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Current State of the Art in DNA Vaccine Delivery and Molecular Adjuvants: Bcl-xL Anti-Apoptotic Protein as a Molecular Adjuvant

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Abstract

DNA vaccines (nucleic acid vaccines) have been gaining importance as promising therapeutics against infectious diseases, cancer, autoimmune disorders and allergy for the past two decades. However, the immune responses elicited by the DNA vaccines are not at the desired level to stimulate a protective immune response. Thus, studies are focused on the enhancement of DNA vaccine-induced immune response by different approaches. The most common approach is to use biomaterial-based adjuvants for enhanced antigen delivery and uptake by antigen-presenting cells. Some of these adjuvants are alum, saponins, microspheres, nanoparticles, liposomes, polymers, etc. used in vaccine formulations. In addition, molecular adjuvants like cytokines, chemokines and heat shock proteins have been shown to be promising in designing DNA vaccines. In this chapter, molecular adjuvants to improve DNA vaccination-induced immune responses will be summarized with a special focus on Bcl-xL anti-apoptotic protein.

Keywords: DNA vaccine, molecular adjuvant, delivery systems, antigen-presenting cells, Bcl-xL anti-apoptotic protein

1. DNA vaccine

DNA vaccine is a third-generation vaccine, which encompasses a vector with eukaryotic cell promoter, and a gene, which encodes for an immunogenic protein. These vaccines have been shown to elicit a robust cytotoxic T cell in comparison with subunit vaccines. Also, DNA vaccine has the capacity to induce both cellular and humoral immune responses by utilizing MHC I and MHC II antigen presentation by DCs [1–3]. Although DNA vaccines are licensed for use in veterinary vaccines since 2005, they have their own limitation due to low transfection efficiency. As a result, they perform poorly in human clinical trials and require multiple booster doses to achieve desirable immune response [2, 4, 5]. With the advent of new adjuvant systems such as nanoparticles, the immunogenicity of DNA vaccines can be enhanced considerably [1]. DNA vaccines are being currently used against a wide variety of infectious diseases as well as cancer [3].

1.1 Antigen presentation to T lymphocytes

Surface receptors of T lymphocytes interact with antigens to mount an immune response [6]. For the antigenic features; alienation, molecular weight, the delivery route to the organism is very important. At the molecular level, these receptors interact with the phagocyte cell or infected target cell, which carries antigen on its surface bound to the major histocompatibility complex (MHC). While T cells do not recognize natural antigens, antigens must first be processed by antigen-presenting cells (APCs) and then presented to the T cells with the relevant MHC protein. T cells (cytotoxic T cells—Tc) with CD8 (cluster of differentiation 8) receptors recognize MHC I antigens, while T cells (helper T cells—Th) with CD4 receptor recognize antigens on MHC II. Macrophages are the first identified antigen-presenting cells. Then, dendritic cells and B cells were identified. T-cell receptors (TCRs) are proteins that have spread through the membrane extending from the cell surface to the outer periphery. Each cell carries thousands of the same receptor surface. TCRs recognize and bind only bound peptide antigens on MHC [7, 8].

1.1.1 Major histocompatibility complex (MHC)

MHC proteins are encoded by the respective gene in the genome of all the vertebrate animals. The human MHC proteins are called human leukocyte antigens (HLAs) (human MHC I antigens, HLA-A, B and C, human MHC II antigens, HLA-DR, DQ and DP). Because of the difference in MHC proteins between the tissue donor and recipient, they were first discovered with tissue transplant rejection. Even within a species, these proteins are not structurally the same because of differences in amino acid sequences, also known as polymorphisms. For this reason, it forms an important antigenic barrier in organ transplantation. MHC genes encode two classes of MHC proteins, class I and class II. While MHC class I protein is located on the surfaces of all the nucleated cells, MHC class II protein is located only on the surface of antigen-presenting cells (APCs), including B lymphocytes, macrophages and dendritic cells. The structure of the MHC I protein comprises of a relatively small size of the β-2 microglobulin protein with α1, α2 and α3 domains linked to each other by disulfide bonds. The α1 and α2 domains constitute the variable antigen-binding domain. MHC II protein is formed by α1, β1, α2 and β2 domains, each of which is attached to one of the non-covalent bonds, and α1 and β1 domains constitute the antigen-binding domain, which is a variable part [7, 9, 10].

MHC proteins carry proteins in the cell which they are in, on themselves. Thus, if the cell is not infected, it will carry its own peptides on the MHC proteins. On the other hand, if the cell harbors a foreign pathogen or protein, it will contain foreign peptides on MHC proteins. The function of these MHC proteins is to allow T cells to recognize foreign antigen. The T cells constantly control the surface of other cells for foreign antigen presence and do not recognize foreign antigens unless they are presented through MHC proteins. No T cell interacts with MHC on a healthy cell surface, and self-attacking cells are eliminated during the development of tolerance. During antigen presentation, the MHC and peptide complex come out of the cell membrane and thus are recognized by the T cells. There are two different antigen presentations to T cells, MHC I and MHC II antigen presentation [7–9].

1.1.2 Antigen presentation via MHC I protein

Peptides that are processed endogenously in the cytoplasm of non-phagocytic cells are presented with MHC I proteins. These antigens are derived from viruses or intracellular pathogens that infect cells, also known as internal antigens. In this

pathway, for example, in a virus-infected cell, virus-associated proteins are primarily digested in the cytoplasmic proteasome. Peptide antigens of about 10 amino acids are delivered to the endoplasmic reticulum (ER), and a pore protein (TAP) produced by the two proteins acts in this stage. The peptides are bound to the MHC I protein, which results in the chaperone protein, which retains the MHC protein at that site. The resulting complex is released from the ER and goes to the cell surface and integrates into the membrane. This complex is recognized and bound by cytotoxic T cells. The CD8 receptor on the Tc cell surface strengthens it by adding the binding complex. This binding allows cytotoxic T cells to produce perforin and cytotoxic proteins that kill infected cells [7, 8, 10, 11].

