Review

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Haobo Kang , Zilu Ma , Yubo Sun , Fanzhuo Kong , Kun Yang * , Dongbo Jiang *

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DNA Vaccines: The Future of Immunization

Haobo Kang [1,†], Zilu Ma [1,†], Yubo Sun [1], Fanzhuo Kong [1], Kun Yang [1,2,3,*] and Dongbo Jiang [1,2,*]

[1] Department of Immunology, Basic Medicine School, Air Force Medical University (The Fourth Military Medical University), Xi'an710032, China; 2175325084@qq.com(H.K.); 13930296799@163.com(Z.M.); sunyubo000103@163.com(Y.B.); 1364078740@qq.com (F.K.).
[2] The Key Laboratory of Biohazard Damage and Prevention Medicine, Basic Medicine School, Air Force Medical University (The Fourth Military Medical University), Xi'an 710032, China
[4] Fourth Military Medical University, No. 169, Changle W. Rd., Xi'an, 710032, China; 1364078740@qq.com
[3] Department of Rheumatology, Tangdu Hospital, Air Force Medical University (The Fourth Military Medical University), Xi'an 710038, China
* Correspondence: superjames1991@foxmail.com (D.J.); yangkunkun@fmmu.edu.cn (K.Y.)
† These authors contributed equally to this work.

Abstract: This article mainly discusses the prospects of DNA vaccines as a future form of immunization. DNA vaccines, also known as nucleic acid vaccines or gene vaccines, inject genes encoding specific protein antigens directly into animals. By utilizing recombinant eukaryotic expression vectors, they activate the immune system to produce specific humoral and cellular immune responses, providing comprehensive protection against specific pathogens. Compared with traditional vaccines, DNA vaccines have shown new promise in addressing many viral infections. Although no DNA vaccines have been approved for use in humans at present, research on DNA vaccines for specific human diseases, such as prostate cancer, is ongoing. Additionally, the delivery methods of DNA vaccines, including oral vaccines and particle-mediated epidermal vaccination, as well as the challenges and directions for improvement in their clinical application, are explored. Finally, despite these challenges, the research and application prospects of DNA vaccines are broad.

Keywords: DNA vaccine; immunization; immunogenicity; delivery; customization

1. Introduction

DNA vaccines have demonstrated remarkable efficacy in diminishing disease mortality and infection rates. Recognized as a significant advancement in the vaccine domain, they have shone as a beacon in public health since the 1990s, owing to their distinctive benefits and impressive outcomes. As the third generation of vaccines, which include live attenuated and genetically modified vaccines, the evolution of DNA vaccines has been marked by numerous challenges and innovative strides. In 1990, pioneering work by the Wolff group experimentally showed that direct injection of plasmid DNA into muscle tissue could lead to the successful expression of protective protein antigens in eukaryotic cells. This pivotal discovery revealed the potential of DNA vaccines and established robust groundwork for subsequent extensive research. As investigations have progressed, the unique advantages of DNA vaccines have become apparent, particularly their ability to elicit long-lasting, specific humoral and cellular immune responses within the body. DNA vaccines outperform traditional vaccines by triggering immune responses more directly and efficiently, resulting in rapid, robust immune protection. Furthermore, they can stimulate cytotoxic T lymphocytes (CTLs), which are crucial for combating viruses, intracellular bacteria, and parasites. Owing to these distinctive attributes, DNA vaccines hold vast potential for application in public health. They are poised to be formidable defenses against both known infectious diseases and emerging or unknown pathogens. Consequently, there is good reason to anticipate that DNA vaccines will assume an increasingly vital role in global public health in the foreseeable future.

2. Briefs to DNA Vaccines

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The introduction of genes encoding specific protein antigens into animals via recombinant eukaryotic expression vectors enables the active expression of these foreign genes. This, in turn, leads to the production of the corresponding antigens and stimulates the body's immune system. This process can elicit specific humoral and cellular immune responses, offering comprehensive protection against particular pathogens. In numerous instances where traditional vaccines, including inactivated and attenuated vaccines, have failed to effectively combat various viral infections in livestock and poultry, DNA vaccines have emerged as a beacon of hope. By utilizing plasmid DNA that encodes antigens from diverse sources, such as viruses, bacteria, and parasites, this type of vaccine can provoke robust and enduring immune responses in vertebrates, including mammals, birds, and fish. This capability underscores the significant potential and application value of DNA vaccines in the prevention and treatment of viral infections in livestock and poultry.

The lineage of DNA vaccines dates back to the 1960s, when it was observed that naked DNA could be transfected into mammalian cells. This finding opened new avenues for subsequent vaccine research. In 1992, scientists discovered that this transfection process could induce antigen-specific antibody responses, offering substantial evidence and support for the evolution of this technology into a novel vaccine platform.

The rapid advancement of this technology has garnered considerable attention and anticipation within the vaccine community. It has demonstrated not only high immunogenicity but also efficacy in providing protection against a range of diseases across multiple animal models. This technology is regarded as a major breakthrough in the field of vaccine science.

