==========REVIEWS==========

DNA VACCINE TECHNOLOGIES: DESIGN AND DELIVERY

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Abstract. The COVID-19 pandemic has triggered the development of new directions in vaccine development, among which DNA- and mRNA-based technologies are particularly noteworthy. The platform based on DNA vaccines is developing particularly intensively due to their high stability at ambient temperature and the ability to activate both humoral and cellular immunity. The full cycle of DNA vaccine creation, which includes the construction of plasmid DNA, obtaining a producer strain, fermentation and purification, takes 2–4 weeks. In addition, the production technology of such vaccines does not require working with dangerous pathogens, which significantly simplifies the process of their production and reduces the overall cost. Over more than 30 years of rapid development, DNA vaccine technology continues to undergo changes. Currently, there is a licensed DNA vaccine for the prevention of COVID-19, and many candidate prophylactic vaccines against viral and bacterial diseases are in clinical trials. The review covers not only the principles of constructing plasmid DNA vaccines, but also new technologies for obtaining DNA constructs, such as minicircular DNA, MIDGE DNA and Doggybone™ DNA. New types of DNA vaccines are interesting because they consist only of the most essential elements for activating the immune response. Such constructs completely lack the sequences necessary for the production of plasmid DNA in bacterial cells – for example, the antibiotic resistance gene. One of the key problems in the development of a DNA vaccine is the method of its delivery to target cells. Currently, various delivery methods are used, both chemical and physical, which are rapidly developing and have already proven themselves to be reliable and effective. The characteristics of some of the most promising methods are also presented in the review.

Keywords: DNA vaccine, minicircular DNA, MIDGE DNA and Doggybone^{TM} DNA, delivery methods

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INTRODUCTION

Recently, research on the development of nucleic acid-based vaccines (mRNA and DNA vaccines) has been actively progressing. This type of vaccine has several advantages compared to classical ones: live, attenuated, and inactivated. Nucleic acid-based vaccines activate both arms of immunity - cellular and humoral. They can be administered multiple times since they do not induce anti-vector immune responses. The production technology of such vaccines does not require working with dangerous pathogens, which significantly facilitates their creation process and reduces its overall cost. And the ability to quickly and easily replace the target gene in mRNA or DNA vaccines without changing the production technology itself provides an opportunity to rapidly respond to the emergence of mutants or new pathogens.

A DNA vaccine is a genetic engineering construct that carries a gene for an immunogenic protein under the control of a eukaryotic promoter and, after introduction into the cell, ensures the synthesis of the target antigen, inducing a specific immune response. The first report about the possibility of target protein synthesis following intramuscular (i.m.) administration of DNA appeared in 1990. John Wolff et al. [1] demonstrated that i.m. injection of "naked" plasmid DNA with a foreign gene under the control of a cytomegalovirus (CMV) promoter in mice provided synthesis of the corresponding protein in muscle tissues. Shortly after, in 1992, Tang D. et al. [2] showed that i.m. injection of plasmids encoding human growth hormone and alpha-1-antitrypsin genes induces antibody production against these proteins, predominantly IgG and IgE. Ulmer J. et al. [3] found that DNA constructs can also induce cellular immune responses; particularly, the formation of cytotoxic lymphocytes was shown in response to i.m. administration of plasmid DNA encoding influenza virus nucleoprotein in animals.

The COVID-19 pandemic has become a trigger for the development of new directions in vaccine development, among which nucleic acid-based technologies, including DNA vaccines, have become leaders [4]. According to the World Health Organization (WHO), as of March 30, 2023, there were 17 DNA vaccines aimed at combating COVID-19 at different stages of clinical trials (https://www.who.int/publications/m/item/draft-landscape-of-covid-19-candidate-vaccines). The

ZyCoV-D vaccine for COVID-19 prevention, developed by the Indian company "Zydus Lifesciences Limited", became the world's first DNA vaccine approved for human use [5-8].

Currently, DNA vaccines are being developed not only for the prevention of infectious diseases but also for the treatment of oncological diseases [9, 10].

Interest in DNA vaccine technology has led to the development of new approaches to their design, delivery methods, immune response research techniques, as well as the need to regulate the quality and safety of clinical trials. In 2005, the WHO developed "Guidelines for Assuring the Quality and Preclinical Safety Evaluation of DNA Vaccines" (WHO. Guidelines for Assuring the Quality, Safety, and Efficacy of DNA Vaccines, 2007; https://cdn.who.int/media/docs/default-source/biologicals/vaccine-quality/guidelines-for-assuring-the-quality-and-non-clinical-safety-evaluation-of-dna-vaccines70ee1b3e-88a6-40af-8989-fbff8304a377.pdf?sfvrsn=521ee591_1&download=true), which was supplemented in 2020 based on new research [11, 12]. The amendments focus on aspects such as production control, requirements for preclinical and clinical trials, as well as providing information that may be required by national regulatory authorities for approval of clinical trials and licensing [13]. Currently, the Food and Drug Administration, FDA has approved four DNA vaccines for veterinary use [14]. These include the DNA vaccine against West Nile fever for horses RECOMBITEK® Equine West Nile Virus ("MERIAL Ltd.", USA); the vaccine against infectious hematopoietic necrosis Apex-IHN®, produced by "Novartis" (Switzerland), licensed for use in Salmonidae family fish; the commercial plasmid DNA LifeTideSW5®, carrying the somatoliberin sequence, used to increase the productivity of sows; and approved by the United States Department of Agriculture , USDA, the first therapeutic vaccine for the treatment of malignant tumors Oncept® ("Boehringer Ingelheim Animal Health USA Inc.", USA), designed for dogs with oral melanoma [15].

