# Chapter 7
# The Application of DNA Nanostructures in Vaccine Technology


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**Abstract** Vaccine is a biological agent for preventing and curing disease, which inducts both innate and adaptive immune mechanism to be effective. Facing potentially unknown pathogens, the current vaccine technologies have problems such as (1) prolonged development time, (2) limited production capacity, and (3) inability to guarantee biosafety. To address these issues, DNA nanostructures as carrier platforms, featured with strong immunogenicity, excellent biosecurity, and promising programmability, have attracted much attention in the development of vaccines nowadays. These DNA nanostructures, including DNA tetrahedra, DNA hydrogel, DNA nanotube, DNA dendrimer, and DNA nanoflower, could not only directly induce macrophages to secrete immune factors by modifying sizes and structures but also indirectly stimulate TLR9 immune response as carriers of CpG ODNs. In addition, DNA sequences can be combined with different antigen molecules to form an antigen presentation system to participate in the body's adaptive immune response. This review summarizes the role of various DNA nanomaterials in the field of immunity and aims to provide new ideas for enhancing the body's immune response against diseases and treating various immune system diseases.

**Keywords** Vaccine · DNA nanomaterials · Immune response · Immunoadjuvant

## Abbreviations

| | |
|---|---|
| anti-dsDNA | anti-double-stranded DNA |
| APC | Antigen-presenting cells |
| ASCs | Antibody-secreting cells |
| BSA | Bovine serum albumin |
| BS-nanow | Bead-chain DNA nanowires |

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Y. Lin, R. Zhou (eds.), *Advances in Nanomaterials-based Cell Biology Research*,
https://doi.org/10.1007/978-981-16-2666-1_7

| | |
|---|---|
| CFA | Complete Freund's adjuvant |
| cGAMP | Cyclic GMP-AMP |
| CpG | Cytosine-phosphate-guanine |
| CTL | Cytotoxic T lymphocyte |
| DL-DNA | Dendrimer DNA |
| DNase I | Degradation of endonuclease |
| DNO | DNA nano-octahedron |
| DOX | Doxorubicin |
| DSHV | DNA supramolecular hydrogel vaccine |
| dsODN | Double-stranded ODN |
| E-DNO | Encapsulated DNO |
| ELISPOT | Enzyme-linked immunosorbent spot |
| FDG | 18F-fludeoxyglucose |
| iDR-NC | DNA-RNA nanocapsules |
| IFN | type I interferon |
| INH-ODN | Immunosuppressed oligodeoxynucleotides |
| IRF3 | Interferon-regulatory factor 3 |
| JAK/STAT | Janus kinase/signal transduction and transcription activation |
| JNK/SAPK | Jun N-terminal protein kinase/stress-activated protein kinase |
| KK | KK1B10 |
| MAPK | Mitogen-activated protein kinase |
| MDR | Multidrug resistance |
| MYD88 | Myeloid differentiation primary response 88 |
| N-DNO | Nonencapsulated DNO |
| NF-κB | Nuclear factor-kB |
| OVA | Ovalbumin |
| pDC | Plasma cell-like DC |
| PLG | Polymeric nanomaterials including poly (d, l-lactide-co-glycolide) |
| PLGA | Poly(d, liter-lactic acid-hydroxy acid) |
| RCR | Rolling cycles |
| RGC | Retinal ganglion cells |
| ROS | Reactive oxygen species |
| shRNA | Short hairpin RNA |
| ssODN | Single-stranded ODN |
| STINGs | Stimulator of interferon genes |
| STV | Streptavidin |
| TDN | DNA tetrahedron |
| TLR9 | Toll-like receptor 9 |
| TNF | Tumor necrosis factor |
| VLPs | Virus or virus-like particles |
| VLPs | Virus-like particles |
| Y-DNA | Y-type DNA |
| Y-ODNs | Y-shaped oligodeoxynucleotides |

## 7.1 Introduction

Ever since the invention of the first vaccine, vaccination have helped save many lives and significantly improved the quality of life. As the most effective medical intervention to control or even eliminate a disease, vaccination can be considered as one of the greatest breakthroughs in modern medicine [1, 2]. Like nature infections, vaccines act by initiating both innate immune and adaptive immune response [3]. Innate immunity occurs within hours of pathogen recognition, followed by an adaptive immune response over several days, leading to immune memory [4]. Currently, live attenuated vaccines usually produce an effective and durable immune response. However, in the case of inactivated vaccines, adjuvants are often required to enhance the efficacy of antigen. Therefore, researches on vaccines in recent years have focused on adjuvants which enhanced the activity of vaccine delivery systems. Adjuvants can be broadly divided into three types of delivery systems: immunomodulatory molecules, non-immunostimulating component antigen delivery systems, and delivery systems that have both functions [5].

The most widely used immunomodulatory molecule in the field of immunity is cytosine-phosphate-guanine (CpG) oligonucleotide. It can activate the myeloid differentiation primary response 88 (MYD88) signaling pathway by interacting with the host's own CpG DNA, through which type I interferon (IFN) and other pro-inflammatory cytokines can be produced [6]. In addition, some clinical trials in humans to evaluate the activity of CpG ODN adjuvants showed that CpG ODNs can induce a T1 immune response and become potential cancer vaccine adjuvants [7]. Among different types of CpG ODNs, D-type ODN can effectively induce plasma cell-like DC (pDC) to produce type I interferon, but cannot activate B cells to produce antibodies. Due to the presence of multiple G tails, D-type ODNs may form aggregates, which limits their applications. K-type CpG ODN (or B-type ODN) (such as K3 CpG) does not form aggregates in solution and can effectively activate B cells for the production of antibodies and IL-6, but only weakly induces pDC to produce IFN. Based on the different properties of various kinds of CPGs, modifying the surface structure of CPG could solve the problem that antibodies and interferons cannot be induced in large quantities at the same time. Linking HIV TAT peptide with K-type CpG ODN to form a CpG ODN nanoring can not only enhance the adjuvant uptake but also produce IFN [8]. Moreover, Y-type, X-type and hexapod-like CPG patterns can be generated to promote the uptake of immune cells and then promote TLR9-mediated production of IFN [9].

Non-immunostimulating component antigen delivery systems which directly activate immune systems are a hot spot in current immune research. Immune response is more effectively induced by nanomaterials, because they have the size equivalent to pathogens, and they can be more easily recognized and absorbed by antigen-presenting cells [10]**.** Nanomaterials currently used for immunization mainly involve (1) polymeric nanomaterials including poly (d, l-lactide-co-glycolide) (PLG) [11] and poly(d, liter-lactic acid-hydroxy acid) (PLGA) [12–14], (2) inorganic nanostructures covering gold nanoparticles [15, 16] and carbon nanoparticles

[17, 18], (3) organic ingredients containing liposome [19, 20], autonomous protein [21, 22], and self-assembled DNA nanostructures [23–25]. The mode of antigens loaded and delivered by nanomaterials is mainly the construction of virus-like particles (VLPs), which induce a long-term production of antibodies specific to many proteins displayed on the surface of these viral particles. But when comparing with the whole cell vaccines, VLPs often show a low-level and short-lived production of antibodies [26, 27]. Therefore, many studies have focused on size control and surface modification of VLPs to enhance the VLP-mediated immune response [26]. Despite many synthetic nanoparticles have been exploited as vaccine carriers to assembly particulate antigens, DNA nanostructures stand out because they can activate both antigen-dependent signal and accessory signal to generate high-quality B-cell responses. As a result, DNA nanostructures harness the engineering potential of particulate antigens for rational design and construction of effective DNA-based vaccines by mimicking biophysical and biochemical cues from viruses [28–31].