1.1.3 Antigen presentation via MHC II protein

The MHC II protein is produced only in cells that present phagocytic antigen. For example, if an extracellular pathogen such as a bacterium is engulfed and then peptide antigens are provided, then MHC II proteins are produced in the ER and accumulated therein blocked with Li proteins, which inhibit binding with other peptides. The MHC II-Li protein complex is transferred to lysozyme and then combined with the phagosome to form the phagolysosome. There are foreign pathogen antigens, pathogenic proteins including connective chaperones are digested to form peptides of 10–15 amino acids. The resulting pathogenic peptides are transferred to the cell surface by binding with MHC II. This complex is recognized by the TCR on the helper T cells; the CD4 coreceptor also binds to this complex and, through interaction with the Th cells, activates to produce cytokines. The produced cytokine activates antibody production by specific B-cell clones or causes inflammation [7, 8, 11].

1.2 Advantages and disadvantages of DNA vaccination

The structure of plasmid DNA provides an advantage over other traditional protein-based or carbohydrate-based grafts in which it inherently possesses DNA vaccination. Immunogenesis from DNA vaccination takes a long time, and there is no pathogenicity caused by inactivated virus in DNA vaccines. The vaccine containing plasmid DNA (pDNA) can encode many immunogenic proteins of the same virus and can also encode similar proteins belonging to different infective agents [12]. Another important advantage is that the production of DNA vaccines is easier when compared to recombinant protein vaccines [13]. DNA vaccines prepared to make the cytokines more desirable to direct the immune system are more potent and suitable for stimulating the type of immunological response by cloning genes encoding the target cytokine and antigens into the same expression plasmids. DNA vaccines lead to sustained stimulation of antigen expression and the immune response leading to prolonged protective immunity [7]. Easy construction and manipulation of plasmid DNA are another important advantage of DNA vaccines [14].

DNA vaccines are stable at room temperature, are easy to obtain, are economical and are relatively more reliable than other vaccines. Plasmid DNA vaccines have been shown to be highly evaluated, safe and immunogenic in human clinical trials, even though they have no mental status [15]. However, it is necessary to increase transfection efficiencies of naked DNA vaccines, facilitate intracellular uptake, target into cells, and perform these operations with small amounts of DNA. Various gene delivery systems and adjuvant systems in the nanosphere are used to overcome these problems. During the use of nanotechnological adjuvant systems, the degradation of DNA is prevented, resulting in ultra-rapid delivery by targeting to desired cells [9, 16].

2. Delivery systems for DNA vaccine

2.1 Viral gene delivery systems

Viral-based gene delivery systems are carriers on which modifications are made to transfer therapeutic genes to target cells without creating viral disease. Viral gene carriers such as adenoviruses, adeno-associated viruses, lentiviruses and retroviruses exhibit an effective ability to transfer genetic material to the target cell with high gene transfer efficiency [17–19]. In viral gene delivery systems, the transferred genetic material either remains an episomal element or is integrated into the host chromosome. Desired genetic placement leads to persistent and stable protein expression but increases with the introduction of oncogenic potential mutagenesis [18, 20]. In addition, viral vectors being targeted to specific cell types, the limited availability of DNA, and the laborious and expensive large-scale production have led to a growing disincentive for the development of non-viral gene carriers. The safety risk is lower for non-viral carriers compared to viral gene carriers [18, 21–24].

2.2 Non-viral gene delivery systems

2.2.1 Physical delivery systems

Physical gene transfection delivery systems are consisting of Non-viral gene delivery systems are in which pDNA is usually applied alone, which involves mechanical processes such as microinjection, biojector, pressure and particle bombardment (gene gun), ultrasound, magnetofection, photoporation (laser assisted), hydroporation (hydrodynamic forced), droplet-based microfluidic platforms for in vitro transfection and electrical processes such as electroporation [10, 11, 21, 23, 25]. In addition, ultrasound and microbubble-mediated plasmid DNA uptake is a fast, global and multi-mechanisms involved process [26, 27].

2.2.1.1 Microinjection

In microinjection, cell membrane or nuclear membrane is penetrated by simple mechanical force using a microneedle diameter of 0.5–5 μm, at a specific and reproducible depth with less physical pain than conventional DNA delivery. This gene delivery system is mainly used to inject DNA constructs in vivo. Application of DNA by this method leads to constant expression of the antigen encoded in the skin. This method can also be used to deliver DNA for a prolonged period of time, similar to the administration of drugs at a constant rate. In the method, usually the stratum corneum and the viable epidermis are breached by microneedles, after which DNA can be delivered into the dermis. There are several different microneedle methods for DNA delivery; solid microneedles can be coated with DNA prior to skin penetration, uncoated microneedles can be used to damage the epidermis prior to application of a transdermal patch containing the DNA of interest, solid microneedles constructed with biopolymers can be coated with DNA such that the needles dissolve upon contact with the fluid in the dermis to release DNA into the skin and hollow microneedles can deliver DNA into the dermis through the needles [28–30]. Quantitative introduction of multiple components into the same cell is an advantage of this technique, while technical skills are required to prevent cell damage [31].

2.2.1.2 Gen gun

The ballistic DNA delivery or DNA-coated particle bombardment (gene gun) that was first used for gene transfer to plants in 1987 uses heavy metal microparticles (e.g., gold, silver microparticles or tungsten, 1–5 μm in diameter) to hold nucleic acids and penetrate the target cells. Momentum allows penetration of these particles to a few millimeters of the tissue and then cellular DNA release. Gas pressure, particle size and dose frequency are critical factors in determining the degree of tissue damage and penetration effectiveness of the application [18, 28]. This method has various advantages such as safety, high efficiency against parenteral injection, total amount of DNA required for delivery is low, no receptor is required, size of DNA is not a problem and production of DNA-coated metal particles is easy to generate. A major disadvantage is that it induces greater immune responses than microinjection due to tissue damage with intradermal delivery, even in low doses, and also, gene expression is short term and low. This technique is a widely tested method for intramuscular, intradermal and intratumoral genetic immunization. The use of gene gun for gene therapy against various cancers in clinical trials has also been demonstrated [18, 28–30].