This review examines the currently known types of DNA vaccines, technologies for their construction, as well as delivery methods.

GENERAL PRINCIPLES OF DNA VACCINE DESIGN

Most commonly, plasmids are used as DNA vaccines, which are circular double-stranded DNA containing sequences necessary for its replication and expression of the target gene both in bacterial cells and *in vivo* in mammalian cells. A classic DNA vaccine contains the following structural

elements: a target gene under the control of a eukaryotic promoter, an origin of replication (ori), a polyadenylation signal, as well as recognition sites for restriction endonucleases for genetic engineering manipulations and an antibiotic resistance gene as a selective marker [4] (Fig. 1).

Fig. 1. Schematic representation of plasmid DNA vaccine.

DNA vaccines most frequently use the human CMV promoter, as it provides high levels of antigen synthesis in various cells of the organism [16, 17]. However, viral promoters are rapidly inactivated due to hypermethylation, which leads to suppression of the expression of transgenes controlled by them [18].

To overcome these problems, hybrid promoters were developed (for example, a combination of the CMV promoter and the human elongation factor-1α promoter), which prevents deactivation caused by gene silencing both at the transcription and translation levels [4, 19]. Additionally, promoters specific to a particular cell type are used - for example, when it is necessary to restrict the expression of antigen or genetic adjuvants to antigen-presenting cells [4].

Typically, non-coding regions responsible for regulating the expression of the antigen encoded in the DNA vaccine are located at the 5′ and 3′ ends of the target gene [20]. To increase the expression of the target gene, an enhancer can be placed in these non-coding regions [21]. In addition, the insertion of certain tissue-specific sequences that bind transcription factors into the plasmid DNA sequence can lead to its tissue-specific nuclear import [4, 22].

One of the factors affecting vaccine immunogenicity is the translation rate of mRNA synthesized from vaccine DNA. To increase translation efficiency, a technique called codon optimization is used, which involves changing the nucleotide sequence without altering the amino acid sequence of the encoded protein. The issue is that codons used by pathogens often differ from those of the host (human), so their optimization is usually required to achieve the most effective synthesis of the target antigen in the body's cells.

Studies in mouse models have shown that optimizing the codon composition of the target gene typically leads to enhanced CD8 T-cell responses $^{+}$ and increased neutralizing antibody titers [23, 24]. However, this is not always the case. Dobaño C. et al. [25] found that *in vitro* expression of a codon-optimized gene encoding the circumsporozoite protein (CSP) of the rodent malaria parasite

Plasmodium yoelii was lower than the non-optimized version, and there was no enhancement of humoral response during DNA immunization. Varaldo P. et al. [26] reported that codon optimization, which led to increased antigen synthesis, did not affect the level of immune response. This may be due to the fact that extremely high or low GC content resulting from codon optimization leads to changes in the secondary structure of mRNA, which contributes to slower translation and reduced protein production. However, high translation rates are not always beneficial, as some proteins require slow translation for proper and effective folding [27]. This is why codon optimization in the open reading frame needs to be controlled to ensure optimal speed and high accuracy of translation [28].

Plasmid DNA can also be modified to stimulate the immune response. For example, a strategy of introducing CpG motifs into DNA constructs is often used. These motifs are known to be unmethylated in bacteria and DNA viruses. In humans and higher primates, conversely, cytosine in most CpG dinucleotides contains a methyl group. As a result, unmethylated CpG motifs are recognized by the human body as pathogen-associated molecular patterns . The presence of CpG motifs is considered a type of evolutionary adaptation to enhance innate immunity against bacterial infection. The presence of CpG motifs in DNA vaccine composition leads to enhancement of both B-cell proliferation and expression of costimulatory molecules of the major histocompatibility complex (MHC) class II, however, these motifs are not antigen-specific [29].

When designing a DNA vaccine, it is important to eliminate restriction sites in the antigen sequence and *cis* -factors that affect the protein synthesis process - such as Rho-independent transcription terminators. Ideally, plasmid DNA as a vaccine construct is a molecule resistant to breaks, rearrangements, denaturation during fermentation, extraction, and subsequent purification. Unusual DNA sequences, such as homopurine-homopyrimidine tract regions in supercoiled DNA, inverted or direct repeats, can lead to structural instability of the plasmid. Palindromic sequences are also considered factors of instability and can lead to a decrease in the copy number of plasmid DNA in the cell. AT-rich regions and cruciform structures increase the frequency of breaks in the plasmid, while Chi sites mediate the multimerization of plasmid DNA [30].