Due to Watson-Crick base-pairing principle, the self-assembled DNA nanostructure is autonomous and programmable, and this unique feature makes it possible to utilize computer programs to design and simulate its structure and geometry [32, 33]. Furthermore, the chemical modification of DNA offers different methods to conjugate DNA to functional ligands, such as covalent cross-linking at 5′ or 3′ ends or nucleic acids base pairing [34]. DNA tile which is assembled as building block was constructed into several nanoscale devices for nanomedical applications in ligand delivery and immunization filed. While nucleic acids need transfection agents to penetrate into the cells, it has been shown that DNA nanoparticles were naturally internalized by antigen-presenting cells (APCs) in a shape- and size-dependent manner, even if they are not targeted ligands [35]. Additionally, the similarity between DNA sequences of the delivery platform and nucleic acid adjuvants, such as CpG DNA, enables DNA nanomaterials to simultaneously activate innate immunity. DNA nanomaterials currently used in immune engineering are mainly DNA tetrahedral [23], DNA hydrogel [24], DNA nanotubes [25], DNA dendrimer [36], and DNA nanoflower [37] (Fig. 7.1). These DNA nanomaterials not only share the common characteristics of nano-vaccines in terms of size and structure but also show their unique advantages. First, DNA nanomaterials are highly biocompatible. Antibodies against double-stranded DNA or DNA nanostructure are not detected in hosts after immunization [38]. It may be due to the presence of the double-stranded DNA genome in the host, which makes the host avoid the immune response against DNA that would cause autoimmune diseases [39]. Second, some DNA nanomaterials without being loaded with any immunoregulatory molecules can regulate the innate immune response by acting on immune-related signaling pathways without producing any toxic side effects [25, 40]. Third, DNA nanomaterials also have structural controllability to be used as a platform for organizing various immune adjuvants, such as CpG ODN and proteins/peptides. Various experiments in combination with immunomodulatory molecules have proven that DNA nanomaterials could (1) protect immunomodulatory molecules from degradation by enzymes and prolong the half-life in the body, (2) improve the efficiency of cells in absorbing immunomodulatory molecules, (3) deliver target

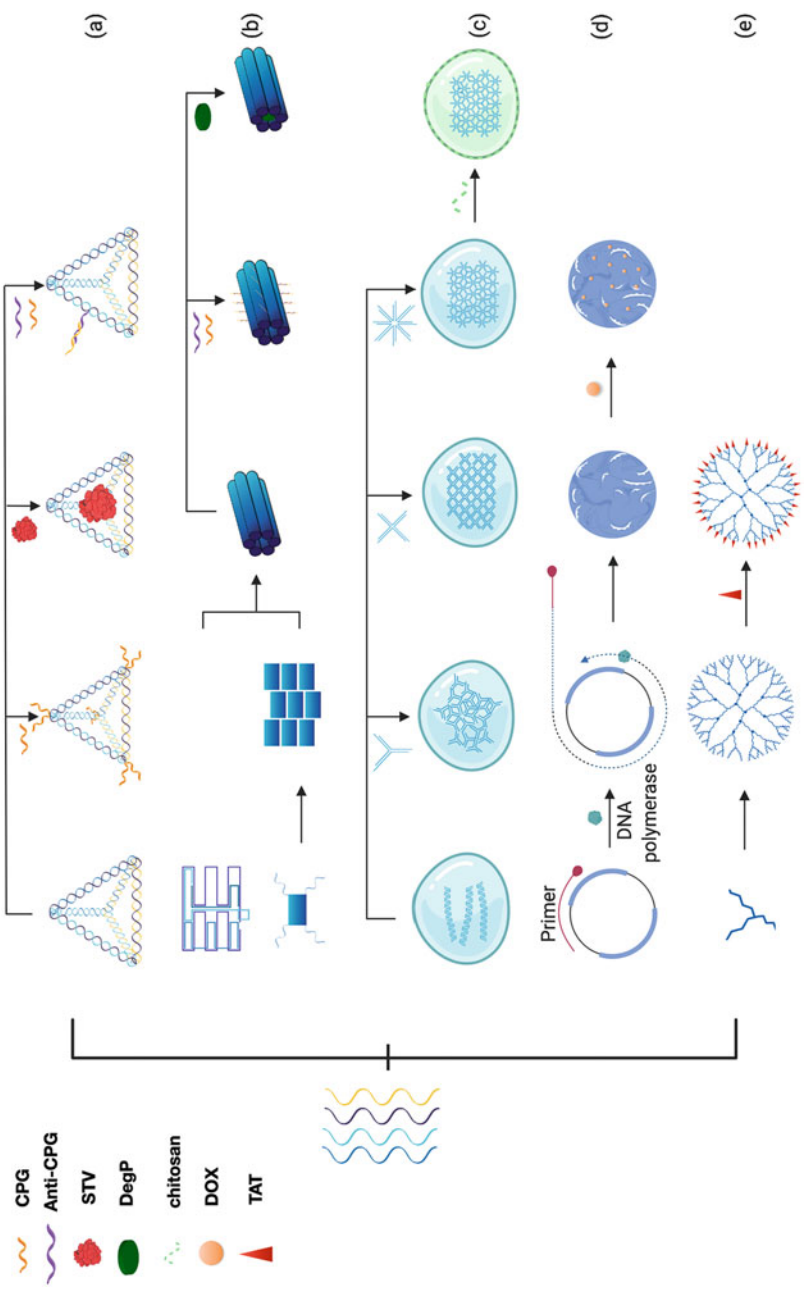


**Fig. 7.1** Syntheses and structures of DNA vaccines: (**a**) DNA tetrahedron, (**b**) DNA nanotube, (**c**) DNA hydrogel, (**d**) DNA nanoflower, and (**e**) DNA dendrimer

immunomodulatory molecules to tissues, (4) change intracellular localization, and (5) change target physical form and induction of cytokines [41–44]. On the basis of the above, the performance of DNA nanomaterials can be further improved by estimating receptor/ligand interactions [38]. For example, the distance between the antigen and the agonist can be reasonably adjusted according to the controllability of its structure. When the spatial positions of the antigen and the adjuvant are close, the immunogenicity of the adjuvant is enhanced [45]. In addition, DNA nanomaterials incorporate nuclease-sensitive sequences to regulate its sensitivity to nuclease degradation, thereby reducing the host's immune resistance to the vaccine [46]. In summary, DNA nanomaterials can be used as a carrier to transport antigens and play a synergistic role with antigens to maximize the influence of vaccine adjuvant.

## 7.2 DNA Nanostructures

### *7.2.1 DNA Tetrahedron*

DNA tetrahedron (TDN) is formed via self-assembly of four or more DNA single strands, based on Watson-Crick pairing principle [47]. For instance, four predesigned single strands are molar—equally added to TM buffer (Tris and $MgCl_2$) and then heated at 95 ° C for 10 min and cooled down to 4 ° C for 20 min. In one DNA tetrahedron, each single-stranded DNA forms one triangle, and three sides of the triangle are complementary with one of the other triangle. Usually, single-stranded DNA molecules with a length of 63 nt are used to construct a DNA tetrahedron with a side length of 20 bp. This DNA tetrahedron is not a solid structure but a framework nucleic acid with cavity, which could carry objects between the edges formed by the double-stranded DNA. DNA tetrahedrons are featured with excellent biosecurity and promising biocompatibility and controllable programmability. Currently, DNA tetrahedrons show promising potentials in promoting proliferation and migration of multiple types of stem cells and cell lines, such as human corneal epithelial cells [48], mouse L929 fibroblasts [49], and rat adipose-derived stem cells [50], with working concentrations below 250 nM [48], which demonstrated excellent biological safety. The biocompatibility of DNA tetrahedra is referred to its transmembrane capacity. To date, accumulating studies have found that DNA tetrahedrons can be efficiently taken up by various types of cells without any transfection agent. DNA tetrahedron was found to minimize electrostatic repulsion through corner attack mechanism and thereby quickly go across the membrane [51], depending on caveolin-mediated pathway [52]. This process usually requires the size of DNA tetrahedron to conform to initiative of cell intake. Inspiringly, after entering mammalian cells, DNA tetrahedron can remain intact for at least 48 h [53]. Based on Watson-Crick base-pairing principle, DNA tetrahedron can be modified via mainly three methods to form upgrading structural and functional transformations (Fig. 7.1a): (1) pre-linking the modifiers, like nucleic acids, at the 5 ′ or 3 ′ end of single strands before self-assembling of DNA tetrahedra;

(2) designing an overhang which would not interfere with DNA tetrahedron formation but combining with modifier through complementary sequences [54]; and (3) physical conjugating modifiers (e.g., proteins) in the DNA double helices [55]. Generally, there are two ways to dissociated the modifiers and carriers: (1) base pair mismatch, of which the degree is often related to the degree of dissociation [54], and (2) arrangement of fragile gaps between modifier and DNA tetrahedron. Such gaps are usually composed of consecutive identical bases [56, 57].