In particular, its ability to induce a T-cell response in inactivated vaccines has caused a sensation in the vaccine community[1]. However, after extensive research, scientists began to realize that while DNA vaccines are highly effective in small animal models, their effectiveness in recent clinical trials has been disappointing. Therefore, current research has shifted to understanding the different performances of DNA vaccines in mouse and large animal models and how to transfer the success of DNA vaccines in small animals to larger animals and humans[2]. Owing to their stability, low cost, and high customizability, DNA vaccines are currently under development or have been successfully developed for various diseases, such as toxoplasmosis[3], cancers[4–6], Flavivirus infection[7], and visceral leishmaniasis[8]. Even individually customized DNA vaccines have achieved molecular mechanisms[9].

3. Advantages and Applications of DNA Vaccines

3.1. DNA Vaccines Are Cost-Effective and Easy to Store

This vaccine is produced through a simple process, resulting in low production costs and making it highly suitable for mass production. At ambient temperatures, they remain stable and do not lose their activity due to temperature fluctuations, greatly simplifying the difficulty of storage and transportation. Furthermore, the production speed of this vaccine is very fast, enabling it to quickly meet market demand. Owing to the flexibility of the production process, we can produce various vaccines for infectious diseases to respond to constantly changing epidemic situations. For the carrier of vaccination, plasmids are the most common choice. The simple structure of a plasmid makes its purification process very straightforward, further reducing production costs. Additionally, plasmid DNA has good biocompatibility, making its application in the human body safer. Owing to its cost-effective production and long shelf life, plasmid DNA has become the preferred carrier for vaccine production. In summary, this vaccine is an important weapon in our response to infectious disease outbreaks, as it is easy to manufacture, low in cost, stable, fast in production, and highly flexible. We can continue to explore the advantages of this vaccine and its application in the field of public health. First, the low cost of this vaccine allows for its widespread use in resource-limited areas. Many developing countries and regions cannot afford the high cost of vaccines for economic reasons. The emergence of this low-cost vaccine has brought good news to these people, enabling them to enjoy the health protection provided by vaccines. Second, the fast production speed and high flexibility of this vaccine enable it to respond quickly to changes in the epidemic situation. When a new infectious disease outbreak occurs, we can quickly adjust the production process and produce a

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vaccine specific to this outbreak, providing strong support for epidemic prevention and control. This rapid response ability is crucial for controlling the spread of the epidemic. Furthermore, as a carrier for vaccination, plasmid DNA offers many conveniences because of its good biocompatibility and long shelf life. This means that vaccines can maintain their activity for a long time during storage and transportation, reducing waste caused by expiration. Moreover, owing to the good biocompatibility of plasmid DNA, the immune response triggered by vaccines in the human body is more moderate, reducing the occurrence of adverse reactions. Finally, this vaccine has broad application prospects in the field of public health. In addition to responding to known infectious diseases, we can use this technology platform to develop new vaccines to respond to possible future outbreaks. With the continuous advancement of biotechnology and improvements in vaccine production processes, we can expect this vaccine to play a greater role in the future and make greater contributions to human health.

With the development of modern biotechnology, remarkable progress and applications have been made in many fields, such as drug treatment, preventive or therapeutic vaccination, and regenerative medicine induction by recombinant DNA technology. This progress undoubtedly drives the demand for high-purity DNA, making it increasingly important in the field of biopharmaceuticals. In this context, how to obtain high-purity DNA effectively, especially plasmid DNA in the form of supercoiled covalent closed circles (CCCs), has become an important research topic. Traditional plasmid purification methods often require a series of complex processes to separate DNA from RNA and other contaminating organic components. While these methods can meet the needs of experiments and industrial production to a certain extent, they often suffer from low efficiency, high cost, and insufficient purity in practical operation. Therefore, finding a simple, efficient, and economical plasmid purification method is highly important for meeting the increasing demand for DNA. In recent years, the emergence of capillary gel electrophoresis (CGE) as an innovative technology has provided a sensitive and effective means for quality control of clinical-grade plasmid DNA. As an efficient separation and analysis technique, capillary gel electrophoresis can achieve high-resolution separation of DNA in a short time, thereby ensuring the purity and quality of plasmid DNA. Additionally, capillary gel electrophoresis has the advantages of simple operation, high sensitivity, and good resolution, indicating that it has a wide range of application prospects in the fields of plasmid DNA purification and quality control. In conclusion, with the widespread use of recombinant DNA technology in the field of biopharmaceuticals, the demand for highly purified DNA continues to increase. Although traditional plasmid purification methods can meet certain needs to a certain extent, they still have many shortcomings. As an innovative technology, capillary gel electrophoresis provides new ideas and methods for the purification and quality control of plasmid DNA and is expected to play a greater role in the field of biopharmaceuticals in the future[10,11]. The administration of a plasmid DNA vaccine based on the pathogen of tuberculosis is widely recognized as an extremely efficient and simple strategy that plays a crucial role in activating the immune system of mice and effectively triggering a strong humoral immune response. This strategy promotes the production of a series of Th1 cytokines by CD4+ T cells, thereby activating other immune system components. Moreover, it also significantly enhances the activity of CD8+ T-cell-mediated cytotoxic T lymphocytes (CTLs), which are crucial for resisting the invasion of tuberculosis. Numerous scientific studies have confirmed the effectiveness of plasmid DNA in activating Th1- and CD8+ T-cell-mediated immune responses, which can be further enhanced by the subsequent use of recombinant proteins or recombinant poxviruses to improve the immune response. In particular, in the field of tuberculosis prevention and treatment, the BCG vaccine prepared from *Mycobacterium bovis* is used for initial immunization combined with subsequent reinforcement immunization programs, showing great application potential. Therefore, more in-depth exploration of this topic is needed in the future so that this method can be successfully applied in clinical practice and provide a new and powerful means for the prevention and treatment of tuberculosis[12]. In addition, owing to the stable double helix structure of DNA molecules, they can be prepared into lyophilized vaccines, which can restore activity in a salt solution when used[13].