In addition to the processes associated with antigen synthesis, the immune response can be influenced by the localization and accumulation of protein in the cell. To regulate this process, a sequence encoding a signal peptide is introduced into the antigen-coding region of plasmid DNA, usually at the N-terminus of the protein, which determines its localization in the cell or secretion into the extracellular space. Signal peptides typically have the following structure: a short positively charged N-terminal region (n-region); a central hydrophobic area (h-region) and a more polar C-

terminal region (c-region) containing a site that is cleaved by a special enzyme – signal peptidase, after the signal peptide fulfills its function [31–34]. Often, to enhance the immunogenicity of DNA vaccines encoding T-cell immunogens, N-terminal ubiquitin or C-terminal tyrosine motif LAMP-1 are used as signal peptides. The sequence containing N-terminal ubiquitin ensures targeting of the immunogen to the proteasome for its processing and presentation of released peptides via the MHC class I pathway to $CD8^+$ T lymphocytes. The sequence containing the C-terminal tyrosine motif LAMP-1 ensures targeting of the immunogen to the lysosome for its processing and presentation of released peptides via the MHC class II pathway to $CD4^+$ T lymphocytes [35].

So, after the DNA vaccine enters the cell, synthesis of the target immunogen occurs, which can then either remain in the cell and be broken down into shorter fragments (for example, in the proteasome) or be secreted from the cell (Fig. 2). When the protein-immunogen is localized inside the cell, cellular immune response is predominantly activated. MHC molecules participate in the activation of the cellular immune response, that is, in antigen recognition by T cells. MHC class I molecules bind peptides 8-10 amino acids in size that are formed during the degradation of intracellular proteins. MHC class II molecules bind peptides approximately 15 amino acids in size of intracellular and extracellular origin. MHC class I components bind to peptides in the lumen of the endoplasmic reticulum. Then, the resulting MHC I-peptide complex is transported to the cell membrane, presented on the cell surface, and recognized by $CD8^+$ T cells [36]. MHC class II in the vesicle fuses with a lysosome carrying short peptide fragments of the target protein. Then, the resulting MHC II-peptide complex is also transported to the cell membrane, presented on the cell surface, and recognized by $CD4^+$ T cells, which are important for the induction of specific $CD8^+$ T-cell and humoral responses.

Fig. 2. Scheme of humoral and cellular immunity activation in response to DNA vaccine administration. Designations: pDNA - plasmid DNA (here and further in the figures); CTL (cytotoxic T cells) - cytotoxic T lymphocytes.

In the case when the target protein is secreted from the cell, it can either bind to B-cell receptors and activate the humoral immune response, or directly bind to MHC class II molecules located on the cell surface and trigger the T-cell component of the immune response [31, 37]. Thus, with the help of

signal peptides, it is possible to regulate the localization of the target immunogen and influence the activation of one or another component of immunity.

The next element of the DNA vaccine is the polyadenylation signal, poly(A), which is necessary for proper termination of transcription, stabilization of mRNA transcripts, and export of mRNA from the nucleus [19]. The polyadenylation signal is a hexamer, most commonly AAUAAA, located 20-30 nucleotides before the 3' end of the mRNA. The consensus sequence of the hexamer can be represented as NNUANA, its variants: AAUAAA, A(U/G)UAAA and UAUAAA are present in 79% of mRNAs [38]. The polyadenylation sequence used in a DNA vaccine can significantly influence antigen expression. For example, it has been demonstrated that the poly(A) of SV40 mRNA is less effective than that of rabbit β-globin and bovine growth hormone, BGH , and when a second SV40 enhancer was introduced after the SV40 poly(A), the target gene expression increased to a level comparable with other signals [39]. Thus, enhancer sequences integrated not only in the promoter region but also in the polyadenylation signal area can positively affect antigen expression and, consequently, stimulate the immune response.

If the task is to express multiple genes (for example, when developing multivalent DNA vaccines or expressing a genetic adjuvant in combination with a transgene), the following strategies can be used. The first involves using different expression cassettes with separate promoters for each gene (independent expression of multiple transgenes). The second involves using bicistronic/multicistronic vectors with a single promoter for the expression of multiple genes, which are separated by internal ribosome entry site (IRES) elements. The third strategy involves using hydrolysis sites in the target protein, the sequence of which in plasmid DNA is located between genes instead of ribosome binding sites. Subsequently, these sites are recognized and cleaved by endogenous protease [4].

In addition to the elements mentioned above, plasmid DNA typically also carries an antibiotic resistance gene, which serves as a selective marker. It also facilitates the production of plasmids in bacterial cultures [19]. However, most often the presence of antibiotic resistance genes, as well as regulatory regions that function in bacterial cells, becomes a problem when using plasmid DNA as vaccine constructs [40, 41].