2.2.1.3 Biojector

This device is commonly used to deliver medications through the skin for intradermal, subcutaneous and intramuscular applications. Usually, CO_2 pressure is used to force medications (e.g., vaccine) loaded in the device through a tiny orifice, which creates a high-pressure stream capable of penetrating the skin in the absence of a needle [28–30]. Biojectors have been used to deliver different kinds of vaccines such as DNA vaccine, in preclinical studies and human clinical trials to elicit significantly higher antibody responses and cell-mediated immunity (CMI) to the conventional (needle and syringe) vaccine delivery systems [29]. Because biojector-based delivery systems can increase the uptake of DNA in tissues of the skin and muscle, efficacy of the DNA vaccine is considerably increased. In a phase 1 trial for HIV vaccine, the success demonstrated with biojector used to enhance the efficiency of DNA transfection coupled with the fact that biojectors do not use needles that will most likely lead to the increased use of biojectors for DNA delivery in the clinic [28–30].

2.2.1.4 Electroporation

Electroporation was first studied on the degradation of cell membrane with electric induction in the 1960s. The first reported study is transfection of eukaryotic culture cells through electroporation in 1982 [18, 28]. In many subsequent studies, transfection was performed on animal and plant cells via electroporation [18]. This physical gene delivery technique uses electrical pulse to generate transient pores in the cell plasma membrane allowing efficient transfer of DNA into the cells. Pore formation occurs very rapidly, in approximately 10 ns. The size of the electric pore is estimated to be smaller than 10-nm radius. If the molecule is smaller than the pore size (as in oligonucleotides and chemical compounds), it can be transferred to the cell cytosol through diffusion [18, 28]. This method has been effectively applied in humans in order to enhance gene transfer and tested in several clinical trials such as prostate cancer [28, 30], leukemia [28], colorectal cancer [28], malignant melanoma [28, 30], brain carcinomas [28], Parkinson's disease [28], Alzheimer's

disease [28] and depression [28]. When the parameters are optimized, this method is equally effective as viral vectors for in vivo application. But, the disadvantage is that it often results in a high incidence of cell death because of high temperature due to high voltage application. And also, transfection of the cells in large regions of the tissues is difficult [18, 28–30].

2.2.1.5 Ultrasound

Ultrasound (US) is a promising tool for gene delivery that has been able to facilitate DNA transfection of cells. US-mediated delivery is of interest due to its potential for repeated application, organ specificity, broad applicability to acoustically accessible organs, low toxicity and low immunogenicity. Different kinds of studies have examined gene transfection in various types of cells in vitro and with various organs and tissues in vivo, including brain [26, 30], cornea [30], pancreas [30], skeletal muscle [26, 30], liver [26, 30], heart [26, 30] and kidney [26, 30]. The advantages are that only acoustic energy is introduced into the cellular environment, which avoids possible safety concerns associated with chemical, viral or other materials introduced and left behind by other methods. Also, US-mediated delivery has been seen in many kinds of cell types and so may be broadly applicable, in contrast to other methods that often require reformulation for specific cell types. Unlike chemical delivery systems, US-mediated DNA uptake is often shown to be non-endocytotic. Acoustic cavitation plays a major role in the cell membrane permeabilization that facilitates DNA uptake. US can possibly deliver plasmid DNA to the periphery of the cell nucleus and facilitate rapid transfection by altering the cytoskeletal network. However, US-based gene transfection studies are still in preclinical trials and have the major challenge of relatively low transfection efficiency compared to optimal complexed chemical formulations and viral gene delivery systems [26, 28, 30].

The use of ultrasonication with the microbubble technique has shown great potential for intracellular gene delivery. The microbubble-cell membrane interaction serves as the key element bridging the acoustic conditions and the endpoint delivery outcomes. However, since the fundamental mechanical question of how plasmid DNA enters the intracellular space mediated by ultrasound and microbubbles is not fully understood, gene transfection efficiency is much lower than the potential for large-scale clinical needs [26, 27]. In one study, the gene transfection of human prostate cancer cell line (DU145) with fluorescently labeled DNA (pDNA gWiz-GFP) was studied after ultrasound exposure. In this process, different sonication conditions have been studied. DNA uptake, location of DNA during its intracellular trafficking and gene transfection efficiency after ultrasound exposure were followed for various periods by confocal microscopy and flow cytometry. As a result, ultrasounds delivered DNA into cell nuclei shortly after sonication and that the rest of the DNA cleared by autophagosomes/autophagolysosomes [26]. Also, ultrasound application combined with microbubbles has shown good potential for gene delivery. In one study, to unveil the detailed intracellular uptake process of plasmid DNA stimulated by ultrasound and microbubbles, the role of microbubbles in this process was investigated. So, targeted microbubbles were used to apply intracellular local stimulation on the cell membrane, and high-speed video microscopic recordings of microbubble dynamics were correlated with post-ultrasound 3D fluorescent confocal microscopic images fixed immediately after the cell. Two ultrasound conditions (high pressure, short pulse and low pressure, long pulse) were chosen to trigger different plasmid DNA uptake routes. Results showed that plasmid DNA uptake evoked by localized acoustically excited microbubbles was a fast (<2 min), global (not limited to the site where microbubbles were attached) and multi-mechanisms involved process [27].

2.2.2 Chemical adjuvant systems

The term adjuvant is derived from the Latin word "adjuvare" which means "help" or "develop." Adjuvants are used to increase the life, quality and degree of the specific immune response developed against the antigens. At the same time, they have low toxicity and are capable of sustaining the immunological activity alone by inhibiting polymer accumulation in the cell [11, 32, 33]. So, they are preferred in vaccinations for newborns or adults. Also, they can stimulate a long-term immune response by reducing the amount of antigen that must be given in a single dose of vaccine [9, 34].

