmid in the host, promotor sequence, multiple cloning sites (MCS), the sequence for a selectable marker, and Transcription stop site (but it is not necessary, in the context of mRNA production via IVT, when transcription could end by linearization, i.e. by cutting the plasmid immediately after the open reading frame).

The backbone of the pDNA vector contains origins of replication (ORI) which allow the vector to be replicated along with the host cell's genome. This ensures that the vector, along with any inserted DNA sequences, can be reproduced reliably with the compatible host organism's cellular machinery. The ORI and other regulatory elements determine the number of plasmid copies within a host cell. Striking the right balance in copy number is critical for achieving optimal gene expression without overburdening the host cell. In most cases, a high-copy plasmid vector is the best approach to produce the highest yields. For example, pBluescript has a copy number 300-500 while pUC based vectors can reach 700 copies. Those high copy numbers pDNA vectors can lead to increased expression but may also impose a metabolic burden on the host, gene and polyA instability. To address this, more stable large-size and low copy number vectors (value of 10-12 copy number, as is the case with pACYC) and linear (Grier et al., 2016) and bacteriophage vectors are being considered as alternative construction options.

The backbone of a vector contains multiple cloning sites (MCS), which enable precise insertion of DNA fragments via specific restriction enzyme recognition sequences. Traditional cloning methods comprise digesting PCR products and plasmids with restriction endonucleases, followed by ligation. The design of an optimized MCS with a variety of unique, compatible restriction sites is crucial to ensure flexibility in cloning given that certain restriction sites can be found in the gene of interest. Combining restriction enzyme digestion with high separation power allows determination of the gene insert, regulatory regions, nuclease sites, or encoded poly-A tails of pDNA with confidence.

Achieving efficient and specific digestion of both the vector and insert DNA with restriction enzymes requires optimization. Incomplete digestion or "star" activity can complicate the process. Therefore, it is necessary to confirm the use of appropriate restriction enzymes for vector and fragment digestion and to check the compatibility of the desired cloning sites with the chosen restriction enzymes. This is not so problematic nowadays, as most modern vectors include an artificial stretch of DNA with many different restriction endonuclease cleavage sites. It is also important to ensure proper heat inactivation of the enzymes before ligation. Another step, ensuring efficient ligation of the insert into the vector without unwanted rearrangements or self-ligation can be challenging. It is important to ensure that the molar ratio of vector to insert is appropriate, use of an efficient ligase buffer and enzyme, and optimal reaction conditions, including proper incubation times and temperatures (Matsumura, 2015).

The backbone often includes regulatory elements, such as promoters, enhancers, and terminators, that respond to specific signals or conditions that control the transcription and translation of inserted gene sequences. RNA polymerases specifically bind to

promoters and form transcription initiation complexes, which initiate downstream gene transcription. Promoters act as "switches", determining the activity of genes and controlling the initiation and extent of gene expression. The duration of transcription is dependent on the strength of the interaction with the promoter and the affinity of the DNA site (Cozzolino et al., 2021; Jensen & Galburt, 2021; Klein et al., 2021). The best outcomes are usually achieved through potent promoters that can generate high rates of transcription. Promoters like T7, T3, and SP6 are commonly used. Also, it is necessary to conduct expression studies over a range of promoter strengths to determine the optimal conditions for production and to ensure proper transcription of RNA (Cazier & Blazeck, 2021; Gong et al., 2020; Rad et al., 2020).