Recently, systems have emerged that allow for avoiding the use of antibiotic resistance [42]. This is a rather attractive strategy for selective screening of bacterial clones, as the problem of antibiotic resistance becomes more acute each year. The complete absence of antibiotic resistance

genes is the only way to guarantee the non-spread of antibiotic-resistant microorganisms in the environment. Different types of such systems are classified according to their mechanism of action, namely: systems based on "post-segregational killing" (PSK), RNA interference, chromosomal integration, and other processes. The essence of all these mechanisms is similar: if a bacterial cell captures plasmid DNA, it reproduces; otherwise, it does not. For more details on antibiotic-free selection processes, see works [43, 44].

The level of immunogenicity of a DNA vaccine can also be influenced by adjuvants. These may be aluminum salts, long known for their immunostimulatory properties [45], but cytokines and signaling molecules are more commonly used. The peculiarity of adjuvants used for DNA vaccines is that they can be delivered directly as part of the vaccine or on a separate expression plasmid DNA. Adjuvant molecules are expressed either from full-length genes of cytokines or signaling molecules, or from their fragments. As a result, proinflammatory cytokines are synthesized in the area of immunogen administration. In addition, components of the complement system, protein aggregation domains, chemokines, or costimulatory molecules can be used as adjuvants [46].

Thus, when developing a DNA vaccine preparation, it is necessary to consider all the factors listed above that affect its immunological efficacy. But in any case, the effectiveness of each genetic engineering construct as a vaccine can only be evaluated experimentally.

TYPES OF DNA VACCINES

Plasmid DNA vaccines

Currently, despite the emergence of new methods for obtaining vaccine DNA constructs, plasmid DNA vaccines still occupy leading positions. One successful example is the ZyCoV-D vaccine approved for human use, developed by Cadila Healthcare Limited (India) and aimed at combating COVID-19. The ZyCoV-D plasmid DNA vaccine contains a gene encoding the full-length codon-optimized spike (S) glycoprotein of SARS-CoV-2 with an IgE signal sequence. The efficacy of the construct was demonstrated in three animal species: mice, guinea pigs, and rabbits [47], after which it passed all three phases of clinical trials [6, 48]. The pVAX1 vector used in the vaccine is a plasmid with a size of 3 000 bp, which is constructed by modifying the pcDNA™3.1 vector and contains the following elements: early CMV promoter for high-level antigen expression in a wide range of mammalian cells, BGH polyadenylation signal for efficient transcription termination and mRNA polyadenylation, kanamycin resistance gene for selection in *Escherichia coli* and unique

restriction endonuclease recognition sites for gene cloning within the vector (https://www.thermofisher.com/order/catalog/product/V26020?SID=srch-srp-V26020).

In addition to the example considered, more than 80 variants of DNA vaccines based on plasmid DNA are currently being actively studied. The main advantages of plasmid DNA vaccines lie in their ability to activate both humoral and cellular immunity, the speed and scalability of production; however, as noted above, plasmid DNA vaccines carry an antibiotic resistance gene, which may be a limitation for mass use due to the possible transfer of the antibiotic resistance gene to human microflora.

New promising platforms for the rapid production of DNA vaccines have also been developed: minicircle DNA , MIDGE DNA and Doggybone ™ DNA. These technologies have several advantages over plasmid DNA, the main ones being: the target product contains only the sequences of interest and does not carry bacterial elements, antibiotic resistance genes, or bacterial culture residues [49, 50].

Minicircle DNA vaccine

Minicircle DNA vaccine (mcDNA) is a circular DNA molecule that has been designed with the imperfections of plasmid DNA in mind. The mcDNA lacks bacterial sequences, specifically the antibiotic resistance gene and bacterial origin of replication; it contains only the eukaryotic promoter and the target gene. The synthesis of mcDNA begins with plasmid DNA, called the parental plasmid (PP), which contains specific sequences - recombination sites, such as att, loxP, MRS, or attP/attB. PP can be transformed into mcDNA and miniplasmid using site-specific recombinases, such as Phage λ integrase, Phage P1 Cre recombinase, ParA resolvase, PhiC31 integrase/I-SceI [51]. Note that the miniplasmid resulting from the recombination process contains a sequence recognized by a restriction endonuclease, which subsequently leads to the degradation of the miniplasmid (Fig. 3).

Fig. 3. Technology for producing minicircle DNA.

The most popular system uses phage phiC31 integrase in combination with the restriction endonuclease I-SceI. The serine recombinase C31 mediates unidirectional recombination between the attP/attB binding sites, while the I-SceI endonuclease cleaves miniplasmid molecules and PP that have

not undergone recombination. The genes of both enzymes must be encoded in the genome of the bacterium used for mcDNA production; typically, this is *E. coli* [52, 53].