Adjuvants are classified according to the source of their constituents, their physicochemical properties or their mechanism of action and are generally grouped into two subheadings. One of them is molecular adjuvants that are immunostimulants (and also genetic adjuvants) (e.g., TLR ligands, cytokines, saponins and bacterial exotoxins that stimulate the immune response) and act directly on the immune system to enhance immune response against antigens. The other one is carrier systems; they are systems that promote vaccine antigens in the most appropriate way to the immune system while also exhibiting controlled release and depot effects, thereby increasing the immune response (e.g., mineral salts, emulsions, liposomes, virosomes, biodegradable polymer micro/nano particles and immune stimulating complexes—ISCOMs) [1, 9, 11, 34–39].

Cytokines can also be delivered directly with the DNA vaccine, either on the same or on a separate expression plasmid as adjuvant duty. The effects of plasmid encoding cytokines such as interleukin IL-10, IL-12 or IFN-γ together with DNA vaccines have been studied in a variety of animal and disease models, up to clinical trials in humans [38]. Also, various studies describe the usage of plasmids coding for immune-signaling molecules, either as partial or as full-length genes. Many adjuvants function by activating the innate immune system via binding to Toll-like receptors (TLRs). Another innate immune mechanism, which is being explored for improving DNA vaccination, is the sensing of viral infections via pathogen recognition receptors (PRRs). Both proteins detect the presence of viral RNA in the cytosol. Co-delivery adjuvants with the vector coding for antigenic proteins result in significantly higher antibody titers as compared to the non-adjuvanted controls. Other strategies for genetic adjuvants include components of the complement system, protein aggregation domains, chemokines or co-stimulatory molecules. Whereas DNA vectors encoding certain cytokines have already entered clinical testing in humans, studies with many other genetic adjuvants were mostly performed in mice. Therefore, these promising studies should be optimized into powerful strategies to boost DNA vaccines in more complicated animals and humans [38].

Adjuvants can easily be internalized by the antigen-presenting cells (APCs) (macrophages and dendritic cells) because of their size being similar to pathogens (<10 μm) [11, 22, 25, 40]. The internalization and presentation of the adjuvant depends on the chemical and physical properties of the adjuvant system. It has been shown in various studies that the particles with cationic properties are more efficiently taken up by macrophages and dendritic cells [21, 24, 25, 41–43].

The characteristics that should be present in the adjuvants can be listed as follows: stability in an acidic, basic and enzymatic environment; retention of retained antigen or nucleic acid by constant release; systemic and mucosal immunity being effectively induced and balance between effective immunity and immunological tolerance at high doses [9, 44]. Also, the immunogenicity of weak antigens should increase the speed and duration of the immune response, cause only minimal local and systemic side effects, and be capable of a wide variety of vaccination with stability and ease of production. The ability to be effective in the living system of

adjuvants depends on its ability to stimulate antigen-presenting cells (dendritic cells and macrophages) and T and B lymphocytes of the natural defense system [11]. There is a need for definitive information on the structure, stability, safety and immunogenicity of the adjuvant to be used in an effective vaccine development process [32, 33]. For this purpose, the choice of adjuvant depends on factors such as antigenic structure, immunization scheme, mode of administration and desired immune response pattern [33].

2.2.2.1 Nanoparticulate adjuvant systems and DNA vaccine delivery

Nanoparticles are matrix systems called nanospheres or nanocapsules according to the method of preparation, which vary in size from 1 to 1000 nm and are prepared with natural or synthetic materials. They are also the matrix systems in which the active substance nucleic acid is solubilized, adsorbed or electrostatically interacted with the surface [23, 45].

In vaccine development studies, nanotechnology is becoming increasingly important. Numerous nanoparticles of varying composition, size, shape and surface properties are used to both increase vaccine efficacy and target vaccination. With these properties, nanoparticles play an active role in in vitro/in vivo drug and gene delivery systems. Because nanoparticles are biodegradable and easily recognizable by antigen-presenting cells (APCs), they can be easily endocytosed and used as successful vaccine carriers in vaccine delivery systems [22, 25, 40].

Nanoparticle-mediated delivery of DNA vaccines has the advantages of increasing transfection efficiency and immunogenicity, inducing both cellular and antibody responses, and not requiring special equipment during administration; there are some disadvantages, such as, the long-term effects of nanoparticles in the body are not yet known [1]. As a DNA vaccine carrier, natural polymeric nanoparticles such as chitosan, alginate, pullulan and inulin (2–1000 nm); synthetic polymeric nanoparticles such as PLGA, dendrimer, PLA, PHB and PEI (2–1000 nm); inorganic nanoparticles such as gold, silica-based and carbon-based nanoparticles (2–1000 nm); nano-liposomes (100–400 nm) which are phospholipids that can be organized in nanoscale and non-infectious virus-like particles (VLP) (20–800 nm) generated by packaging the nucleic acid into biocompatible capsid proteins [1, 9, 22] are used. In recent years, the use of complex nanoparticles to overcome the disadvantages of enhancing the function of single-source nanoparticles has been discussed. Polyethylene/PLGA, polyg-glutamic acid/chitosan, polyethylene/chitosan, dendrimer/polyethylene glycol, polyg-glutamic acid/polyethyleneimine (PEI), chitosan/tripolyphosphate and polyethylene glycol/liposome nanoparticles are remarkable DNA transport systems [1, 22, 25, 40, 46, 47]. The overall efficiency of nanoparticle-based DNA delivery systems depends on four basic factors. These factors explain the necessity of nanoparticle-based adjuvant systems. Accurate plasmid DNA is integrated with the nanoparticle into the target cell correctly, the nanoparticle is removed from the endosomal vesicles and is transferred to the cytoplasm, then is transferred to the mitochondria or nucleus [23, 25].