Inspired by the above three modification methods, researchers assembled CpG ODN on the DNA tetrahedron to form a complex in the field of immunoengineering. CpG ODNs are well-known immunostimulatory agents, which can be recognized by Toll-like receptor 9 (TLR9) that activates downstream pathways to induce immunostimulatory effects, secreting various pro-inflammatory cytokines including tumor necrosis factor (TNF)-R, interleukin (IL)-6, and IL-12. This TDN-CpG ODN complex can be taken up by APCs to enhance immunity. The unique properties of TDN-CpG ODN complex are, firstly, in the preparation of vaccines, the biosafety of the complex needs to be premiere considered. Li et al. tested the biocompatibility of low concentration TDN-CpG complex in cells, and the results showed that cell viability was not affected. The immune system maintains a critically organized network to defend against foreign particles. The immune system becomes active when TDN-CpG complex is applied to organisms. Many DNA nanomaterials are greatly restricted in their applications due to the potential to induce autoimmune diseases. For example, anti-double-stranded DNA (anti-dsDNA) antibodies are implicated in the pathogenesis of many autoimmune diseases. However, a study showed that after DNA tetrahedron injection for 18 days, researchers observed no detectable level of anti-dsDNA antibodies [38]. Moreover, a recent study found that tetrahedron DNA can significantly regulate the balance of NO (an inflammatory mediator) production, particularly at the dose of 250 nM. TDN can also work as a potentially useful candidates in immunomodulation to inhibit mitogen-activated protein kinase (MAPK) phosphorylation to attenuate the expression of NOIL-1β (interleukin-1β), IL-6, and TNF-α in RAW264.7 cells induced by LPS. In addition, researchers have also found that DNA tetrahedron inhibit LPS-induced reactive oxygen species (ROS) production and apoptosis by upregulating the mRNA expression of antioxidants [40]. The anti-inflammatory and anti-oxidative stress abilities of DNA tetrahedrons dispel concerns that they may cause autoimmune diseases and further proved the biosafety of DNA tetrahedron. In addition, DNA tetrahedrons are synthesized from single-stranded DNA and can be degraded and metabolized by endonuclease in organism. The metabolic products are deoxynucleotide monomers, which will not produce more toxic side effects. Secondly, the TDN-CpG ODN complex needs to be efficiently taken up by APC. Ohtsuki et al. designed and compared the uptake rate of tetrapod-like structured DNA (tetrapod DNA), tetrahedral DNA, tetragonal DNA, and single-stranded DNA (ssDNA) into macrophages [58], and results showed that DNA tetrahedron was taken up by cells nearly twice as fast as tetrapod DNA and tetragon DNA and nearly five times as fast as ssDNA, thus confirming that TDN-CpG ODN complex possesses the capacity of efficient cell uptake. Thirdly, TDN-CpG ODN complex needs to be stable for a period of time in

organism, which requires certain resistance to endonuclease. DNA tetrahedral nanostructures have been proven to be stable against nuclease degradation in biological media. The stability of TDNs has been further quantitatively analyzed by incubating the same concentration of TDN and double-stranded DNA in 50% non-inactivated fetal bovine serum [23]. Weakened TDN band could still be observed after 24 h, while the DNA double-strand was completely degraded after only 2 h. Additionally, co-localization study using dual-labeled nanostructures (Cy3 and Cy5 labeled on different vertexes) showed that the two fluorescent colors were present nearly in the same place even after 8 h, which further confirms that DNA nanostructures are intracellular stable. Compared with other CpG carriers, such as liposomes, the free arrangement of the four bases provides a high degree of freedom for DNA tetrahedrons and can be programmed to design sequences that meet different needs. Using the programmability of DNA tetrahedrons, Zhang et al. incorporated a biotin moiety at the 5′ end of DNA single-strand and self-assemble the DNA upward. Each surface of the DNA polyhedra displays three biotin moieties, related by a threefold rotational symmetry [59]. Overall, the excellent properties of the CpG-TDN complex suggest its potential for application in immunoengineering. To further assess its ability of stimulating immunity, Ohtsuki et al. incubated 6 μg/mL CpG-TDN and CpG ODN with human PBMCs; as a result, cell treated with CpG-TDN for 24 h expressed twice the amount of IFN-α by comparison to the CpG-ODN-treated cells [58]. But after adding chloroquine, an inhibitor of endosomal TLR signaling and IFN-α releasing from human PBMCs were strongly inhibited, highly suggesting that the IFN-α release after addition of CpG-TDN complex occurred through TLR9 pathway. This result indicated that loading on TDN directly or indirectly enhanced the immunostimulatory capacity of CpG ODN. To investigate the impact of different concentrations of CpG-TDN, two sets of varying CpG-TDN concentrations (2 μg/mL and 6 μg/mL) were constructed by incubating with RAW264.7 cells for 8 h, and it has been found that CpG-TDN concentration was positively correlated with TNF-α expression [58]. Another study showed that the expression of TNF-α induced by the CpG-TDN complex was more than five times higher than that of the CpG carried by Lipofectin. In addition to TNF-α, other cytokines also play a role in CpG-TDN-mediated immune activation. After adding CpG-TDN complex to RAW264.7 cells for 8 h, high levels of IL-6 and IL-12 expression were also detected. The results of ELISA assays showed that the expression level of IL-6 can reach more than 60 pg/mL and the expression of IL-12 can reach more than 200 pg/mL [23]. TNF-α, IL-6 and IL-12 were all secreted by the activation of the TLR9 pathway, which suggested that the CpG-TDN complex can produce a stronger immunostimulatory capacity through the TLR9 pathway than the CpG ODN, but whether there are any other signaling pathways or cytokines involved in the immune-activation process remains to be elucidated. Cellular uptake efficiency and stability significantly enhances the immunostimulatory capacity of the CpG-TDN complex, but this cannot be taken as strong evidence for its significant difference from Lipofectin. Some studies elucidated the mechanism of CpG-ODN's powerful immune-stimulating ability from multiple perspectives. Exposure of the 5′ end of CpG ODN is closely related to its immunostimulatory activity. Conjugation at

the 5′ end will significantly inhibit the immunostimulatory activity of CpG DNA, while the conjugation at the 3′ end won't, and this difference does not owe to the difference in cell uptake capacity, which indicates that the receptor reads the DNA sequence from the 5′ end [60, 61]. Further research showed that CpG ODN was often composed of stimulatory and structural domains. Different combinations of stimulatory and structural domains can stimulate the immune activation of different cell lines, suggesting that the secondary structure formed by the CpG-TDN complex may be one of the reason for the strong immune stimulation ability [62, 63]. In addition, the physical aggregation state of CpG ODN is also related to its immunostimulatory ability [64]. Studies showed that CpG aggregates can induce bone marrow-derived monocytes to secrete more IL-12 than CpG ODN, indicating that it has stronger TLR9 binding ability [65]. Recently, attention had been paid to the relationship between the number of CpG motifs and the immune activity of CpG-TDN. Li et al. found as the number of CpG motifs increased the immune stimulatory effect was enhanced [23]. The enhancement was not only due to the increased concentration of CpG which leads to an increase in the affinity of TLR9 but also due to the common effect of the four CpG motifs caused by the spatial structure of DNA. Because the DNA tetrahedron has a uniform size and precise structure, the CpG motif can be accurately placed at any specific position of the tetrahedron for predetermined sequence number and sequence design. The accurate correspondence is beneficial to the recognition between the CpG sequence and TLR9. Based on this, the efficacy of DNA nanostructures can be further improved.