Given that mcDNA is much smaller than plasmid DNA and therefore can penetrate the nucleus much more efficiently, it is logical to assume that this will also lead to higher expression levels. Additionally, the removal of all bacterial sequences from the plasmid vector, including any antibiotic resistance genes, ensures the safety of mcDNA. It has been reported that mcDNA provides prolonged transgene expression *in vivo* [54], as well as increased stability in serum [55].

The use of this type of construct as a therapeutic agent has proven to be much more effective compared to plasmid DNA [52, 56-59]. For example, when mcDNA was administered to mice, a high level of expression of human blood coagulation factor IX (FIX) was recorded for 7 weeks: a sharp rise on the second day after mcDNA administration with a gradual decrease over the next 3 weeks [40].

Due to the fact that mcDNA molecules are synthesized *in vivo* in *E. coli* - by inducing intramolecular recombination of PP, - the question of their purification from other DNAs arises, particularly from residual non-recombined PP with corresponding topoisomers. And solving this experimentally is not simple. The issue is that mcDNA and all variants of the remaining PP have similar physicochemical properties. Moreover, the sizes of mcDNA and miniplasmids are usually similar. The efficiency of the recombination process is especially important in this context, as 100% recombination of PP prevents contamination of mcDNA [60].

MIDGE DNA

MIDGE DNA (abbreviation from Minimalistic Immunogenically Defined Gene Expression) is a linear double-stranded DNA consisting of a promoter, target gene, and RNA-stabilizing sequences, which are in turn surrounded by two short oligonucleotide sequences in the form of hairpins, forming a covalently closed dumbbell-shaped molecule. This approach to designing DNA vaccines allows minimizing the non-coding part of the target gene [61-63] (Fig. 4). The technology for producing such constructs consists of several stages: 1) production of plasmid DNA containing an expression cassette flanked by restriction endonuclease recognition sites; 2) treatment of plasmid DNA with restriction endonuclease to form single-stranded sticky ends; 3) ligation of the sticky ends of the expression cassette with pre-synthesized oligonucleotides forming a hairpin structure; 4) purification of linear DNA from by-products [62].

Fig. 4. Technology for producing MIDGE DNA.

Among the advantages of MIDGE DNA constructs are their small size and linearity of the DNA molecule, which avoids folding and gives a conformationally homogeneous final product. In addition, the ends of MIDGE vectors are available for chemical modifications, making it possible to bind peptides, proteins, sugars, or other DNA molecules [63]. This technology allows obtaining the target product completely *in vitro* , which significantly facilitates the purification process [49, 50]. MIDGE vectors have been successfully used in several studies as experimental DNA vaccines. Here are some examples of such studies: a) the use of a DNA construct encoding glycoprotein gp140 of feline immunodeficiency virus (FIV) [64]; b) the use of MIDGE vectors encoding glycoprotein D of bovine herpesvirus-1 [65]; c) investigation of MIDGE vectors containing the hepatitis B virus surface antigen gene (HBsAg) [61]. These works have shown that the use of such DNA constructs leads to extended and stable expression of the target gene. However, the main limitation of the technology may be the rapid elimination of the target MIDGE DNA. For instance, Nele Galling et al. [66] showed that 6 hours after administration of MIDGE-CMVhTNF, a rapid decrease in its content in tumor tissue was observed.

Doggybone ™ DNA

Doggybone ™ DNA (dbDNA ™) is another technology for producing minimalist DNA constructs, developed by the British biotechnology company "Touchlight". This technology involves enzymatic amplification of plasmid DNA containing an expression cassette flanked by telLR repeats, *in vitro* using two enzymes: a highly processive DNA polymerase (typically Phi29), which amplifies the DNA template into concatemers via rolling circle amplification, and a protelomerase (TelN from bacteriophage N15), which recognizes specific telLR sites and catalyzes cleavage at the junction of concatemers. The result is linear molecules with covalently closed ends (Fig. 5). The obtained DNA is fully functional, stable, and contains only the necessary sequences, including the target antigen, promoter, and polyadenylation signal; bacterial sequences are completely absent [4, 67]. Plasmid DNAs containing nucleotide sequences of the antigen, promoter, polyadenylation signal, and other regulatory regions flanked by telLR sequences can serve as templates for the enzymatic synthesis of dbDNA ™ . The protelomerase enzyme recognizes the palindromic telR sequences and catalyzes cleavage-joining reactions. Due to the action of the TelN protelomerase, dbDNA ™ constructs have

an unusual appearance of a linear, closed DNA molecule resembling a "doggy bone" [4]. Like MIDGE DNA, this technology allows for the production of the target product entirely *in vitro* , which significantly simplifies the purification process [49, 50].

Fig. 5. Technology for producing Doggybone ™ DNA.

Experiments on laboratory animals have demonstrated the effectiveness of the Doggybone ™ DNA against pathogens such as influenza viruses, human immunodeficiency virus, and human papillomavirus [68–70]. Scott V. et al. [68] showed that the use of dbDNA ™ constructs activates both humoral and cellular immunity against the influenza virus, comparable to the responses to plasmid DNA. Mucker E. et al. [71] compared the immune response in Syrian hamsters induced by the administration of dbDNA ™ construct and plasmid DNA encoding the SARS-CoV-2 S protein, and found no statistically significant differences in their immunogenicity, concluding that these approaches have comparable effectiveness.