3.2. High Safety of DNA Vaccines

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A DNA vaccine is an innovative immunization method that can effectively stimulate both the humoral and the cellular branches of the human immune system. Humoral immunity primarily neutralizes foreign pathogens through the secretion of antibodies, whereas cellular immunity directly attacks and destroys infected cells by activating specific immune cells. Together, these two branches provide double insurance for the human immune system to fight diseases, which is crucial for preventing a variety of infectious diseases, including AIDS. Moreover, the stimulation of this dual-arm immune response provides possibilities for vaccine application in therapeutic scenarios. Traditional attenuated pathogen vaccines are prepared by weakening pathogen pathogenicity, effectively triggering both cellular and humoral immune responses. However, there is a risk that the attenuated pathogen may regain its pathogenic ability and return to its virulent state during storage, transportation, and use, necessitating strict regulatory and control measures. In contrast, DNA vaccines use artificially synthesized DNA fragments that encode specific proteins of the pathogen, inducing the immune system to produce immune responses to these proteins. Since DNA vaccines do not contain live bacteria or viruses, they do not have the risk of returning to a virulent state from an attenuated state, providing significant safety advantages and facilitating vaccine storage and transportation. Furthermore, DNA vaccines can be tailored to prevent or treat multiple diseases by adjusting the types of encoded proteins, broadening their application scope. Additionally, we can delve into other advantages and potential applications of DNA vaccines. First, DNA vaccines are highly customizable. As they are based on DNA sequences, they can be easily modified through genetic editing techniques to match different pathogens or variants. This flexibility enables DNA vaccines to respond quickly to emerging outbreaks or pathogen mutations, providing strong support for disease prevention and control. Second, DNA vaccines have long-term immune effects. Owing to their ability to continuously express antigens in the body, they can continuously stimulate the immune system to produce long-term immune memory. This means that once an individual is vaccinated with a DNA vaccine, they will maintain immunity to specific pathogens for a long time, reducing the risk of reinfection. Moreover, DNA vaccines exhibit good tolerability and safety. As they do not contain live bacteria or viruses, they do not trigger severe immune reactions or side effects. Additionally, the production process of DNA vaccines is relatively simple and cost-effective, making them more widely accessible. In terms of potential applications, DNA vaccines can be used not only for preventive measures against infectious diseases but also for treating other diseases. By encoding specific cytokines or antibodies, DNA vaccines can be used to treat cancers, autoimmune diseases, etc.etc. Furthermore, DNA vaccines can be developed as multivalent vaccines that can prevent multiple diseases simultaneously, greatly increasing vaccine efficiency and coverage. In conclusion, as an emerging immunization method, DNA vaccines have high customizability, long-term immune effects, good tolerability and safety, and a broad range of application prospects. With continuous technological development and improvement, DNA vaccines are expected to play an increasingly significant role in disease prevention and treatment in the future[14]. For example, in the case of a multivalent DNA vaccine for HIV, Ian Frank and other researchers in the US used the HVTN 124 method (a randomized, phase 1, placebo-controlled, double-blind study involving HIV-seronegative subjects with low infection risk aged 18--50 years) in 2024. The results revealed that most of the reactions were mild but still need to be evaluated with large-scale data[15]. Another example is the safety of an Andes virus DNA vaccine for needle-free injection. Grant C Paulsen and other researchers used a phase 1, double-blind, dose-escalation trial to randomly assign 48 healthy adults to a placebo group or an ANDV DNA vaccine group and administered needle-free jet injection. Cohorts 1 and 2 received 2 mg of DNA or placebo in a 3-dose (day 1, day 29, day 169) or 4-dose (day 1, day 29, day 57, day 169) regimen, respectively. Cohorts 3 and 4 received 4 mg of DNA or placebo in a 3-dose and 4-dose regimen, respectively. The safety and neutralizing antibodies of the subjects were monitored through a pseudovirus neutralization assay (PsVNA50) and a plaque reduction neutralization assay (PRNT50). The results showed that the safety of DNA vaccines can be effectively guaranteed[16]. Experiments such as these have demonstrated that DNA vaccines are relatively safe.

4. Application of DNA Vaccines on the Basis of the above Advantages and Characteristics

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Many disease vaccines are customized on the basis of the above advantages. For example, Table 1.

Table 1. The diseases, time and related scientists for which basic research and development work are fully carried out.

