

# Nanotechnological Approaches for Genetic Immunization

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**Abstract** Genetic immunization is one of the important findings that provide multifaceted immunological response against infectious diseases. With the advent of r-DNA technology, it is possible to construct vector with immunologically active genes against specific pathogens. Nevertheless, site-specific delivery of constructed genetic material is an important contributory factor for eliciting specific cellular and humoral immune response. Nanotechnology has demonstrated immense potential for the site-specific delivery of biomolecules. Several polymeric and lipidic nanocarriers have been utilized for the delivery of genetic materials. These systems seem to have better compatibility, low toxicity, economical and capable to delivering biomolecules to intracellular site for the better expression of desired antigens. Further, surface engineering of nanocarriers and targeting approaches have an ability to offer better presentation of antigenic material to immunological cells. This chapter gives an overview of existing and emerging nanotechnological approaches for the delivery of genetic materials.

**Keywords** DNA vaccines • Lipid carriers • Polymer carriers • Non-invasive route • Nanotechnology

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## 1 Introduction

Vaccine development offers an attractive and cost-effective preventive approach against deadly disease. New advances in immunology, molecular biology and biotechnology as low as for the development of unique, safe and effective against some dreadful diseases like HIV, cancer, hepatitis, tuberculosis, etc. (Table 1). Genetic immunization holds potential to discover new vaccines and may be an efficient vaccine delivery system. In the early 1990s, DNA vaccines burst into the scientific limelight. Tang and Johnston described the delivery of DNA using a gene gun into the mice skin and felt that this could be a useful technique to generate antibody responses against specific transgene product (Tang et al. 1992).

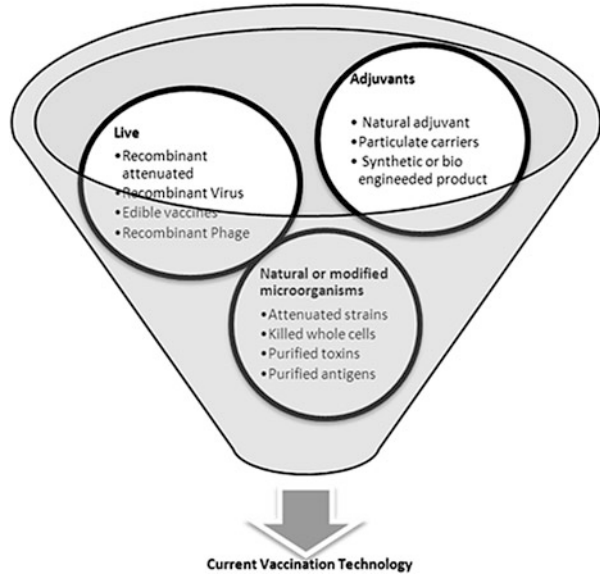
In 1992, at the annual vaccine meeting at the Cold Spring Harbor Laboratory reported to drive both humoral and cellular immune responses against pathogens or tumor antigens *in vivo* by the use of DNA vectors. Merck pharmaceutical company reported that developed immune responses against influenza virus antigens in mice after injecting the naked plasmids intramuscularly (Ulmer et al. 1993). Similarly, Robinson proved the ability of DNA plasmids against influenza virus antigens (Fynan et al. 1993). The capability of plasmids carrying HIV antigens or tumor antigens to generate immune responses and protection from tumor in mice has been described (Wang et al. 1993). Importantly, a DNA vaccine affects humoral as well as cellular immunity. The use of the DNA approach also promised to overcome the safety concerns associated with live vaccines—their reversion risks and their potential spread to unintended individuals, avoids the risks linked to the manufacture of killed vaccine (Ruprecht 1999).

Vaccines are generally composed of whole organism—either live and weakened or killed forms (first-generation vaccines). Live, attenuated organisms such as smallpox and polio vaccines are able to induce killer T-cell ( $T_C$ ) responses, helper T-cell ( $T_H$ ) responses, and antibody immunity (Fig. 1). First-generation vaccines providing maximum protection but associated with a risk that attenuated forms of a pathogen can revert to a dangerous form and may still be able to cause disease, especially in immune compromised vaccine recipients (e.g., AIDS patients). Killed vaccines cannot generate specific killer T-cell responses and will be effective against limited diseases where cellular response is not essential (Alarcon et al. 1999). These were the reasons which initiated the research for second-generation vaccines.