DNA VACCINE DELIVERY METHODS

One of the factors playing a key role in the effectiveness of DNA vaccines is the reliability and safety of the delivery method that ensures a high level of nucleic acid penetration into target cells. Generally, nucleic acid delivery systems are divided into viral and non-viral (Fig. 6). Non-viral delivery methods, in turn, are subdivided into physical and chemical.

Fig. 6. DNA vaccine delivery methods.

In this review, we do not focus on viral delivery systems, which are well-studied and described in detail [72–75].

Chemical methods of DNA vaccine delivery

Chemical delivery methods are based on the ability of negatively charged nucleic acid molecules to bind to positively charged molecules used as delivery agents. This allows the creation of compact and stable structures (nanoparticles) that can overcome tissue and cellular barriers for successful

delivery of nucleic acid into the cell. Nanoparticles provide protection of nucleic acids from degradation in environmental conditions during transportation to target cells. Compared to viral vectors, nanoparticles provide a higher level of safety; their size ranges from 10–500 nm, which allows them to penetrate cells. By varying the composition of nanoparticles, their biocompatibility and biodegradability can be altered. Delivery systems using nanoparticles have advantages in terms of design and production, as they are easier to develop and modify compared to viral vectors [76].

Lipid Nanoparticles

The most common and characterized chemical method of delivering mRNA and DNA vaccines is based on the use of liposomes [76, 77]. Liposome technology was used by "Pfizer" (USA) and "Moderna Inc." (USA) for packaging mRNA vaccines against COVID-19, which were approved for use in humans [78]. Liposomes are cationic nanoparticles consisting of cholesterol, phospholipids, and other lipids, which in a certain ratio can bind and encapsulate nucleic acid, providing effective protection and transportation of mRNA or DNA into cells [79–81]. The high transfection efficiency of liposomes and lipid-based delivery systems is usually explained by their compatibility with lipid bilayers forming the cell membrane, which facilitates cell penetration [82].

For many years, research has been conducted to improve lipid composition. One such development is called a "niosome." This liposome consists of cholesterol or cholesterol-like molecules and non-ionic surfactants that form highly stable bilayer vesicles protected from oxidation [83, 84]. It has been shown that the stability of niosomes increases when linear mannose polymers are attached to the particle surface, which simultaneously enables targeted delivery to antigen-presenting cells [85]. The possibility of using niosomes as carriers for hepatitis B DNA vaccine for topical epidermal administration has been demonstrated [86]. Additionally, it has been shown that the introduction of niosomes into the body using hollow microneedles for epidermal vaccination induced humoral and cellular immune responses to the antigen encoded in the DNA vaccine [87].

Despite their high popularity, liposomes have a number of disadvantages, including high cost, the need to maintain a cold chain for storing the finished product, rapid elimination from the body, and induction of immunostimulatory reactions [88].

Polymer Nanoparticles

Polymer nanoparticles are also widely used for the delivery of DNA and mRNA vaccines due to their versatility, safety, and ability to enhance immune response [89]. However, compared to lipids, they have several additional limitations, primarily the difficulty of biodegradation of polymers

associated with their high molecular weight. The release rate of nucleic acid from polymer nanoparticles can be controlled by designing their chemical structure in such a way that the particle behavior is regulated by the composition of the surrounding environment. For example, a pH-stimulated control system includes a structure that provides penetration through the dissociation of various surface ligands when pH changes. Due to the possibility of modification and alteration of chemical and biological properties, polymer nanoparticles find wide application in various fields [90]. Let's briefly consider some of them.

Chitosan is a widespread polysaccharide that is a biocompatible, biodegradable, practically non-toxic polymer suitable for biomedical applications [91-93]. Chitosan is suitable for the delivery of DNA vaccines due to its cationic nature, allowing it to electrostatically bind to the anionic structure of DNA. This results in polymer-DNA complexes that protect DNA from enzymatic degradation. Chitosan is also an inert and hypoallergenic compound with favorable mucoadhesive properties, facilitating vaccination through mucous membranes [76, 92, 93].

Despite its unique physicochemical and biological properties, chitosan has not yet found widespread clinical application due to its low solubility [38, 39]. However, various modification methods have already been developed to address these problems [40, 41]. Free amino and hydroxyl groups have been used to create a wide range of chitosan derivatives with improved solubility, based on its high affinity for functional proteins and ability to self-assemble [43, 94, 95].

Research on chitosan-based nanoparticles has been ongoing for many years and has led to numerous developments, including a potential therapeutic DNA vaccine against human papillomavirus infection, as well as DNA vaccines against viral myocarditis and other mammalian diseases [76].