In order to further explore the application of DNA tetrahedrons in the field of immunity, researchers used the structural properties of DNA tetrahedrons. Since these cage-like nanostructures are hollow structures, they are able to assemble with subunit proteins into virus-like particles (VLPs) [66]. VLP represents a major breakthrough in vaccine development. It is considered to better induce immune response. Previous studies have shown that the size, shape, surface charge, hydrophobicity, hydrophilicity, and receptor interactions of an antigen can affect APC's absorption [26]. Although direct connection of CpG ODN with antigen has been shown to induce a strong B-cell response [45], it is not feasible to use it to prepare vaccine directly. Therefore, effective carriers carrying CpG ODN and antigen are required to prepare more complex and useful vaccine. Recombinant DNA technology assembles subunit proteins into VLPs [66, 67], which is similar to natural virus structures, but without viral genetic material. The immunogenic epitopes displayed on VLPs can induce a strong immune response. Therefore, VLPs were widely studied as an effective and safe platform for assembling target epitopes against many pathogens and tumors [68]. At present, DNA tetrahedrons are used to construct VLPs. CpG ODN is connected to the vertices of tetrahedrons, and antigens are connected to each face of the tetrahedron through biotin. By increasing the number of biotins, this connection can be strengthened, which can solve the great challenge for DNA-directed guest organization [69–71]. TDN-VLP is constructed by three steps: (1) conjugation of CpG ODN and biotin moiety at the end of DNA single strand, (2) the programmed self-assembly of DNA tetrahedron, and (3) immobilization of proteins onto the DNA scaffolds [59]. The currently reported TDN-VLP only

carries streptavidin (STV) as antibody and CpG ODN as adjuvant. But the successful deployment of STV also highlights that DNA tetrahedron scaffold has the potential to organize a wider range of objects, which can be applied to develop other VLP vaccines by mounting other antibodies as needed. To verify the immunostimulatory ability of TDN-VLP, particularly in elicit an antibody response against the model antigen, BALB/c mice were treated with experimental artificial immunization protocol including three steps: (1) primary immunization, (2) secondary immunization, and (3) antigen challenge. The time intervals between primary and secondary immunization and antigen challenge were 28 days and 24 days. Compared to those immunized with free CpG + STV and STV only, the TDN-VLP complexes induced a stronger and longer lasting anti-STV antibody response, partially due to the generation of STV-specific memory B cells. Quantitative analysis of anti-STV IgG antibodies expression level was processed by ELISA, and results showed that TDN-VLP induced twice the antibody secretion of free STV + CpG, even after 60 days of antigen challenge. To directly evaluate the long-term immunity induced by various immunization regimes, researchers applied an enzyme-linked immunosorbent spot (ELISPOT), assay resulted that significantly elevated levels of specific antibody-secreting cells (ASCs) were found in mice immunized with the TDN-VLP complex compared to those immunized with free CpG + STV and STV only, and ASCs were transformed from memory B cells after STV stimulation in vitro, which indirectly proved that TDN-VLP can induce the generation of memory B cells. The CpG-TDN complex can only elicit a short-acting immune response because it mainly acts on T cells and only induces upregulation of multiple cytokines, but does not promote the generation of memory B cells. TDN-VLP can induce the generation of memory B cells and establish long-term and efficient artificial immunity, which is the goal pursued by vaccination. The reason for such a significant difference may be that the TDN-VLP complex better mimics the natural virus structure. Through the programming of TDN, the spatial arrangement of each immunogenic component can be controlled to meet the needs of receptor recognition. However, the recognition receptors and downstream signaling pathways that induce long-term immunity by TDN-VLP still need to be further studied. The influence of the spatial arrangement of various components on immunogenicity has not yet been elucidated, which is of great significance for the rational design of VLP.

Overall, DNA tetrahedron as a carrier, its size, charge, and other physical properties meet the requirements of internalization by APC. Base pairing can also allow DNA tetrahedra to inherently carry CpG motifs, which has unique advantages over other vaccine vectors. Because the close proximity of antigens and adjuvants is essential to enhance the immunogenicity of vaccines, programmable DNA tetrahedrons provide multivalent and three-dimensional configurations. Therefore, DNA tetrahedrons can be considered as an excellent platform for constructing vaccines that mimic virus-like particles. Additionally, the 3D spatial arrangement of each immunogenic component can be easily controlled through the rational design of the tetrahedral sequence, so that the DNA tetrahedrons can meet the spatial structure requirements for inducing the optimal immune response. Most importantly, DNA

tetrahedrons have better safety because they can regulate the oxidative stress and inflammatory response of macrophages and can effectively prevent the occurrence of autoimmune diseases. The above characteristics complement each other, making DNA tetrahedron a potential vaccine preparation platform in the field of immune engineering.

### *7.2.2 DNA Nanotubes*

Among various artificially synthesized nanotubes, biomimetic DNA nanotubes have attracted widespread attention due to their design flexibility. Two methods can be used to prepare structurally stable DNA nanotubes. One is programmable assembly of DNA magnetic tiles [72] (Fig. 7.1b). The DNA tile consists of a DX molecular core and four single-stranded sticky ends which allow it to bind to other tiles. Given an appropriate set of sticky ends, DNA tiles will form a lattice sheet by adjusting the curvature of the phosphate skeleton and the location of the sticky ends. After assembly, DNA tiles form an angle with each other, and the flat sheet becomes tubular. The other method is to plicate layers of double helices to a honeycomb lattice [73]. With the help of caDNAno software, honeycomb DNA origami tubes can be easily designed [32]. As one of the candidate carriers of nano-vaccine, DNA nanotubes have excellent stability, flexible loading capacity, and remarkable biocompatibility. The robustness of Watson-Crick base pairing ensures a programmable and sophisticated design of various types of DNA nanotubes. DNA nanotechnology allows bottom-up assembly of complicated nanotube structures ranging from a few nanometers to micrometers in size, able to load functional nucleic acids, proteins, peptides, and organic and inorganic materials. Additionally, DNA nanotubes also show promising biological properties. Upon exposure to multiple endonucleases [33], including DNase I, T7 endonuclease I, T7 exonuclease, *Escherichia coli* exonuclease I, lambda exonuclease, Mse I restriction endonuclease, and lysates from various cell lines [74], DNA nanotube can still maintain its structural integrity for 12 h. Furthermore, a higher cell-permeable efficiency of DNA nanotubes with greater rigidity was observed compared to that of spherical, circular, or other DNA nanostructures [75, 76]. Due to the larger contact area with cell surface and cross-linking membrane receptors, CpG-modified DNA nanotubes are more easily to be internalized than single spherical DNA-adjuvant complexes or single-stranded CpG motifs. Recent study suggests that the efficient internalization of cells is also due to the corner attack mechanism which indicated that the cell entry of DNA nanostructures in the range of several tens of nanometers is not related to their size but to the shape, and the anisotropic structures are more likely to enter cells than isotropic structures [51]. Overall, these characteristics make DNA nanotube an efficient vehicle for the delivery of CpG.