Name of disease	Time	Relevant scientist
Alzheimer's disease[17]	2008	Yoshio Okura , Yoh Matsumoto
Kidney Disease[18]	2009	Debbie Watson , Guoping Zheng, Huiling Wu, Yuan Min Wang
HIV[19]	2021	Paul Munson
Dengue[20]	2015	Kevin R Porter , Kanakatte Raviprakash
Decayed Tooth[21]	2020	M Patel

5. Limitations and Potential Solutions of DNA Vaccines

Although many data have been accumulated from various clinical trials thus far, which consistently indicates that DNA vaccines are well tolerated in humans and show excellent safety, the immunogenicity of DNA vaccines in humans has not been fully demonstrated in the early design stage. Nevertheless, scientists have not stopped researching DNA vaccines. They are striving to improve vaccine design in the hope that they can demonstrate sufficient immunogenicity in future trials. Scientists are deeply aware that immunogenicity is the key for vaccines to successfully induce immune responses in the body and prevent diseases. Therefore, they have conducted in-depth analysis and discussion on the early design of DNA vaccines, seeking to identify any shortcomings and propose improvement plans. In the process of improvement, scientists first focused on the selection of vectors and antigen genes for DNA vaccines. They reported that different vectors and antigen genes may affect the expression and immune response of vaccines in the human body. Therefore, through many experiments and screenings, more suitable vectors and antigen genes to improve the immunogenicity of DNA vaccines have been identified. Additionally, scientists have optimized the production process and administration methods of DNA vaccines. They reported that different production processes and administration methods may affect vaccine stability and immune responses. Therefore, through repeated experimentation and adjustments, they finally determined the best production process and administration method to ensure that DNA vaccines can achieve the maximum effect in the human body. With further research, scientists' understanding of DNA vaccines is becoming more profound. They believe that with continuous efforts and improvements, DNA vaccines will become an important tool for disease prevention in the future. At the same time, they also call on the public to maintain confidence in vaccines and actively participate in vaccination to jointly contribute to building a healthy society[22]. When applied to human subjects, the lack of immunogenicity remains the greatest challenge in the practical use of DNA vaccines. To address this issue, many different strategies have been tested in preclinical models, including novel plasmid vectors that increase antigen expression and codon optimization, new gene transfection systems or electroporation that improve delivery efficiency, protein or live virus vector enhancement schemes to maximize immune stimulation, and the formulation of DNA vaccines using traditional or molecular adjuvants[23]. These measures aim to improve the immunogenicity of DNA vaccines, which is relatively weak owing to the need for cellular transfection to express antigen proteins, and currently, increased dosing or frequency is needed to improve success rates. In the future development of DNA vaccines, three promising approaches are expected to advance DNA vaccines: delivery technology, the addition of molecular adjuvants, and improvements to DNA vaccine vectors.

5.1. Low Immunogenicity and Improved Delivery Efficiency

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Thus, the greatest challenge faced by DNA vaccines in the process of research and application is their relatively low immunogenicity. The root cause of this issue lies in the difficulty of effectively delivering the plasmid used in DNA vaccines into host cells. Specifically, to successfully transport the plasmid of a DNA vaccine to the nucleus, a series of complex biological barriers need to be overcome. First, the vaccine plasmid must rely on endocytosis or pinocytosis, two ways for cells to take in foreign substances, to pass through the phospholipid bilayer structure of the cell membrane. This is a challenging process because the plasmid needs to avoid being recognized and degraded by two types of organelles called endosomes and lysosomes. These organelles are similar to "garbage processing plants" inside cells and can decompose various foreign substances. Therefore, to successfully enter the cell nucleus, the plasmid must survive under the "inspection" of these two types of organelles. Additionally, after the plasmid passes through the cell membrane and enters the cytoplasm, it still faces the threat of nucleases in the cytoplasm. Nucleases are enzymes that specifically decompose nucleic acids, and a plasmid must possess a certain degree of antienzyme ability to survive in the cytoplasm. Finally, the plasmid also needs to cross the nuclear membrane as the final barrier to enter the cell nucleus. The nuclear membrane is an outer wrapping of the cell nucleus that strictly controls the entry and exit of substances. To enter the cell nucleus, the plasmid must find a way to cross the nuclear membrane smoothly. To improve the transfection efficiency of DNA vaccines, researchers have tested various methods. Compared with physical delivery systems, chemical delivery methods use biopharmaceuticals to increase the transfection efficiency of DNA vaccines. This approach has achieved good results to some extent but still faces many challenges. Although chemical delivery methods can improve the transfection efficiency of plasmids, their safety and stability still need further research[23]. In 2010, Mohan Karkada et al. tested a liposome-based vaccine platform, VacciMax (VM), and its modified anhydrous version, DepoVax (DPX), to improve the ability of plasmid DNA (pDNA), mRNA, and siRNA to be delivered in vivo. Subcutaneous injection of pDNA for IL12 and pDNA for green fluorescent protein (GFP) in VM/DPX significantly increased their expression in vivo. Enhanced IL12 secretion and GFP expression were limited to CD11b(+) and CD11c(+) antigen-presenting cells but not B cells. Additionally, significant inhibition of plasmid/antigen-induced IL12 secretion was observed after injecting IL12-siRNA into VM. These results suggest that VM and DPX are promising tools for delivering nucleic acid vaccines in vivo, and further studies are warranted to explore their role in inducing effective immune responses[24]. In 2012, Rebecca J. Grant-Klein et al. evaluated the immunogenicity and protective efficacy of DNA vaccines expressing codon-optimized envelope glycoprotein genes from Zaire Ebola virus, Sudan Ebola virus, and Marburg Marburg virus (Musoke and Ravn). The vaccine was delivered intramuscularly or intradermally via a TriGrid™ electroporation device in BALB/c mice. Mice receiving DNA vaccines targeting individual viruses produced robust glycoprotein-specific antibody titers measured by ELISA and survived lethal viral challenges without showing clinical symptoms of infection. Survival curve analysis revealed a statistically significant increase in the survival rate compared with that of the control group for the Ebola virus and Ravn virus challenges. These results indicate that multidrug filovirus DNA vaccines delivered by intramuscular electroporation can completely protect mice from Ebola virus and Marburg virus attacks.