In recent years, there has been a tremendous improvement in the area of gene delivery system with the advent of cationic polymers. These polymers bind with nucleic acid(s) to form complex structures known as polyplexes, which have increased transfection efficiency [10, 48]. Some of the examples of the polycationic polymers are chitosan, polyethyleneimine (PEI), poly(2-hydroxy ethyl methacrylate) (pHEMA), polyamidoamine (PAMAM) dendrimers, polylactic-co-glycolic acid (PLGA), polyethylene glycol (PEG) and poly-L-lysine (PLL) and the negatively charged phosphate group of pDNA, which have cationic properties. [10, 23, 42, 43, 49–51]. Many factors such as molecular weight (MW), surface charge, charge

density, hydrophilicity and morphology significantly influence the gene transfection efficiency of cationic polymers. For this reason, in general, optimization of various forms of these cationic polymers with pDNA is required to increase transfection efficiency. The polyplex structure electrostatically formed with DNA packs it into smaller structure, pDNA, that interacts effectively with the negatively charged cell membrane and is endocytosed easily into the cell. In addition, cationic polymers are suitable for specific applications like conjugation of targeted ligands and various moieties, which provide them with specific characteristics [21, 22, 24, 25, 52].

2.2.2.1.1 Liposomes

Since their discovery in 1964, liposomes are extensively studied and used in the pharmaceutical and cosmetic industries. It is a generic name for single- or multi-lamellar vesicles in lipid-based nano- and micro-dimensions, whose surface charge can be changed by lipid formulation. Thanks to biocompatibility, biodegradability, low toxicity and low immunogenicity, liposomes have been used as potential nucleic acid carriers both in vitro and in vivo systems. In the composition of cationic liposomes, neutral lipids, cationic lipids and/or anionic lipids can be complexed in different ratios for creating ideal co-lipid. Neutral lipids (DOPE, DPPC, DOPC and Chol) are also often involved in the cationic liposome structure for more efficient transport systems, although sometimes only cationic lipids (DOTAP, DC-Chol, MMRIE, DODAP, DDTAP and DDA) are used [8, 36, 53]. The choice of ideal co-lipids is important during the formulation phase [54–56], since they significantly affect the overall performance of cationic liposomes.

There is a strong correlation between morphology and performance of lipoplex (liposome and DNA complex). There are several models that determine morphology such as the external model (DNA is attached to the cationic liposome surface), the internal model (DNA is surrounded by the cationic liposomes), the cationic liposome beads model on the DNA sequence and the spherical model. In addition to the liposomal composition, cationic lipid/nucleic acid (N/P) load ratio, preparation methods, ionic strength and temperature also affect lipoplex formulation and morphology. While high N/P ratios accept that the lipid/DNA complex is compatible with the global model with effective DNA concentration [22, 54, 57, 58], the low N/P ratio accepts the model of cationic liposome beads on the DNA sequence. In addition, high concentrations of cationic liposomes can cause cytotoxicity. The particle size of the lipoplexes is also influential on transfection efficiency. In general, large lipoplexes are more effective with in vitro transfection of nucleic acids because large pieces allow rapid sedimentation, maximum cell contact with the cell membrane and easier separation after endocytosis. Small particles, on the other hand, are much more effective, safe and suitable for in vivo transport of nucleic acids [57, 59].

Various studies have shown that cationic liposomes (DC-Chol/DOPE) formed at various ratios of 3β-[N-(N′,N′-dimethylaminoethane) carbamoyl] cholesterol (DC-Chol) and 1,2-dioleoyl-sn-glycero-3-phosphatidylethanolamine (DOPE) are effective pDNA carriers in the preclinical as well clinical trials for plasmid transfection [55, 57, 60]. In another study, it was reported that the vaccine adjuvants with high immunogenicity were obtained with cationic liposomes formed at 50:50 ratio (50 mol%) with DC-Chol/DPPC (3β-[N-(N′,N′-dimethylaminoethane) carbamoyl] cholesterol/1,2-dipalmitoyl-sn-glycero-3-phosphocholine) [53]. In many studies, it has been reported that surface modifications to cationic liposomes (PEG-polyethylene glycol coating, etc.) increase transfection efficiency due to more stable plasmid retention, longer circulation time and lower immunological response. Studies have shown that transfection efficiency is increased by about 55% with PEG coating to protect surface charges [23, 48, 54, 56, 57, 61].

2.2.2.1.2 Chitosan

Chitosan is the best-known working polymer among the therapeutic natural polymers. It consists of D-glucosamine and N-acetyl-D-glucosamine units linked by β-(1,4) glycosidic bonds. The chitosan is derived from the chitin molecule and the thermoacetal deacetylation process forms the bonds. The chitin is a biopolymer in abundance in nature and is the cell wall of most of the fungi and bacteria of the shellfish and the outer shell of insects [62]. One of the most important applications of chitosan is its application as non-viral vector in gene therapy. For this reason, chitosan has recently been used for gene delivery systems for therapeutic purposes [50, 63]. The first chitosan/DNA complex was made about 25 years ago, and was composed of plasmid and chitosan in size of 150–600 nm. Positive charge, which allows it to complex easily with negatively charged DNA, allows the formation of nanocapsules (80–500) at various sizes that protect DNA from nuclease activation [64, 65].

Chitosan nanoparticles are formed by a wide variety of methods, by the formation of different bonds, by different polymer conformations and by various internal and external molecular interactions. In addition to covalent cross-linking and desolvation methods, ionic gelation is one of the most useful methods used in the handling of chitosan nanoparticles and is especially considered for polyelectrolyte sodium tripolyphosphate (TPP) [42, 43, 66]. In chitosan-based adjuvants, DNA is involved in structure determination, electrostatic interaction, encapsulation and surface adsorption [42, 50]. Chitosan has excellent biocompatibility, acceptable biodegradability, high biosecurity, low cytotoxicity and low immunogenicity. However, limited application of gene transport due to poor solubility at physiological pH, insufficient positive charge and low transfection efficiency can be prevented by various surface modifications on chitosan nanoparticles [25]. For example, TPP is useful in preparing chitosan nanoparticles and enhances its non-toxic nature. Due to the self-regulation nature of polycations and polyanions, it leads to the formation of linking complexes between TPP groups and chitosan amino groups. In the process of the protonation of the chitosan at physiological pH, TPP is covalently bound to chitosan amino groups providing structural change and better provocation [23, 50, 61, 67, 68].