Currently, methods to modify CpG onto DNA nanotubes are (1) adding single-stranded DNA handles that protrude from the wall of the DNA origami tube to the defined position, meanwhile combining anchor sequences which complementary to

the handles with CpG, and CpG is connected to the nanotube by base pairing. (2) Wrapping modifiers (e.g., proteins) in the hollow structure. Studies showed these CpG-modified DNA nanotube complex could trigger immune responses. Mammadov R et al. [77] conducted nanotubes with a diameter of 10–15 nm and a length >200 nm, via using CpG ODN and β-sheet-forming peptides. Compared to spherical nanostructures and CpG ODN, the nanotubular structures induced higher levels of IFN-γ expression and lower levels of IL-6 expression. More importantly, the nanotubular structure can also synergize with CpG ODN itself and induce higher levels of CD86 expression, which proves that the immune response to Th1 phenotype induced by CpG-DNA nanotube is more effective in defending against intracellular pathogens. The role of the nanotubular structure and the CpG ODN is not superimposed on each other but rather a synergistic effect of mutual promotion. Under this effect, using the nanotubular structure of the CpG ODN will improve the adaptive immune response to the vaccine complex by allowing more CpG ODN loaded and spatial synergies. Currently, researchers have developed a hollow tube-shaped DNA origami structure consisting of 30 parallel double helices with maximized surface area for both 62 inner or 62 outer binding sites for CpG anchor sequences (CpG-H0s) [25]. These nanotubes can be efficiently internalized by antigen-presenting cells, while protecting CpG sequences from degradation and inducing high local concentration of CpG in vivo, suggesting a high-intensity immune response. As entering antigen-presenting cells, CpGs dissociated from carrier tubes and bound to TLR9 receptor of endosomal membrane. Compared with the equivalent amount of free CpG-H0s, CpG-H0-modified DNA nanotubes triggered a higher cytokines secretion with more than fivefold of CD69 expression by dendritic cells. Compared to Lipofectamine, a commonly used lipid transfection reagent, DNA nanotubes can induce higher levels of IL-6 and CD69 expression but lower cell viability. Interestingly, DNA nanotube itself was reported with the ability to activate innate immunity through a non-TLR9-mediated pathway. However, if the CpG sequence is decorated in the DNA tube, immune stimulation is mainly performed through the TLR9-mediated pathway. These traits should be considered when DNA nanotubes are used in future vaccine vectors.

Besides inducing immuno-related cytokine expression, DNA nanotubes can also induce the recruitment of leukocytes. Forty-eight different oligonucleotides are temperature-controlling assembled into eight parallel double helices to form a DNA nanotube, combined with 20 nt CpG ODN [78, 79]. This complex is found stable in serum at different normal tissue-like concentrations and can significantly increase TNF-α expression levels in RAW 264.7 macrophages. In vivo study suggested that NF-κB pathway and TLR9-mediated immune response were involved. Within 5 min after venous microinjection in the cremaster muscle, DNA nanotubes were rapidly internalized by resident cells attached to blood vessels and tissues around the injection site. Inspiringly, a significant recruitment of leukocytes into the target tissues, depending on the activation of mast cells, was also observed. Mast cells were close to the inner side of capillary cavity, quickly degranulated after receiving cytokines secreted by macrophages [79, 80], then released pro-inflammatory mediators [81, 82], and increased leukocyte' stickiness [83–85],

allowing leukocytes to be expelled from the venules behind capillaries. This phenomenon could not be caused by ordinary DNA nanotubes or CpG ODN, indicating DNA nanotube as a potential vehicle for targeted macrophage recruitment, but its mechanism is not clear. For a long time, the low affinity of proteins to DNA nanotubes limits its application in the field of vaccine. Recently, Sprengel et al. wrapped DegP protein in an envelope-like hexagonal DNA prism, with weak non-covalent interactions on protein surface, which protected its natural state [86]. Such DNA nanotubes are theoretically suitable for any type of protein recognition motif and are able to overcome the low affinity for ligand binding. It is expected that this structure can be used to encapsulate specific antigens and adjuvants after being modified in the lumen and plays a role in the assembly of vaccine.

In summary, with high biocompatibility, accurate design of the nanoscale cavity, and multiple ordered modification sites to facilitate the deployment of immunostimulants and the ability to recruit leukocyte, DNA nanotubes have opened up broad prospects in the field of immune engineering.

### *7.2.3 DNA Hydrogel*

DNA hydrogels are formed by cross-linking different DNA monomers into a 3D network [87] (Fig. 7.1c). By changing the type and concentration of DNA monomers, DNA hydrogels have been designed to enable a variety of biomedical applications, including drug delivery, cell encapsulation, and immune regulation [88, 89]. Sequence-based immunostimulatory and immunosuppressive effects have been identified in DNA hydrogels [88, 90]. Compared to its DNA strand components, cross-linked DNA gels are more physically and chemically stable which often take longer to be degrade. Extending the retention time of DNA hydrogels in the body may help to enhance immune response and develop adaptive immunity in cancer treatment [88].

The DNA hydrogel structure which consists of Y scaffolds with three CPG ODN single chains and linkers were confirmed to be rapidly formed without any chemical treatment and can thermally stimulate by switching between gel and sol states within the transition temperature. Therefore, the local temperature changes between normal tissues and tumor areas have shown huge potential in the concentration and induction of immune responses at tumor sites and exert antitumor effects [91]. DNA supramolecular hydrogel vaccine (DSHV) which was formed from Y-type DNA hydrogel with P1 antigen was applied to the top of macrophage RAW264.7 cells which stained with CM-Dil dye at 37 ° C for 30 min. DSHV system inherited the self-healing properties of DNA supramolecular hydrogels which can ensure sufficient mechanical support for close contact between cells and immunostimulants/antigens, which was able to induce a strong immune response. The migration of RAW264.7 cells was observed that the cells migrated up 100 μm into the DSHV about 1 h with a turntable confocal laser scanning microscope. This antigravity movement of the cells proved that DSHV can effectively recruit macrophages. At the

same time, after the cells passed through the DSHV, no obvious channels were left. Detecting with ELISA reagent, it was found that the DSHV group could strongly produce 365 pg/mL IL-6 and IL-12 pg/mL IL-1, which exerted the most influence on cytokine-inducing effect compared to the control group [92]. Two types of X-DNA were constructed using four oligodeoxynucleotides; one contains six valid CpG motifs (CpG X-DNA) and the other not (CpG-free X-DNA). CpG X-DNA hydrogel was more effective than its components and the hydrogel without CpG on the production of TNF-α from mouse macrophage-like RAW264.7 cells and the maturation of mouse dendritic DC2.4 cells. The cytotoxic effects of X-DNA, doxorubicin (DOX), and their complexes (DOX/X-DNA) were examined in colon26/Luc cells which are murine adenocarcinoma clones stably expressing firefly luciferase and RAW264.7 cells co-culture systems. Among of them, DOX/CpG X-DNA showed the highest ability to inhibit colon 26/Luc cells proliferation and colon26/Luc subcutaneous tumor growth by slowly releasing DOX from CpG DNA hydrogel. These results indicated that CpG DNA hydrogel was an effective continuous system that transmits CpG DNA to TLR9 positive immune cells and DOX to cancer cells [24]. Hexapod-like DNA (hexapodna) hydrogels were composed of six ODNs with unmethylated CpG sequences. An in vivo study showed DNA hydrogels were more resistant to degradation than hexapodna in DNase buffer solution and had a higher level to induce IL-6 released by cells than hexapodna and CpG-ssDNA. IL-6 expression was present at the site where the hexapod or DNA hydrogel was injected for 6 h. After 24 h, the IL-6 expression remained high only in DNA hydrogels and was observed in draining lymph nodes. However, after injecting DNA hydrogels into the skin, the IL-6 concentration in serum did not increase significantly, indicating that the DNA hydrogels only induced IL-6 expression in the local location where the hydrogel aggregated. When loading ovalbumin (OVA) with DNA hydrogels, the OVA/DNA hydrogels were found significantly increased the content of OVA-specific IgG in mouse serum and stimulated spleen cells to produce higher amounts of IFN-γ. Besides, OVA/DNA hydrogels could induce cytotoxic T-lymphocyte (CTL) response against EG7-OVA tumors in mice. Compared to complete Freund's adjuvant (CFA) and alum-injected OVA used in some vaccine formulations, OVA/DNA hydrogels did not cause significant changes in the injection site or spleen weight. However, the formation of the hydrogel delayed the clearance of CpG DNA and OVA to increase the activity of CpG DNA immunostimulatory and enhance the immune response of OVA, which indicates that the OVA/DNA hydrogel can act as an antigen, and did not cause obvious harm in vivo [88]. Chitosan is a biocompatible cationic polymer that can electrostatically interact with DNAs, which is further studied by mixing OVA/hexapod-like DNA hydrogels and chitosan (chitosan-OVA/DNA hydrogel) and injecting into mice. Compared with simple sDNA hydrogel, the structure of chitosan-OVA/DNA hydrogel was more stable and tougher, which lead to OVA antigen released more slowly and remained longer in the injection site. Compared with the OVA/DNA hydrogel, the chitosan-OVA/DNA hydrogel had higher level of serum OVA-specific IgG induction by intradermal immunity. These results indicated that chitosan-OVA/

DNA hydrogel was an improved sustained release preparation for effectively inducing an antigen-specific immune response [93].