However, the application of this model in clinical practice is limited. Further research is needed to explore the most effective and safe methods for delivering DNA vaccines and improving their immunogenicity[25].

Owing to the inefficient degradation of nucleases to DNA and delivery to immune cells, the immunogenicity of DNA vaccines delivered in the form of naked plasmid DNA is usually weak. Therefore, biomaterial-based delivery systems based on particles and nanoparticles encapsulating plasmid DNA represent the most promising DNA vaccine delivery strategy. Microparticle delivery systems allow the passive targeting of antigen-presenting cells through size exclusion and the continuous presentation of DNA to cells through the degradation and release of encapsulated vaccines. In contrast, nanoparticle encapsulation can increase internalization, improve overall transfection efficiency, and increase uptake on mucosal surfaces. Additionally, selecting appropriate biomaterials can increase immune stimulation and activation by triggering innate immune response

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receptors and targeting DNA to professional antigen-presenting cells. Finally, selecting materials with appropriate properties and achieving efficient delivery through patient-friendly administration routes that can generate systemic and local (i.e., mucosal) immune responses can lead to more effective humoral and cellular protective immune responses[26]. Therefore, future DNA delivery techniques are likely to be based on the development of novel material-based delivery systems to improve the efficiency of DNA vaccines. Polymer materials have been extensively explored in the field of nanomedicine; among them, polylactic-co-glycolic acid (PLGA) plays a significant role in micro- and nanotechnology due to its biocompatibility and controllable biodegradability. The combination of PLGA with different inorganic nano-materials to form nano-composites overcomes the limitations of polymers and expands their application fields[27]. The use of PLGA materials to encapsulate DNA vaccines can increase systemic antigen-specific antibody responses[28].

5.2. DNA Vaccine Administration Routes

To maximize transfection efficiency, researchers have attempted various approaches for vaccination, such as intravenous injection[29], which allows drugs to enter the bloodstream directly, thereby improving the distribution and efficiency of drug action. At the same time, they have also explored oral administration methods[30], which are easy to perform, are well accepted by patients, and can avoid the first-pass effect of the liver. Additionally, the lung administration method[31] can quickly deliver drugs to the whole body via the rich capillary network in the lungs. Another method involves local application through the skin[32], which can be used to treat specific lesion areas and reduce damage to other normal tissues. Furthermore, there is also intramuscular injection, which allows drugs to be slowly released in muscle tissue, prolonging the duration of drug action. These explorations and applications of vaccination routes aim to improve the transfection efficiency and provide more possibilities for clinical treatment[33].

Oral administration: Traditional modes of drug delivery, such as parenteral administration, if not feasible, oral vaccination would be a good alternative. Compared with traditional vaccine administration routes, oral vaccination can reduce the degree of pain associated with vaccination and the risk of wound infection/inflammation. Additionally, owing to the ability to trigger a strong and complete immune response, the cost of oral vaccination is significantly reduced on the basis of a reasonably designed oral vaccine system[34]. There is a theoretical basis for the use of oral vaccines, but the pH and temperature of the gastrointestinal tract are not suitable conditions, which means that they may be degraded by various enzymes and thus lose the immunological properties of the vaccine. This limits antigen presentation through the mucosal barrier and necessitates competition with endogenous or exogenous microorganisms[35,36]. Therefore, only a few products are available for oral vaccination to prevent certain infectious diseases. Most licensed oral vaccines or vaccines in production are based on attenuated viruses or bacteria that can survive in the gastrointestinal tract, which is not a suitable environment, and continue to produce antigens to enhance immune responses and achieve the final effect of the vaccine. Currently, the vaccines under development include rotavirus[37], DPT[38], and cholera[39]. However, oral administration is hindered by the difficulty of transferring vaccines to the body. Synthetic materials can improve the efficiency of oral vaccination, so selecting and developing suitable nanoparticles and particles with adjuvant properties are important for oral administration.

5.3. Delivery of the DNA Vaccine

The main drawback of DNA vaccines administered through needles and syringes is their poor immunogenicity, which is related to the low efficiency of the cellular uptake of DNA. Many research teams have focused on the most basic vaccination methods, exploring the effectiveness of physical vaccination methods such as electroporation, particle-mediated epidermal delivery, NFIS, and lipid particles[40].