Polyethylenimine (PEI) is considered a versatile material with properties depending on molecular weight and degree of branching [96]. High molecular weight PEI typically has a branched structure, which leads to higher transfection efficiency but also higher cytotoxicity. Conversely, low molecular weight PEI, especially with a linear structure, has a lower surface charge, which reduces its cellular toxicity. However, due to its inability to form stable structures with DNA and protect it from enzymes and aggressive biological environments, low molecular weight PEI is characterized by low transfection efficiency. To increase transfection efficiency and minimize toxicity, various modifications are used, such as conjugation of high molecular weight branched PEI with polysaccharides, hydrophilic polymers, disulfide bridges, and lipid residues [97]. For instance,

Torrieri-Dramard L. et al. [98] demonstrated that immunization of mice with DNA encoding influenza A virus (H5N1) hemagglutinin and encapsulated in PEI generates high levels of specific IgA antibodies.

Polyethylene glycol (PEG) is an FDA-approved polymer commonly used for attachment to the surface of nanoparticles [99]. The main function of PEG is to protect the surface charge of nanoparticles and provide steric stabilization, which leads to reduced charge-related cytotoxicity, prevention of non-specific interactions with serum proteins, and makes them undetectable to phagocytes. PEG attachment results in increased systemic circulation time of nanoparticles, improved stability in the bloodstream, and reduced immunogenicity of the delivery system, although there is also a proven disadvantage – reduced transfection efficiency [100]. Liu Y. et al. [101] showed that PEG coating improves biodistribution, exogenous protein expression, and enhances the immune response induced by a DNA vaccine. Intramuscular administration of the DNA vaccine led to increased levels of ovalbumin-specific antibodies and epitope-specific T-cell activity *in vivo.* Based on these results, the authors concluded that the delivery of PEGylated DNA vaccine is highly effective.

Dendrimers are highly branched macromolecules with a tree-like structure with symmetrical branches. They are characterized by high degree of molecular uniformity, controllable size, functional surface groups, controlled physicochemical properties and high water solubility . Due to their unique properties, dendrimers have found wide application in medicine [102]. Dendrimer-based nanosystems for drug delivery are usually based on polyamidoamine dendrimers (PAMAM) or polypropylenimine derivatives, which can be modified depending on the chemical nature of the drug to be transported. Moreover, dendrimers can be used as conjugates delivering several substances simultaneously [103, 104]. Karpenko L. et al. [105] demonstrated the effectiveness of dendrimers as delivery systems for nucleic acids. H owever, dendrimers also have disadvantages that limit the possibility of their widespread use at the moment. For example, their high toxicity associated with low biodegradability due to their branched structure [106].

Dextran belongs to one of the most interesting non-toxic, biocompatible macromolecules for the delivery of pharmaceutical and medical preparations. It is a branched glucose polymer that can be modified with various functional groups to improve its properties. Dextran-based compounds are naturally biodegradable and can serve as biologically active carriers for many biomolecules [107, 108]. In recent years, numerous variants of dextran-based delivery systems with individual properties and geometry have been developed, such as self-assembling micelles and nanoparticles, nanoemulsions, magnetic nanoparticles, microparticles, and hydrogels [109]. In particular, at the

FBUN SSC VB "Vector," a conjugate of dextran (polyglucin) and polyamine (spermidine), called polyglucin-spermidine (PGS), was developed for the delivery of nucleic acids *in vivo* . The research has shown that this conjugate can be considered as a promising and safe delivery vehicle for nucleic acid-based vaccines. Moreover, nucleic acid in the PGS conjugate shell can be lyophilized, which makes it possible to store DNA vaccines encapsulated in PGS at a temperature of 4°C without loss of specific activity [110-112].

Cell-penetrating peptides

Cell-penetrating peptides (cell-penetrating peptides, CPP) represent a class of diverse peptides, usually positively charged, less than 30 amino acids in length. They possess a unique ability to penetrate through cell membranes without interacting with specific receptors. In recent decades, CPPs have become a frequently used tool for delivering nucleic acids into intracellular space due to their high efficiency and low toxicity [113].

Although the exact mechanisms of CPP internalization are not fully understood, two main mechanisms have been proposed: endocytosis and direct translocation (or non-endocytic translocation). Endocytosis, which is considered the main mechanism of CPP penetration, includes macropinocytosis, clathrin-mediated and caveolae/lipid raft-mediated endocytosis [113]. It is also suggested that CPPs can promote clustering of negatively charged glycosaminoglycans on the cell surface, which, in turn, trigger macropinocytosis and lateral diffusion or directly disrupt the lipid bilayer. It has been reported that CPPs with arginine-rich repeats can be used as an mRNA delivery system to dendritic cells [114].

Currently, research is being conducted on other chemical delivery systems besides those mentioned above. For example, such as inorganic nanoparticles, which can be read about in reviews [76, 101].