DNA hydrogel not only plays a role in the stimulation of immune response but also suppresses immune responses for the treatment of autoimmune diseases. It has reported that activation of TLR9 can exacerbate autoimmune diseases such as rheumatoid arthritis and systemic lupus erythematosus [94]. Therefore, TLR9 inhibitors have great potential as therapeutic agents for such inflammatory diseases. TLR9 antagonistic ODNs which called immunosuppressed oligodeoxynucleotides (INH-ODNs) combined to a structure similar to the Chinese character Takumi and then processed it into a higher-order hydrogel. Flow cytometry analysis and confocal microscopy revealed that TNF-α and IL-6 activity were reduced in mouse macrophage-like RAW264.7 cells and DC2.4 dendritic cells. Compared to iTakumi and iODN1 which is a sense of iTakumi, iTakumiGel more effectively inhibited the release of TNF-α, and iTakumiGel showed the highest inhibitory effect, which may relate to the decrease of CpG uptake by immune cells [90]. The more complex the structure of the nucleotide molecule is, the greater the absorbing efficiency of immune cells have when the total number of nucleotides is the same [95], so the complex structure of the iTakumiGel promote cells to uptake INH-ODNs. This result indicated that Takumi-based DNA hydrogels could be used to deliver INH-ODNs to macrophages and dendritic cells to inhibit TLR9-mediated over-induction of pro-inflammatory cytokines, which showed its potential for treating autoimmune diseases [90].

In summary, different types of DNA monomers have diverse qualities, which result in two capabilities of DNA hydrogels that enhancing the innate immunity and adaptive immunity by prolonging the action time in the body and inhibiting the immune response to treat autoimmune diseases.

### *7.2.4 DNA Nanoflower*

DNA nanoflower (NF) is formed with two types of DNA strands (a template and a primer) and replicates through rolling cycles (RCR) which is an isothermal enzymatic reaction involving many circular genomic DNAs (such as plasmids or viral genomes) to generate long components (Fig. 7.1d). Without relying on Watson-Crick base pairing, NFs are not self-assembled using conventional short DNA, but long structural units are obtained through liquid crystal synthesis, which helps NFs maintain high stability. The main reasons of their stability are the following: (1) long structural units avoid other nicking sites being sensitive to nuclease cleavage; (2) extensive inter-strand and intra-strand weaving of stable DNA building blocks to prevent denaturation or dissociation; (3) each NF is equipped with high density DNA, thereby reducing the probability of nucleases access to NF; and (4) even if the outer layer of NF is dissociated, its inner layer can maintain its function [96]. Because of its assemblability and biosecurity, it is widely used in drug loading, transportation [97], and biological imaging [98]. In order to optimally deliver CpG ODN to

stimulate the immune cell response, CpG ODN must be internalized into the cells, especially to the endosome. Due to suitable size of NF is from 100 to 300 nm, CpG NF which consist of CpG ODNs is easily captured by macrophages and activate the immune system [99].

DNA nanoflowers can excrete TNF-α and IL-6 by TLR9 immune pathway. After incubating macrophages with 100 and 20 nM CpG-NF, free CpG, and free CpG-liposome for 8 h, ELISA analysis showed that CpG NF induced TNF-α and IL-6 secretion level was significantly higher than that induced by free CpG or CpG-liposome. Even when the concentration of CpG NFs was reduced to 10 nM, their induction still caused the saturation level of TNF-α secretion. Additionally, NFs specifically stimulated the proliferation of immune cells when they were incubated with RAW264.7 cells for 24 h [100]. Moreover, NFs can trigger the proliferation of macrophage-like cells through its immune stimulation, thereby stimulating the secretion of immune-stimulating cytokines that induce apoptosis and necrosis of cancer cells [101]. The efficacy of NFs has been proved by analyzing the co-culture supernatant of CCRF-CEM cells (T-lymphocyte leukemia, suspension cells) and RAW264.7 macrophages with flow cytometry. The result showed that the percentage of CCRF-CEM cells treated with CpG NF was significantly reduced compared with the control NF or free CpG treated groups. In addition, the inhibitory effect increased with extension of treatment time [100]. Cancer chemotherapy is partially hindered by side effects and multidrug resistance (MDR), which are partly caused by drug efflux of cancer cells [102], so that it is urgent to require a targeted drug delivery system to circumvent MDR. NF loading with Dox is a potential platform for circumventing drug resistance during targeted anticancer drug delivery. It has PH adaption capability which is stable at physiological pH and promotes drug release under acidic or alkaline conditions. An experiment transported NFs with leukemia cell aptamers KK1B10 (KK) to deliver Dox showed that the same concentration of KK-NF-Dox was more stable and the release of Dox from NF-Dox was slower under the condition of PH 7.4 compared with the rapid release of free Dox. At pH 5 and pH 9, the release of Dox was greatly accelerated, and its release rate was about half of the free Dox diffusion rate. Therefore, NF-Dox can transport Dox steadily during drug delivery and promote the release of Dox when accessing to acidic subcellular organelles such as endosomes and lysosomes. In short, DNA NFs can prevent drug outflow and strengthen the retention of drugs in MDR cells, thereby avoiding MDR and reducing side effects [37].

Intertwining DNA-RNA nanocapsules (iDR-NC) is consist of DNA CpG and STAT3 short hairpin RNA (shRNA) by using micro-flower nano-systems, subsequently shrunk by PEG-grafted polypeptide (PPT-g-PEG) copolymers. The nanocapsules act as a vaccine carrier based on following characteristics: (1) NC improves the delivery efficiency of lymph node at the tissue level and APC at the cell level; (2) acid-labile PPT not only ensures solubility of the high-level copolymer and effective MF contraction rate but also promotes intracellular delivery by enhancing the proton sponge effect after PEG shedding to expose cationic PPTs in acidic endolysosomes; and (3) hydrophobic PPT allows tumor-specific neoantigens to be loaded into iDR-NC through the hydrophobic interaction between peptide

neoantigens and PPT to co-deliver adjuvants and antigens [103]. Janus kinase/signal transduction and transcription activation (JAK/STAT) pathway has been targets of cancer immunotherapy [104], which can inhibit APC by various mechanisms, such as induction of antigen-specific T-cell tolerance immune response and suppression of CPG-activated immune response [105]. Therefore, it is necessary to activate TLR9 pathway and inhibit STAT3 pathway for clinical cancer immunotherapy [106]. Because of its special property, it usually acts as a vaccine carrier for vaccine delivery. An animal study showed that 18.2% and 25.4% iDR-NCs were effectively delivered to DCs and macrophages, after subcutaneously co-delivering iDR-NC and CSIINFEKL which was an epitope of chicken OVA with a cysteine appended on the N-terminal. In addition, CD80 expression in DCs and macrophages also increased, which indicate APC is activated after iDR-NCs injection. When injecting iDR-NC combined with Adpgk which was a neoantigen generated by MC38 tumor mutations into C57BL/6 mice, the results showed that the compound would elicit a strong and durable antitumor T-cell response. Besides, the compound also exert a negative impact on tumor growth. Compared with the free Adpgk, the mice treated with iDR-NC/Adpgk have five times lighter lung tumor, and the radioactivity of lung and tumor marker $^{18}$F-fludeoxyglucose (FDG) was also significantly lower. Therefore, iDR-NC/Adpgk compound triggers strong and durable tumor-specific antitumor immunity [103].

In conclusion, due to the PH adaptability of DNA nanoflower, it can play a powerful role in drug transport. And after modifying and assembling it, DNA nanoflower can not only activate the immune system but also exert a strong specific antitumor effect.