2.2.2.1.3 Polyethyleneimine (PEI)

Polyethyleneimine is a gene-carrying cationic polymer that has high transfection efficiency in vitro and in vivo with lower cost, but can exhibit high toxicity with concentration pulse [69–71]. PEI receives proton in aqueous solutions and has a high positive charge. Thus, in vitro and in vivo DNA and oligonucleotide transport are promising candidates as non-viral transport systems [71, 72]. PEI and its derivatives are commonly known as an effective dispersant and cationic flocculent used for negatively charged colloids [73].

Adjuvant systems with PEI involve the electrostatic interaction of DNA with the cationic polymer and the formation of the polycation/DNA complex [74]. The DNA/polymer complex (N/P) occurs when the amine group of this polymer interacts with the phosphate group of DNA [73]. In many studies, it has been shown that PEI has the advantages of holding pDNA with electrostatic bonds, binding to the cell surface and its endocytosis, and releasing pDNA into the cell. It also enhances the entry of the gene from cytoplasm to the nucleus [70]. This polymer is used for DNA-based immunotherapy or DNA vaccine delivery to ensure that the immune response is activated to provide a strong immune response [74]. Factors such as molecular weight, branching grade, ionic strength of solution, zeta potential and particle size affect the

efficacy/cytotoxicity of PEI. PEI is coated with PEG and various ligands to increase transfection efficiency and reduce cytotoxicity. Meanwhile, the PEI in the linear structure helps to create an effective transport system than the branched PEI [69].

2.2.2.1.4 Dendrimers

Dendrimers are nanometer-sized (1–100 nm) particles with a unique architectural structure in the form of spherical macromolecules, consisting of a central core, a hyperbranched mantle and a corona containing a peripheral reactive group. Dendrimers can be fabricated by convergent or divergent synthesis. The high-level control system on dendritic architectural synthesis makes dendrimers almost perfect spherical nanostructures with predictable properties. Dendrimers can build up ionic interactions with DNA, creating complexes with high stability and resolution. Costly production is the only disadvantage. Structures such as polyamidoamine and polypropylenimine are included in the dendrimeric classification [25, 75–78].

Of the cationic compounds used in gene delivery, polyamidoamine (PAMAM) dendrimers have been regarded as the most suitable gene carrier, due to the presence of abundant amino groups on the electrostatically interacting surface with negatively charged nucleic acid material and low polydispersity. This association at the nanoscale (nucleic acid-dendrimer pair) is called dendriplex [79]. These particular supramolecular structures not only protect the genetic material from nuclease degradation but also interact with the negative surface of the cell membrane and activate entry into the cell through endocytosis. The high amine content of PAMAM dendrimers provides significant buffering capacity in the endosomal pH range. This extraordinary buffering capacity plays a powerful driving force in the liberation of dendriplex complexes prior to enzymatic degradation to lysosomal enzymes in endosomes. These properties make PAMAM dendrimers the carriers that are obliged to carry out future polycation-based gene delivery studies. On the other hand, long-term storage stability and high biocompatibility make PAMAM dendrimers almost necessary to use as gene carriers in vivo [24, 25, 49, 51, 61, 75].

The number of generations on the transfection efficiency is important. Dendrimer generations G0–G3, low-grade PAMAM dendrimers, exhibit low gene transfection efficiency and low cytotoxicity, while G4–G8 dendrimers show high transfection efficiency and high cytotoxicity. For this reason, dendrimer generations G4–G5, which have low cytotoxicity as well as high transfection efficiency in gene transfer, are preferred [25, 76]. In addition, adding different moieties enhances various features of dendrimers. For example, high amine content on the PAMAM surface allows conjugation of various materials to improve transfection efficiency and reduce target cytotoxicity [24, 25, 49, 61]. PEG conjugation provides positively charged protective sheath, which reduces cytotoxicity, undesired interactions with blood components, and facilitates binding of the ligand. Adding hydrophobic moieties favors hydrophilic-hydrophobic balance, reduced cytotoxicity and facilitation of packaging back into the vector. With glucocorticoid conjugation, nuclear targeting and parental dendrimers (dexamethasone and triamcinolone acetonide conjugates) provide hydrophilic-hydrophobic equilibrium modulation. By cyclodextrin conjugation, increase of endosomal escape and decrease of cytotoxicity as well as oligonucleotides against enzymatic digestion are protected. Finally, by amino acid, peptide and protein conjugation, it is also possible to increase cell penetration (arginine and TAT peptide conjugation), cellular uptake, endosomal escape, serum resistance (histidine conjugation), and nuclear localization, and also target specific receptors [24, 25, 51, 61, 77, 78].

2.2.2.1.5 Poly(lactic-co-glycolic acid) (PLGA)

PLGA is a solid polymeric material approved by the Food and Drug Administration (FDA) for nanoparticle-based drug and gene delivery systems. Their biocompatibility, biodegradability, reliability and high stability characteristics during storage provide advantages in delivery systems [20, 80–82]. PLGA nanoparticles can easily pass through vessels in vivo without damaging the tissues surrounding the tumor and thus accumulate with the mechanism of "enhanced permeability retention" (EPR) in solid tumors. However, PLGA particles are less efficient in encapsulating nucleic acids because hydrophobic properties of PLGA are not compatible with anionic, hydrophilic properties of nucleic acids. In addition, difficult preparation conditions and pH decreases during PLGA hydrolysis inactivate nucleic acid loading and prevent polyplex formation. In order to overcome these drawbacks, effective gene transfer systems can be formed with different formulations made with different molecular interactions [21, 48, 52, 61, 83–86].