Table 2. Common delivery methods, advantages, disadvantages, and prospects.

	Core	Advantages	Cost

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		Transfection rates	Safety	Immune response	Adverse Reactions	
Particle-mediated epidermal delivery (PMED)	Gold particles	H	NE	NE	NE	H
Electroporation (EP)	Electric pulse	H	NE	H	YES	NE
Needle-free Injection System(NFIS)	High-pressure jet flow	NE	H	H	YES	H
Lipid nanoparticles (LNP)	Cationic lipids	L	H	L	YES	NE
Self-assembling peptides	Self-assembling peptides nanostructures.	H	L	NE	YES	NE
Gene gun	Metal particles	H	NE	NE	NE	H

NE, not evaluated; H, high; L, low.

5.3.1. Particle-Mediated Epidermal Inoculation (PMED)

Additionally, "gene gun" or "particle bombardment" uses a specialized ballistic device to generate a cold gas shock wave through compressed gas dynamics and bombards the gold particles carrying DNA into the cells. The immune response induced by PMED is mainly Th2, with a high level of humoral immunity. Owing to the limited amount of DNA carried by gold particles and the small single-dose inoculation, multiple inoculations are needed. This technology has problems such as difficult storage, unstable DNA properties, and uncontrollable inoculation processes, which are not currently used for human DNA vaccination. However, it is being re-evaluated in preclinical studies[41,42].

5.3.2. Electroporation Technology (EP)

The use of electroporation technology involves injecting a DNA vaccine, followed by the application of a current with a needle near the site of vaccination to generate electrical pulses. These pulses open temporary channels in cells, increasing cell membrane permeability and enabling the plasmid to efficiently enter the cytoplasm and transfer into the nucleus. This process can increase the transfection rate of the plasmid 100–2000-fold. The electric pulses induce local cell death and tissue damage, release damage-associated molecular patterns, enhance immune responses, produce inflammatory cytokines, and promote the migration of APCs and T cells. This can amplify immune responses by 10--1000-fold. The most common side effects of EP are pain and bleeding at the injection site. Other drawbacks include the need for access to power, expensive and bulky equipment, difficulty in use for obese patients, the need for individualized electroporation schemes, and potential harmful effects after tissue damage. Recently, EP has been included in clinical trials to promote DNA vaccines for COVID-19 [43] and various cancers[44 – 46].

5.3.3. Needle-Free Injection System (NFIS)

NFIS originated in the 1930s for the prevention and treatment of infectious diseases. It uses high-pressure jet streams to quickly puncture the skin and achieve needle-free injection of vaccines. Early NFIS was limited by productivity and often used reusable syringes, leading to cross-contamination. After the 1990s, it was basically updated to disposable syringes. The new generation of NFIS is more

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portable, easy to operate, and can perform thousands of injections quickly. Studies have shown that NFIS may increase immunogenicity, reduce vaccine doses, and is suitable for responding to emergency public health needs. Additionally, NFIS reduces the use of needles, lowers costs, accommodates patients with trypanophobia, and eliminates the risk of needle stick injuries. Healthcare workers and patients prefer NFIS, which is cost effective in large-scale vaccination programs. NFIS has been used for a variety of preventive DNA vaccines. However, it also has drawbacks such as a lack of understanding by healthcare workers, cost differences, differences in injection penetration rates, the need to preset injection volumes, and unsuitability for intravenous injection. To address these shortcomings of NFIS, scientists and public health experts are actively seeking solutions and optimization strategies.

First, to improve healthcare workers' understanding and ability to use NFIS, extensive training and educational activities can be carried out. Online courses, workshops, and onsite guidance can ensure that healthcare workers are familiar with the working principles, operating procedures, and precautions of NFIS, thereby improving their acceptance and efficiency of use. Second, to address cost issues, ways to reduce costs and improve production efficiency can be explored. For example, through technological innovation and mass production to reduce production costs, governments and international organizations can provide financial support and policy incentives to encourage more countries and regions to adopt NFIS, thereby expanding market demand and the production scale to further reduce unit prices. Additionally, to address issues such as differences in injection penetration rates and injection site pain, researchers can further optimize the design of NFISs. For example, a more precise injection control system that can automatically adjust the injection volume and depth on the basis of the type of vaccine and the characteristics of the target population can be developed; at the same time, more advanced materials and coating technologies can be used to reduce the sense of pain and discomfort during injection. Finally, although NFIS is not suitable for intravenous injection and prefilled forms, this does not affect its widespread use in other vaccination scenarios. Instead, this prompted us to continue exploring and expanding the application scope of NFIS, such as its use in auxiliary vaccination for oral vaccines and rapid immunization in emergency situations.