### *7.2.5 DNA Dendrimer*

Dendrimer is a well-defined synthetic spherical polymer; it is composed of Y-type DNA building blocks (Y-DNA). Y-DNA consists of a rigid arm and a specially designed hybrid region that becomes a sticky end, based on which DNA dendrimer (DL-DNA) is synthesized by a controlled enzymatic ligation method and becomes a highly charged and void-containing macromolecular tree-like architecture (Fig. 7.1e). DL-DNA has a series of interesting chemical and biological properties. The chemical properties include multiple surface functional group ends on its surface, which can be used to couple biological related molecules, and the surface groups can also be precisely heterofunctionalized by programming [107]. Due to the anisotropy and biodegradability of DL-DNA, antigens can be combined with it in various ways, thereby overcoming the problems of low cellular absorption efficiency, insufficient release of intracellular antigen, and low efficiency of antigen targeting in antigen delivery. In addition, the vector has the property of transferring nucleic acid into cells without any other transfection reagents, giving it the potential to target and deliver nucleic acid of pathogen by forming a virus-nonviral hybrid system. It can further adapt to specific cells by binding specific ligands. On account

of its programmability and great flexibility, the system can realize the targeted delivery of antigen components, which may set a promising platform for DNA vaccines [108].

Y-shaped oligodeoxynucleotides (Y-ODNs) were prepared using three ODNs with the halves of each ODN being partially complementary to a half of the other two ODNs. Y-ODN induced greater expression level of TNF-α and IL-6 from RAW264.7 macrophage-like cells than conventional single-stranded ODN (ssODN) or double-stranded ODN (dsODN); therefore, Y-type CpG DNA was more immunostimulating than the other CPG motifs [109]. Subsequently, DL-DNA was prepared by linking Y-DNA monomers and had 12 or 24 efficient CpG motifs in a unit. In order to determine the difference of immunostimulatory between Y-DNA mixture and DL-DNA, researchers mixed Y0-DNA and Y1-DNA at a molar ratio of 1:3 to generate DL-DNA (G1), then connect 6 Y2s at the end of G1 to generate DL-DNA (G2), and generate DL-DNA (G3) in the same way. G1, G2, and G3 were compared under conditions that did not include/include the immunostimulatory CPG motifs. Under non-CPG pattern conditions which contained 24 CG dinucleotide sequences but no potent immunostimulatory CpG motifs, it was found that the addition of DL-DNA (G2 and G3) induced RAW264.7 cells to secret TNF-α 2 to 50 times as much as Y-DNA mixture. And compared to the Y-DNA mixture, DL-DNA induced the cells to secrete IL-6 which is about three to five times. In addition, under the conditions of concentrations of 6 μg/mL and 18 μg/mL, the amount of TNF-α secreted by cells with a larger molecular weight G3 was about 1.3 or two times higher than that with a smaller molecular weight G2. These results indicated that DL-DNA itself had stronger immunostimulatory activity than Y-DNA. Further study also found that the molecular weight of DL-DNA was positively correlated with its immunostimulatory ability in a certain range. The molecular weight determines the size of the dendritic structure, and the particle size of G3 DL-DNA is about 20–36 nm, which is within the optimal radius of spherical granule cells to be absorbed within 27–30 nm, so macrophages can enhance the uptake of G3 DL-DNA. In another group containing CPG motifs, the addition of CpG ssDNA or CpG dsDNA induced a little secretion of TNF-a in RAW264.7 cells, but high concentration of G3 DL-DNA (18 μg/mL) can significantly enhance the secretion of TNF-α and IL-6 by about 100 times in a high concentration-dependent manner compared with Y-DNA. It was indicated that DNA immunostimulatory activity containing CpG motifs could be significantly enhanced by the formation of dendrimer-like structures. After the DL-DNA is taken up by the cell, the mechanism that can induce the cell to release a large amount of cytokines may be as follows: (1) its large branched structure leads to the reduction of active site that the nuclease can contact, thereby slowing the DNA in the cell of degradation. (2) Its unique branch structure increases the chance of being recognized by TLR9. (3) It has more CPG ODN 5′ ends for receptor recognition and subsequent immunostimulation. Therefore, due to the unique advantages of DNA dendritic structure, it can not only enhance the uptake of immune cells but also further induce immune cells to secrete cytokines to maximize the immune response [110]. Besides Y-DNA, researchers also used other monomers to construct the DNA dendrimer.

DNA strands with different combinations of hexapod, tetrapod, and tripod were designed as dendritic nanomaterials to immunize macrophages showed that under the combination of hexapod and tripod, the nanomaterial could be taken up by RAW264.7 cells and induce cytokine liberate TNF-α maximally. For hexapod-tripod dendritic structure, the more number of branches, the less expression of TNF-α by macrophages. Because the immune response induced by hexapod-hexapod dendritic structure was less than that induced by hexapod-tripod dendritic structure, it constitutes an opposite point of view to the previous conclusion that the more branches of the polypods, the stronger immune response was induced [9]. And in terms of the uptake mode, the dot-like distribution of fluorescent signals in the cells indicated that RAW264.7 cells had the same mechanism for taking in dendrimers and polypods. According to the molecular size of the hexapod-tripod structure which was the largest of all structures, this experiment speculated that the ability of nanomaterials to induce immune responses in cells may be related to the molecular size of nanomaterials, indicating that larger DNA assemblies can be more effectively absorbed by cells than smaller DNA assemblies [36].

Dendritic DNA can also interact with other molecules to induce immune responses. TAT peptide is a cell penetrating peptide and can target the endosomes of macrophages. It can be linked to DNA dendrimers to enhance cell membrane permeability and increase the accumulation of nanocarriers in the intracellular and endosomes of macrophages. Loop-CpG consists of a single-stranded loop composed of 30 nucleotides containing three unmethylated CpG motifs, an 11 bp double-stranded stem, and a sticky consisting of 12 nucleotides 5′- end [36], which can induce more TNF-α and IL-6 than Y-CPG alone. In order to combine the advantages of the two, a macromolecular polymer containing TAT and loop was constructed and evaluated. Mixing TAT-DNA conjugate with loop-CpG at 16: 1 to form CPG-loop-TAT to stimulate the immune response of macrophages. The result showed that G2-loop-TAT could induce macrophages to produce more TNF-α and IL-6 cytokines through the TLR9 recognition pathway compared to TAT and G2-loop control groups [111]. The reason why CPG-loop-TAT has stronger immunostimulatory activity may be (1) the hairpin and dumbbell structure of DNA is more resistant to endonuclease degradation than single-stranded DNA [112]. (2) CpG loop DNA on dendrimers can enhance the stability of CpG ODN in the biological environment by blocking the open end of CpG ODN, thus further stimulate the uptake of cells [111]. (3) Dendrimer nanostructures is about 33.6 to 46.6 nm, which will promote the absorption of CpG-loop-TAT by cells [113]. (4) There are 48 CpG motifs in the DNA dendrimer, each ring structure has three adjacent CpG sequences, and the structure of a unit of multiple CpG promotes the interaction with TLR9, thereby enhance the immune response [114].

In summary, DNA dendrimers have the great properties to meet the demands of effective immunostimulatory compounds (adjuvants) and improve the efficiency of vaccines, so that dendrimers can provide molecularly defined multivalent scaffolds to produce highly defined conjugates of small molecule immunostimulants and antigens.

## 7.3 Challenge and Prospect

DNA nanomaterials have splendid assemblability and immunity, so they can be used as ideal vaccine adjuvants in clinical applications. The assembled DNA vaccine particles can not only promote antigen formation but also deliver and retain antigens in secondary lymphoid tissues. When co-delivering with antigen and adjuvant to antigen-presenting cells, the components are able to stimulate adaptive immune response [38] (Table 7.1). Therefore, this article mainly introduces DNA tetrahedra, DNA hydrogel, DNA nanotubes, DNA dendrimer, and DNA nanoflower to explain the application of DNA nanomaterials in the field of vaccines.