For example, PLGA nanoparticles can be processed with materials such as PEI to increase positive charge distribution and provide a stronger penetration of nucleic acid. After penetration of the nucleic acids, PEI, PEG cross-linking material and cell penetration peptides can be used for effective encapsulation and stabilization of the nano-carrier system [48, 81, 85]. It is also one of the systems to produce PLGA-based adjuvants in sizes of 200–300 nm by such means as cationic hydrophilic properties by condensing PLGA with cationic polymers such as polyethylenoxide (PEO) and polyethylene glycol methacrylate (PEGMA), emulsifying solvent diffusion method without shear stress [48, 81, 85].

In one study, DNA-loaded PLGA particles were fabricated by a double emulsion water in oil in water (w/o/w) method, in which energy is introduced to the system typically by either sonication or homogenization, and they were provided with submicron size (generally 0.1–10 μm). Then, conjugation of PLL to PLGA was achieved through the coupling agent at different percentages to create pDNA/PLGA/PLL (poly-l-lysine) complex. This system achieved effective gene transport by acquiring cationic adjuvant property. [87]. In another study, it has been reported that the PLGA particle, which is condensed with the cationic lipid DOTAP, provides efficient pDNA encapsulation by forming a cationic adjuvant system [88].

2.2.2.1.6 Polyethylene glycol (PEG)

Polyethylene glycol (PEG) is a highly hydrophilic, non-immunogenic, semi-crystalline, linear polyether diol used as a non-ionic polymer consisting of ethylene oxide monomers. PEG, a polymer approved by the Food and Drug Administration (FDA), is non-toxic at low density and does not damage active proteins or cells. PEG is excreted completely through the kidneys (<30 kDa PEGs) or stool (>20 kDa PEGs) [89]. In addition, functionalities by conjugation of different terminal groups such as amino, carboxyl and sulfhydryl groups can be increased. It is soluble in aqueous solutions and in most organic solvents like methanol and dichloromethane [89].

The physical properties of the PEG material vary with the molecular weight. With an increase in the molecular weight, viscosity of PEG increases, while the water solubility decreases. Furthermore, the high solubility of PEG in organic solvents provides a great advantage in preparing solid dispersions. PEG provides stability to coating particles. The flexibility of the polymer chain, which allows the polymer units to rotate freely, ensures that the PEG protects the particles. Thanks to its high hydrophilic property, it creates a protective shield around the particulate. Nowadays, PEG is used not only to increase stability and circulation time of particles in vivo, but also to target particles to the desired areas [90].

In vivo transfection experiments with DC-Chol/DOPE liposomes with 1% PEG coating showed that the PEG coating increases the stability and longevity of the adjuvant, while it decreases the pH sensitivity and thus decreases the transfection rate. This pH sensitivity is important for the vaccination strategies carried out in the treatment of an existing tumor tissue [55]. However, there is no indirect disadvantage of PEG coating on the transfection rate, as there is no intention to improve the present tumor tissue, but there is no need for pH sensitivities for adjuvants in vaccine studies designed to protect against tumor formation [23, 54, 56, 61].

2.2.2.1.7 Poly(2-hydroxyethyl methacrylate) (pHEMA)

The pHEMA [poly(2-hydroxyethyl methacrylate)] polymer is the polymerized non-toxic form of HEMA (hydroxyethyl methacrylate) which is a toxic monomer. Hydrogels are hydrophilic and are capable of holding water up to thousands of times more than their own dry mass. For this purpose, pHEMA that is virtually uncharged is a three-dimensional hydrophobic polymer that can swell in water or biological fluids, and it can be used with a large number of pathways [91, 92]. Because of its high water content such as those in body cells, it is used in ureters, cardiovascular implants, contact lenses, tissue restorative surgical materials and many dental applications [93, 94]. pHEMA is also used in the pharmaceutical industry and in tissue engineering because of its biocompatibility and similar physical properties as living tissues [93]. In addition, the pHEMA polymer has been developed by virtue of its high biocompatibility properties and successful complexes formed by a wide variety of cationic compounds. It is also used in DNA purification, RNA adsorption and drug and enzyme transport [91, 95, 96]. These approaches shed light on the creation of new adjuvant systems for the transport of genetic material using pHEMA [91, 93, 97].

In our previous studies, we purposed to develop new pHEMA-based adjuvant systems to increase the immune effectiveness and protectivity of the DNA vaccine. Within this scope, cationic pHEMA-His/PEG, pHEMA-Chitosan/PEG, pHEMA-PEI/PEG and pHEMA-DOTAP/PEG particles were developed. As a result, all pHEMA-based adjuvant systems, which can be produced in nano-sizes and in the desired properties, have been shown to increase in vitro transfection efficiency compared to naked DNA by using them in different pDNA/adjuvant formulation ratios. When compared to Lipofectamine 2000 agent, pHEMA-PEI and pHEMA-DOTAP adjuvant formulations are promising candidates for gene transfection agents [98].

2.2.2.2 Characterization of adjuvant systems

Advances in adjuvant systems have led to the development of biodegradable, environmentally responsive and biocompatible vaccine carriers (e.g., droplet-based microfluidic devices). An ideal adjuvant system should effectively interact with both the pDNA and cellular membrane and should not elicit an immune response or cytotoxicity. Characterization studies of pDNA vaccine-loaded delivery systems are carried out by size and zeta potential measurements, transmission electron microscopy (TEM), scanning electron microscopy (SEM), atomic force microscopy (AFM), gel retardation assay with agarose gel electrophoresis, PicoGreen assays, robustness assays and FT-IR [26, 31, 99–102].