5.3.4. Nucleic Acid Delivery Lipid Nanoparticles (LNPs)

It mainly utilizes advanced nanotechnology to encapsulate negatively charged deoxyribonucleic acid (DNA) through carefully designed cationic lipids, forming stable and efficient complexes. These complexes, as carriers, can accurately deliver drugs into cells. Traditional drug delivery methods often rely on viral vectors. However, the clinical application of viral vectors is greatly limited because of inherent safety concerns, strong immunogenicity reactions, and limited genetic capacity. In contrast, LNP (lipid nanoparticle) technology, as an innovative nonviral vector, has numerous significant advantages. It not only is highly safe, significantly reducing the risk for patients undergoing treatment but also supports repeat dosing, providing convenience for long-term treatment. Moreover, its high genetic capacity allows it to carry more therapeutic genes or drug molecules, increasing treatment efficacy. Additionally, LNPs are easy to design and manufacture, allowing flexible adjustments on the basis of specific treatment needs. Currently, there is an LNP formulation that has been successfully validated in a large population, consisting of four carefully selected lipid components: efficient ionizable cationic lipids that can release positive charges under specific conditions and tightly bind with DNA; stable phospholipids that provide a protective layer for the complex, preventing interference from the external environment; and cholesterol or cholesterol derivatives and polyethylene glycol (PEG)-lipids, each of which play important roles in the stability and targeting of the formulation. The synergistic effect of these four lipid components enables LNP formulations to efficiently deliver drugs to target cells. Notably, although each component of the LNP formulation may affect the overall immunogenicity, the detailed mechanisms and interactions involved have not been fully revealed. In particular, the specific structural features of lipid molecules, such as the number and length of "tails" and the presence or absence of linear head groups and heterocyclic rings, may have profound effects on the immunogenicity of LNP formulations. Therefore, to further increase the safety and effectiveness of LNP delivery technology,

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we need to conduct in-depth research on the mechanisms triggered by each lipid component to better optimize the formulation of the next generation of LNP preparations and provide patients with safer and more effective treatment options[47,48].

5.3.5. Self-Assembling Peptides

Nonviral vectors are designed to efficiently deliver genetic material to cells, especially to the nucleus. The self-called self-assembled peptide refers to the self-assembly of the peptide under various non-covalent driving forces to form nano-fibers, nano-lamellar structures and micelles. The self-assembly design of some drugs or the use of self-assembled polypeptide materials as drug delivery carriers can solve the problems of a short half-life, poor water solubility and a low penetration rate of physiological barriers. The nanoscale drug transport system constructed from peptides via self-assembly can improve the targeted accumulation of drugs in vivo and slow release in tumors, effectively enhancing the inhibition of tumor growth. Self-assembled polypeptide drugs can also be used in tissue engineering and medical imaging. Considering the various factors affecting peptide self-assembly and the hydrophilic/hydrophobic force-driven design and electrostatic force-driven design, peptide drugs can be more rationally designed and constructed, allowing them to not only improve in vivo residence time and targeting but also improve drug delivery methods[49].

5.3.6. Gene Gun

This method is also called the particle bombardment cell method or micro bomb technology. A gene gun is a type of compressed gas (helium or nitrogen, etc.) that produces a cold gas shock wave into the bombardment chamber (to avoid "hot" gas shock wave cell damage), and the sticky DNA fine gold powder moves into cells through the cell wall, cell membrane, and cell cytoplasm structure to the nucleus, completing gene transfer. Only a small number of cells meet such a requirement, and most fail, but this small number of cells is sufficient to complete the gene transfer operation. Future work could explore the application of gene gun delivery in skin regenerative medicine, such as wound healing and scar aesthetics, as well as localized disease treatments, such as oncology and monogenic skin diseases. This convertible and effective biological approach should now be explored as an alternative to conventional nucleic acid delivery methods[50].

5.3.7. Others

In addition to the aforementioned common modes of administration, emerging and innovative methods of drug delivery have been developed.

Table 3. Other delivery methods and characteristics.

Name	High accurancy	high stability	safety	application
Biodegradable Microbots[51]	YES	YES	High	NE
Microneedle drug delivery[52,53]	NE	YES	Low	NE
Transdermal Drug Delivery by Ultrasound[54]	YES	YES	High	NE
Chitosan[55]	NE	YES	High	NE
Polyethyleneimine[56]	NE	YES	High	NE
Dendritic Polymers[57]	NE	YES	High	NE
Polylactic-co-glycolic acid[58]	NE	NE	High	Extensive

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Polyethylene Glycol[59]	YES	YES	High	NE
Polymethyl methacrylate[60]	NE	NE	NE	Extensive

NE, not evaluated.

6. Future Directions

DNA Nanotechnology: Controlling DNA nanostructures is one of the most important branches, where well-defined static nanostructures are constructed from reasonably designed DNA motifs. The diversity and complexity of these DNA nanostructures also endow them with a wide range of applications in nanofabrication, nano-electronics, biological diagnosis, and DNA computation[61]. For example, in 2022, Xu Yuwei, Lv Zhaoyue, Chi Yao, Yang Dayong, and other researchers, in view of the rapid development of DNA nanotechnology, adopted the method of reasonable design of rolling circle amplification (RCA) templates and the introduction of other functional components and concluded that RCA-based DNA materials have structural dynamic responsiveness and diverse biological functions. This allows for the future development of RCA-based DNA nanostructures to provide more theoretical foundations for the further development of precision medicine[62]. As an excellent programmable nanomaterial, DNA can now achieve many innovative functions with custom-designed, user-defined, and accurate DNA structures[63]. Among them, DNA origami is one of the most commonly used techniques[64]. Breakthroughs and research on these cutting-edge basic DNA nanotechnologies have promoted more precise synthesis of the DNA components of certain pathogenic microorganisms in vitro. This further accelerated the arrival of the "custom DNA vaccine era."