Another crucial step in mRNA processing is polyadenylation that enhances stability and translation efficiency of mRNA molecules. The process of polyadenylation involves the addition of approximately 200 adenine residues to the 3' end of mRNA molecules. The poly-A tail is essential for the regulation of gene expression and the efficient functioning of mRNA molecules in the cell. The poly-A tail protects the mRNA molecule from degradation by exonucleases, and facilitates the export of mRNA from the nucleus to the cytoplasm. There are two primary ways to add a poly-A sequence to a DNA molecule (1) including a polyadenylation signal sequence downstream of the gene of interest in the expression vector, the mRNA produced will be polyadenylated; (2) a poly-A tail can be added to DNA molecules *in vitro* using enzymatic methods- common approach is to use poly-A polymerase, which catalyzes the addition of adenosine residues to the 3' end of a DNA molecule. Both methods have their advantages and limitations, and the choice between them depends on factors such as experimental requirements, compatibility with cloning strategies, and the specific application of the DNA molecule. However, sometimes deletion of the polyadenylation sequence may occur. This can be due to formation of 3D structures in repetitive DNA sequence; DNA mutations in the polyadenylation signal sequence or the gene encoding poly-A polymerase); viral interference (viral proteins may target components of the polyadenylation machinery, leading to abnormal poly-A tail synthesis or degradation) or cellular stress (heat shock, oxidative stress, or nutrient deprivation) (Matsumura, 2015).

A selectable marker is often part of plasmid backbone, and represents an effective screening strategy to identify and select cells that have successfully taken up the vector, depends on which host cell type is used. pDNA vectors typically come equipped with selectable markers like antibiotic resistance genes or utilize other markers, such as color or fluorescence, depending on the cloning system. Common antibiotic resistance genes include ampicillin, kanamycin, tetracycline, or chloramphenicol resistance.

Additionally, reporter genes may be included in pDNA sequence. These elements are critical for confirming the presence of the desired insert in the recombinant plasmid and checking for the absence of undesired mutations or rearrangements requires thorough screening and verification (Cazier & Blazeck, 2021; Jensen & Galburt, 2021).

Implement quality control, throughout vector construction, including restriction enzyme digestion, measures verify the accuracy of the cloned insert and the integrity of the plasmid vector, but sequencing and analytical techniques must be employed for final verification. In addition to the vast array of vectors available for commercial use, automated synthesis allows for the construction of oligonucleotides and genes (gene synthesis). Researchers can then use automated analytical techniques to analyze the products obtained. However, it is essential to ensure the full accuracy of the antigen's sequence, especially when it contains numerous epitopes, as in the case of the mRNA vaccine against tuberculosis.

3.5 Scalability and Regulatory Compliance

The success in the commercial, clinical, and regulatory aspects of mRNA vaccines has led to an increased demand for up-scaled production of efficacious and stable GMP-grade mRNA vaccines. Ensuring consistent vaccine quality relies on reproducibility, necessitating plasmid scalability without compromising its quality and consistency. Scalability enables a consistent supply of vaccines and meets the rising demand for effective and safe vaccines. A scalable vaccine should be able to adapt to increasing demands in a short period, have a low dose requirement, and low costs, and not have complex or difficult manufacturing steps that hinder scalability. Herein, plasmid design must include facilitating efficient and scalable production and purification processes.

The regulatory considerations surrounding mRNA vaccines play a pivotal role in their development and production. These guidelines are designed to ensure that vaccines meet strict standards of safety, efficacy, and quality before they can be authorized for use by the general public. They encompass regulatory mandates, thorough documentation of the entire process, including vector design and construction, as well as validation procedures to ensure compliance with regulatory standards. It's imperative to underscore the importance of maintaining the stability of plasmids within host cells for their sustained presence and replication over time. The vector system must meet regulatory

guidelines for vaccine manufacturing, be scalable, and thoroughly documented to facilitate large-scale production. This entails adherence to Good Manufacturing Practices (GMP) and fulfilling regulatory criteria for safety and efficacy.

4. Conclusion

Two essential characteristics of plasmid DNA cloning vector are their modularity and adaptability. The need to apply plasmid DNA cloning vector to a wider variety of organisms is developing as a result of the increased recognition of the diverse roles that microbes play in industry and health. A solution is provided by broad-host-range vectors and shuttle vectors. In the context of modularity, biological components must be well characterized and standardized, both inside and outside of already recognized biological systems.

While a universal vector may remain elusive, the future likely involves establishing design principles across multiple model organisms and applying them to new targets using increasingly affordable DNA synthesis technologies.

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