Physical methods of DNA vaccine delivery

Along with chemical delivery methods, physical methods are also being actively developed, which can facilitate DNA delivery into cells and ensure enhancement of its immunogenicity. One such technology is *electroporation* (EP). EP involves the application of an electrical pulse that induces the formation of temporary pores in cell membranes, thereby facilitating the uptake of DNA by cells *in situ* . The main studies on this topic [115, 116] show that the efficiency of DNA uptake and expression depends on parameters such as voltage, duration, number, and frequency of pulses.

In addition to using electrical pulses, drugs, including DNA vaccines, can be administered using a needleless jet injector. *Jet injection* allows the administration of therapeutic drugs and vaccines using a high-speed jet under high pressure and delivers the preparation to intradermal, intramuscular, or subcutaneous tissue without the use of a needle [117–120]. The DNA vaccine administered in this manner induces a higher level of cellular immune response and antibody production in humans compared to needle administration [4, 106]. The increased effectiveness of DNA vaccination in this case is most likely due to the fact that delivery using an injector can increase DNA uptake by skin and muscle tissues compared to conventional delivery *in vivo* [121]. In recent years, jet injection as a means of delivering nucleic acid-based vaccines has been gaining increasing popularity. For instance, Dey A. et al. [47] used the PharmaJet® Tropis® ("PharmaJet", USA) jet injector to administer the ZyCoV-D vaccine against COVID-19. Houser K. et al. [122] used the Stratis ("PharmaJet") jet injector to deliver a DNA vaccine against influenza. The same method for delivering an experimental DNA vaccine based on Doggybone™ technology was applied by Mucker E. et al. [71]. For intratumoral delivery of MIDGE DNA, Galling N. et al. [66] also used the jet injection method.

Another method of DNA vaccine delivery is based on the use of a *gene gun* . In this case, gold nanoparticles coated with DNA are typically used, which are delivered to tissues using high-pressure gas. Due to the high delivery force, gold particles penetrate through the cell membrane, resulting in more efficient cellular uptake compared to naked plasmid DNA. This delivery method allows for the induction of the same immune response as intramuscular or intradermal injection, with significantly smaller amounts of DNA (a few nanograms instead of several micrograms) [106].

For DNA vaccine delivery, an array of *microneedles* is also used. Microscopic needles (from 25 to 2 000 μm) penetrate the skin to a specific and reproducible depth with minimal pain [123]. DNA delivery using microneedles leads to stable expression of the encoded antigen in the skin. This method is based on breaking through the stratum corneum and viable epidermis with microneedles, after which DNA can be delivered to the dermis [106]. It has been demonstrated that DNA delivery to the skin using microneedles induced a strong cellular immune response in mice, which protected them from influenza virus infection [124].

Most DNA vaccine delivery methods, both physical and chemical, are quite effective. Despite this, each of them requires refinement aimed at increasing the effective efficiency of DNA vaccines and reducing their production costs. For example, using physical methods for vaccine delivery is more advantageous compared to chemical ones, as the reactogenicity of the preparation administered in this way is much lower. This is due to the fact that physical administration methods use only "naked"

nucleic acid without auxiliary components. However, in some cases, chemical methods also have certain advantages. For instance, the use of chitosan as a "carrier" for a DNA vaccine during intranasal administration can lead to the formation of an effective mucosal immune response. Each of the delivery methods presented in this review requires special attention from researchers, as progress in this area will allow another step forward in the creation and implementation of new vaccines.

CONCLUSION

The COVID-19 pandemic, caused by the SARS-CoV-2 virus new to humanity, which rapidly spread throughout the world taking millions of lives, became a shock to the global healthcare system. Rapid development of preventive vaccines was necessary. As a result, many DNA vaccines against SARS-CoV-2 have been developed, including those based on mcDNA and MIDGE technology, and at least 10 of them have entered clinical trials [7 . The DNA vaccine for COVID-19 prevention ZyCoV-D, developed by the Indian company "Zydus Lifesciences Limited," became the world's first DNA vaccine approved for human vaccination [5].

Many candidate preventive vaccines against viral and bacterial human diseases are in the clinical trial stage. DNA vaccines for immunizing animals, fish, and birds have been licensed and used for quite some time. Recently, the US Department of Agriculture licensed a DNA vaccine against highly pathogenic influenza A virus (H5N1) for chickens, developed by "Agrilabs" (Indonesia) [4]. The license allows the company to store large volumes of the vaccine in case of an avian influenza outbreak.

One of the important advantages of DNA vaccines is their low cost and quick production, minimal requirements for transportation and storage, as well as stability at elevated temperatures. The experience of using DNA constructs for human vaccination is still incomparable with the experience of using viral vaccines, although a favorable safety profile has been demonstrated using the example of COVID vaccine ZyCoV-D [5]. Thus, DNA vaccines have a number of properties that allow them to be considered a promising vaccine platform for the prevention and treatment of infectious diseases.

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ETHICS DECLARATION

This article does not contain any studies involving humans or animals as research subjects.

CONFLICT OF INTERESTS

The authors declare no conflict of interest.

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