Molecular adjuvants: Molecular adjuvants effectively and moderately enhance the immunogenicity of DNA vaccines through a series of complex biological mechanisms. These mechanisms include activating the innate immune response of the body, promoting the formation of antigen libraries, increasing antigen uptake and the activity of antigen-presenting cells, and up-regulating the expression of co-stimulatory molecules. Among the various vaccine adjuvants, aluminum hydroxide particles are a common type. In a 2012 study, researchers, including Kamy Hosseinian Khosroshahi, Fatemeh Ghaffarifar, Zohre Sharifi, and Sushila D'Souza, demonstrated through a cocktail mixing method that aluminum hydroxide particles can moderately enhance the immunogenicity of DNA vaccines. The related research was published previously [65]. Another type of adjuvant is the MF59 emulsion, which was developed by Ott et al. in 2002. This novel cationic emulsion can adsorb plasmid DNA, thereby improving the intracellular delivery of plasmid DNA during the immunization process. Additionally, CpG oligonucleotides that activate TLR9 have been developed as adjuvants for DNA vaccines. In 2011, researchers such as Christian Bode and Gan Zhao reported that synthetic oligonucleotides (ODNs) containing unmethylated CpG motifs as vaccine adjuvants can improve the function of professional antigen-presenting cells and promote the generation of both humoral and cellular vaccine-specific immune responses. By maintaining close contact between ODNs and vaccines, these effects can be further optimized. Clinical trials have shown that CpG ODNs are safe and can enhance the immunogenicity of co-administered vaccines. Related research has been published[66]. In the future, with the continuous development of materials science, we believe that more types of molecular adjuvants will be developed to further improve the immunogenicity of DNA vaccines. The application of these new adjuvants will lead to greater improvements in the safety and effectiveness of DNA vaccines, thereby making greater contributions to human immunology. With the continuous progress of technology and in-depth research, the application of molecular adjuvants in the development of DNA vaccines will become more extensive and diverse. In the future, molecular adjuvants may have increased targeting ability, which can more accurately guide the immune system to produce immune responses to specific pathogens. This will greatly improve the effectiveness of vaccines, reduce unnecessary immune responses, and further ensure the safety of vaccine recipients. Additionally, with the integration of nanotechnology and biotechnology, the design and development of new molecular adjuvants will become more flexible

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and efficient. These new adjuvants may have multiple functional properties, simultaneously activating multiple immune pathways to generate more comprehensive and powerful immune responses. This will provide broader space and possibilities for vaccine research and development. Moreover, we need to pay attention to the safety evaluation of molecular adjuvants in vaccines. Although existing molecular adjuvants have undergone rigorous clinical trials and safety assessments, with the emergence of new adjuvants, we still need to conduct in-depth safety studies to ensure that they do not pose any potential health risks to vaccine recipients. Overall, as an important means to improve the immunogenicity of DNA vaccines, molecular adjuvants have broad application prospects in the field of vaccine research and development in the future. We look forward to the continuous progress of technology and research to develop more efficient and safe molecular adjuvants, making greater contributions to human health.

7. Conclusions

On the basis of the above discussion, although there are currently few approved DNA vaccines for human use, this does not mean that the use of DNA vaccines for human immunity is just a product of imagination. Research on DNA vaccines for specific human diseases is in full swing. For example, clinical trials using DNA vaccines to treat prostate cancer[67–69] have begun, and thus far, they have demonstrated safety and immunological effects. Additionally, multiple preclinical models have demonstrated the antitumor efficacy of DNA vaccines, and many efforts are underway to improve the immunogenicity and antitumor effects of these vaccines. For example, sipuleucel-T is used not only for tumor treatment but also for antiviral treatments, such as those against the Zika virus[70], Hantavirus[71], and human papillomavirus (HPV)[16], and for the treatment of AD[72]. As an emerging vaccine technology, its rapid and scalable production capacity, which is highly important for dealing with large-scale epidemic situations, has been demonstrated. Moreover, DNA vaccines have also shown strong potential in targeting a variety of different antigens, which means that they can help the human body fight multiple diseases. Furthermore, DNA vaccines are characterized by repeated reinforcement, which helps improve the human body's immunity to diseases and enables sustained and effective resistance to diseases after vaccination. In addition, the thermal stability of DNA vaccines in storage and transportation is also an important advantage. This feature allows DNA vaccines to maintain their potency and stability during long-term storage and transportation, which is highly important for the distribution and application of vaccines. Although we have encountered some challenges in the production and development of DNA vaccines, such as low transfection rates, these issues require further research and resolution. However, we are full of confidence in the future. We believe that in the near future, more effective DNA vaccines specific to certain diseases will be developed and approved for the market. These vaccines will become important weapons for humanity to fight against diseases, enriching the arsenal of human immunity and providing a solid guarantee for the development of global public health. Overall, the development prospects of DNA vaccines are broad, and they will make important contributions to human health. We look forward to the DNA vaccine playing a greater role in the future and providing more effective protection for humanity.

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