To date, the main challenges faced by DNA nanostructures are the following: (1) DNA nanostructures are structurally unstable in a physiological environment and

**Table 7.1** Various types of DNA vaccine

| DNA structure | | | Immunoreaction | References |
|---|---|---|---|---|
| DNA tetrahedron | DNA ODN | Connect to CPG | TNF-α↑ | [61] |
| | DNA ODN | Connect to CPG | Activate TLR9 pathway | [23] |
| | DNA ODN | Connect to CPG and STV | High-level antibody production, memory B-cell production | [38] |
| | DNA ODN | Itself | Inhibition of MAPK pathway | [40] |
| DNA nanotube | CPG ODN | Itself | IFN-γ↑ | [79] |
| | DNA ODN | Connect to CPG | Activate TLR9 pathway | [25] |
| | DNA ODN | Itself | Activate non-TLR9-mediated pathway | [25] |
| DNA hydrogel | Y-CPG | Connect to P1 | Recruitment of macrophages; IL-6↑; IL-12↑ | [92] |
| | X-CPG | Connect to DOX | TNF-α↑; Inhibit the growth of adenocarcinoma cells | [24] |
| | Hexapod-CPG | Connect to OVA | IL-6↑; IgG↑; Induce CTL response | [88] |
| | Hexapod-CPG | Connect to OVA and chitosan | IL-6↑; IgG↑↑; Induce CTL response | [93] |
| | iTakumi-CPG | Itself | Inhibit TLR9 pathway | [90] |
| DNA nanoflower | CPG ODN | Itself | Activate TLR9 pathway; Stimulates immune cell proliferation | [100, 101] |
| | CPG ODN | Connect to DOX | Antitumor effect; Enhance aggregation in cells | [37] |
| | CPG ODN; shRNA ODN | Itself | Activate APC immune response antitumor effect | [103] |
| DNA dendrimer | Y-CPG | Itself | Activate TLR9 pathway | [109] |
| | Hexapod-tripod-CPG | Itself | Enhance macrophage uptake; TNF-α↑ | [9, 36] |
| | Loop-CpG | Connect to TAT peptide | Enhance aggregation in cells; Activate TLR9 pathway | [111] |

are easily degraded by nucleases to lose their functions. Therefore, DNA nanostructures cannot efficiently reach diseased tissues and organs when intravenously injected into vivo [115]. (2) DNA nanostructures lack targeted delivery methods, resulting in low cell absorption efficiency. Due to the strong electrostatic repulsion between the negatively charged cell membrane and DNA components, DNA nanostructures cannot easily enter the target cells [116], which limits the ability of diagnosing and treating certain types of diseases to hinder their practical application in vivo [117]. In order to make them possess target capability, DNA nanostructures are often modified with specific recognition ligands to upregulate cell receptors or cancer biomarkers, so that they can more effectively across cells through the receptor-mediated endocytosis via [54, 118]. Besides, because the methods which produce specific arrangement between ligands and DNA nanostructures in a precise and controlled manner are absent, the biological activity of incorporated targeting ligands is not significant [119–121]. (3) The limited drug payload capacity and size limitations of DNA nanostructures inhibit their therapeutic effects. For instance, the ratio of encapsulation between drug and ligand has the limitation of molecular pairing such as inserting Dox molecule into G/C bp instead of A/T bp, and their cell uptake rate is also affected by the optimal particle size (20 to 100 nm) of the nanocarriers in targeted tumor drug delivery [118, 122], displayed in weak drug loading capacity of nanosphere DNA nanocarriers which limited by particle size and drug loading. In this case, even if the nanoparticle drug delivery system is specifically internalized into diseased cells, the concentration of anticancer drug released from the nano-formulation cannot reach the therapeutic threshold, resulting in unsatisfactory therapeutic effects.

In clinical applications, DNA nanomaterials serve as double-edged sword. On the one hand, their nanoscale size lead them to penetrate biological tissues and may destroy biological functions [123]. On the other hand, if DNA nanomaterials with reasonable dose range can be completely removed and degraded in vivo, they will have great potential in the field of diagnosis and therapy [124]. Therefore, many studies focused on removing various nanoparticles in renal system [124, 125] and found that the filtration of nanoparticles through the kidney depends on many factors, including surface chemistry and the hydrophobic/hydrophilic nature of the nanoparticles [125]. To sum up, the current problems related to the application of nanoparticles in vivo mainly include the following three points: (1) the effective delivery of nanoparticles in vivo without causing damage to other tissues; (2) the balance between sufficient nanoparticle retention time in the body; (3) clearance of key components of nanoparticles in the body.

DNA nanostructures can not only be used as a vaccine to stimulate the immune response but also can reduce the immunogenicity by encapsulating surface proteins, to serve as a therapeutic agent of autoimmune diseases. The wire-frame DNA nano-octahedron (DNO) was encapsulated in PEGylated lipids resisting to nuclease digestion and injected into primary mouse splenocytes. Flow cytometry showed that the average fluorescence of spleen cells incubated with nonencapsulated DNO (N-DNO) was $111 \pm 8$ times higher than the average fluorescence of encapsulated DNO (E-DNO), suggesting that the spleen cells reduced the uptake quantity of DNA

nanostructures encapsulated in PEG lipid membrane [126]. Besides, DNA origami structure which was smeared with bovine serum albumin (BSA) found that the BSA coating can resist the degradation of endonuclease (DNase I) to significantly improve the stability of origami and enhance transfection of embryonic kidney cells (HEK293). Most importantly, the test also observed that the BSA coating attenuated the activation of immune responses in mouse primary spleen cells [127]. Therefore, surface packaging of DNA nanomaterials can suppress the body's immune response, showing the potential for treating autoimmune diseases.

Based on the activation of interferon gene (STING), it is effective to increase the production of innate and adaptive immunomodulatory proteins such as CXCL10 and TNF-α undergoing the transcription factors interferon-regulatory factor 3 (IRF3), nuclear factor-kB (NF-κB), and Jun N-terminal protein kinase/stress-activated protein kinase (JNK/SAPK) pathway [128]. A novel immunomodulatory molecule called cyclic GMP-AMP (cGAMP) which is an agonist of STING began to bring itself into notice. Tan YS et al. delivered cGAMP into the tumor cells of head and neck squamous cell carcinoma by loading in nanosatellite vaccine, resulting in enhancement of tumor antigen density and powerful and specific antitumor effects [129]. However, the STING ligand DMXAA may induce an unwanted type II immune response when activating the STING-TBK1-IRF3 pathway [130]. In order to deal with the stimulation of type II immune response by STING, there was a study combining 3′3′cGAMP and K3 CPG weakly inducing interferon alone [7, 131] to jointly stimulate the immune response of cells. The compound was demonstrated that could synergistically induce NK cells to produce IFN-γ through the synergistic effect of IL-12 and type I interferon. And further research evaluating the influence of compound in vivo demonstrated it could suppress the type II immune response while inducing strong type I immunization and CTL response [132]. Thus, some unconventional vaccine adjuvants can also be combined with DNA nanoparticles according to the desired immune effect and be used with CPG to offset the adverse effects by agents in a certain immune link while amplifying the specific desired immune link.

More newly developed DNA nanostructures have opened a new path for the development of DNA vaccines. Bead-chain DNA nanowires (BS-nanow) is assembled from DNA tetrahedron units with precise nanometer-scale spatial control, capable of accommodating chemotherapeutic agents with high payload capacity (1204 binding sites) as well as possessing a 60-fold enhanced binding affinity for target cells. Although its application in immunoengineering is rarely explored, its high load capacity, targeted localization ability, programmability, and biocompatibility make it have immunoengineering potential, especially in the field of vaccine preparation [133]. With the development of recombinant DNA technology and biocomputer technology, it is believed that more DNA nanostructures will be developed and costs will gradually be reduced. The "plug and play" of DNA nanostructures as vaccine vectors can be realized, and even intelligent DNA can be manufactured to build artificial immune defense system.

**Acknowledgments** This work was supported by the National Natural Science Foundation of China (Grants 81800947), Sichuan Science and Technology Program (Grant 2020YFS0176), and Postdoctoral Science Foundation of China (Grants 2018M640930 and 2020T130443).

**Authors' Contributions** TZ, TLL, and HS contributed to conception and design. TLL and HS contributed to manuscript writing and figures making. TZ critically viewed, edited, and approved the manuscript. All authors read and approved the final manuscript. TLL and HS contributed equally to this work.

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