2.3 Cellular uptake of delivery systems

Cellular uptake adjuvant/nucleic acid formulations mainly depend on type, size, shape as well as composition, surface chemistry and/or the carrier charge. These are key factors, which affect carrier/cell interactions and the transfection

efficiency. Cellular uptake of nucleic acid-loaded delivery systems and their localization in 2D (monolayer culture) and 3D (multicellular tumor spheroids) in vitro cell culture models and also in vivo models are studied by multi-labeling 3D confocal fluorescence microscopy, flow cytometry, overlaid bright field fluorescence microscopy based on GFP expressions, luciferase assays and fluorescence images [26, 27, 100–102].

3. Molecular adjuvants and Bcl-xL anti-apoptotic protein

Molecular adjuvants can be defined as plasmids expressing cytokines, chemokines or co-stimulatory molecules which can be co-administered with the antigenic DNA vaccine plasmid [103] or vaccine plasmid can be constructed as a bicistronic vector system. The magnitude of immune response after DNA vaccination is very closely related to: (i) the source of Ag presentation, (ii) the immunological properties of the DNA itself and (iii) the role of cytokines in eliciting the immune responses [104]. Thus, with the cells transfected by molecular adjuvant, encoding plasmids secrete the adjuvant into the surrounding region stimulating both local antigen-presenting cells (APCs) and cells in the draining lymph node, especially dendritic cells. The examples of molecular adjuvants as cytokines: GM-CSF (granulocyte-macrophage colony stimulating factor), M-CSF, IFN-γ, IL-2, IL-4, IL-7 and IL-8, IL-10, IL-12, IL-15, IL-18; as chemokines: IL-8, MCP-1 (monocyte chemoattractant protein 1), MIP-1a (macrophage inflammatory protein), RANTES (CCL5); and as co-stimulatory proteins: CD40L, CD80/86, ICAM-1 (intercellular adhesion molecule 1) [103]. In addition, ligands of pattern recognition receptors (PRRs) are described as molecular adjuvants. There are 13 TLR genes (TLR1–TLR13). TLR3 and TLR9 recognize dsRNA and ssDNA, respectively, and their ligands have been shown to act as molecular adjuvants. Poly(I:C) is a classical TLR3 ligand, and CpG is a TLR 9 ligand, showing molecular adjuvant properties via increasing cytotoxic T-cell responses [105]. Bcl-xL anti-apoptotic protein was also described as molecular adjuvant. Inhibiting the apoptosis of antigen-presenting dendritic cells, the cytotoxic T-cell responses (CD8+ T cells) are increased due to the longer survival of the dendritic cells [106]. In addition, rather than co-transfection, expression of Bcl-xL in a bicistronic vector further enhances CD8+ T-cell responses compared to co-transfection [107, 108].

In our previous studies, pIRESEGFP/Bcl-xL is a bicistronic vector bearing CMV (cytomegalovirus) promoter and IRES (internal ribosomal entry site) used as a backbone for DNA vaccination studies. Bcl-xL anti-apoptotic protein in frame with eGFP (enhanced green florescence protein) as a molecular adjuvant was also encoded by the plasmid. It's shown that Bcl-xL anti-apoptotic protein rescued cells from serum deprivation, doxorubicin, camptothecin and staurosporine induced apoptosis [71, 109], induced prolonged expression of the antigen of interest in expressed under CMV promoter that facilitates an increased CD8+ T cell response in DNA vaccination studies encoding foot and mouth disease multi-epitopes [4] and Toxoplasma gondii, SporoSAG antigen [5].

4. Conclusion

In this chapter, we have discussed DNA vaccines, several widely used and emerging gene delivery systems to increase efficacy of DNA vaccines, characterization of these systems and cellular uptake of DNA vaccines yet to be tested in the clinic in the future. Also, as means of molecular adjuvants, several agents are in

consideration like chemokines, cytokines, co-stimulatory molecules, PPR ligands and anti-apoptotic proteins.

Nanotechnology offers new strategies in formulating better adjuvants for DNA vaccines. However, they are very stable and the long-term cytotoxic effects in the body appear to be a potential problem. In order to remove this problem, most effective surface and content modifications of nanoparticles studied are being made. The relatively short history of the use of nanoparticles has led to a lack of understanding of the safety profile of human use. For this reason, many studies are being carried out in this regard today. If a safe profile can be shown as a result of these studies, this new vaccine delivery system will be considered to be an effective method, which will be widely used. In addition, nanoparticle-based DNA vaccines are seen as a strategy for future single-dose applications and the need for needle-free vaccines, as they enhance cell transfection efficiency and immunogenicity and enable targeting strategies.

In future studies, the development of nanoparticle-based gene delivery systems for different purposes will continue to be critical. Modification of toxicity and immunogenicity problems of viral vectors, enhancement of transfection efficiency as much as possible for non-viral vectors, enhancement of vector targeting and specificity, regulation of gene expression and identification of synergies between gene-based agents and other cancer therapies are promising studies. Nevertheless, the safe and efficient transport of plasmid DNA to initiate immunological responses remains an important barrier to human DNA vaccination. The development of new non-viral strategies for DNA vaccines has to continue to serve as biological insight and clinic-related methods. Specific concerns include difficulties with transfection of dendritic cells. This includes methods that target strong antigen signaling, antigen-presenting cell uptake and lymph node transduction without sacrificing biocompatibility. Carriers must deliver the genetic load specifically to the target tissue, while protecting the genetic material from metabolic and immune pathways.

DNA transfection of cells in vitro/in vivo studies requires overcoming both extracellular and intracellular barriers to gene transport from cell plasma membrane which is the barrier of intracellular DNA uptake and hinders DNA trafficking in the cytoplasm, and also into the cell nucleus that is nuclear envelope. Therefore, gene delivery methods including viral, non-viral, physical, chemical and molecular systems should facilitate DNA delivery across these barriers and into the nucleus to enable transcription without any degradation very quickly. Gene delivery systems are so important that besides the characterization of these systems by various methods such as SEM, TEM, AFM and FT-IR, the examination of their cellular uptake by various techniques like confocal complex fluorescence microscopy and flow cytometry and the development of study done in this area are extremely important in the future.

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