**Table 1** Currently using vaccines against infectious disease

Infectious diseases	Type of vaccine	Recommended route of administration
(a) Systemic		
Anthrax	Inactivated organism	Subcutaneous
Diphtheria	Toxoid	Intramuscular
Hepatitis A	Inactivated organism	Intramuscular
Hepatitis B	Inactivated organism/ subunit	Intramuscular
Haemophilus influenzae B type	Conjugate	Subcutaneous
Influenza	Inactivated noninfectious virus	Subcutaneous or intramuscular
Japanese encephalitis	Inactivated mouse- brain derived	Subcutaneous
Lyme disease	Protein subunit	Intramuscular
Measles	Live virus	Subcutaneous
Meningococcal	Purified bacterial capsular polysaccharide	Intramuscular (meningococcal conjugate vaccine), subcutaneous (meningococcal polysaccharide vaccine)
Mumps	Live attenuated virus	Subcutaneous
Pertussis	Whole cell or acellular	Intramuscular
Streptococcus pneumonia	Polysaccharide	Intramuscular or subcutaneous
Poliomyelitis (inactivated (killed) polio vaccine)	Inactivated organism	Intramuscular or subcutaneous
Rabies	Cell-cultured or embryonated egg	Intramuscular or intradermal
Rubella	Live attenuated	Subcutaneous
Smallpox	Live virus	Percutaneous
Tetanus	Toxoid	Intramuscular
Typhoid fever	Vi polysaccharide	Deep subcutaneous or intramuscular
Tuberculosis	Live organism	Intradermal
Varicella (chickenpox)	Live attenuated	Subcutaneous
Yellow Fever	Live viral	Subcutaneous
(b) Mucosal		
Cholera	Toxin B subunit +inactivated organism	Oral
Cholera	Live attenuated	Oral
Influenza	Live attenuated	Nasal
Poliomyelitis (live attenuated (weakened) oral polio vaccine)	Live attenuated organism	Oral
Rotavirus	Live attenuated	Oral
Typhoid fever	Live attenuated mutant	Oral

**Fig. 1** Advances in the vaccination technology



Second-generation vaccines were the subunit vaccines, consisting of defined protein antigens (such as tetanus or diphtheria toxoid), recombinant protein components (hepatitis B surface antigen), or surface proteins (influenza). These vaccines are able to generate TH and antibody responses, but not killer T-cell responses. This reason again restricted the utility of these vaccines to limited number of diseases. Today is the era of genetic immunization, which is next-generation vaccine (third generation), which seems to be highly effective till date. This strategy is based upon improved gene optimization, improved RNA structural design, novel formulations and immune adjuvants, and more effective delivery approaches (Alarcon et al. 1999; Robinson and Pertmer 2000). At the cellular level, introduction of nanotechnology and the development of nanocarrier-based vaccines provide effective immunization through better targeting and by triggering antibody responses. In order to induce an effective protective immunity, these vaccines require boosting with agents called adjuvants. Adjuvants and delivery vehicles have shown to protect antigens from degradation. The current trend toward many efforts to develop novel adjuvants and carrier have persistent on systems at the micro- and nanoscale.

## 2 Genetic Immunization

Immunization by traditional vaccines requires the administration of live attenuated virus, killed organism, whereas DNA vaccines can be constructed to encode specific antigenic determinants. DNA vaccines are highly flexible, stable, easily

**Table 2** DNA vaccines in comparison with other traditional vaccines

Properties	Attenuated pathogen	Inactivated pathogen	Protein vaccine	Peptide vaccine	DNA vaccine
Antibody response	Yes	Yes	Yes	Yes	Yes
Antibody rise	Fast	Fast	Fast	Fast	Slow
CTL induction	Yes	No	No	Variable	Yes
T-helper induction	Yes	Yes	Yes	Yes	Yes
Duration of response	Long	Short	Short	Short	Long
Booster dose	One or more	Multiple	Multiple	Multiple	One or more
Risk of reversion	Yes	No	No	No	No
Ease of production	Variable	Difficult	Difficult	Difficult	Easy
Cost	Variable	Expensive	Expensive	Expensive	Inexpensive
Manufacture	Rapid	Rapid	Moderate	Slow	Rapid
Safety	Low	Low	High	High	High
Stability	Low	Moderate	Low	Moderate	High

stored, manufacture on large scale, encoding several types of genes including viral or bacterial antigens, and immunological and biological proteins (Gengoux and Leclerc 1995; Kutzler and Weiner 2008). Many potential advantages of DNA vaccines are summarized in Table 2.

## 2.1 Mechanism of Action

The gene of interest having the antigenic determinant is inserted into the recombinant vectors like multiple cloning region of plasmid by enzymatically, synthetically or by PCR and delivered to the inoculation site by one of several delivery methods like physical (gene gun, electroporation), viral (virosomes), or nonviral (liposomes, microspheres, nanospheres) to either skin (intradermally), subcutaneum, or muscle. The mechanisms by which DNA vaccines produce antigen-specific immunity *in vivo* are under intense investigation, with an idealized model presented in Fig. 2.

Figure 2 represents the overview of the mechanisms of plasmid uptake and proteinaceous antigen expression by either somatic cells (e.g., myocytes, keratinocytes) at the site of injection or the resident antigen-presenting cells (APCs), the immature dendritic cells. The mechanisms include (a) direct targeting of dendritic cells (Langerhans cells, i.e., skin dendritic cells) in gene gun administration of plasmid DNA, which involves high-speed shooting of gold microbeads coated with plasmid DNA into the upper layers of the skin or (b) “cross-priming,” most likely in intramuscular injections or any parenteral injections where the somatic cells mentioned above primarily express the protein encoded by the

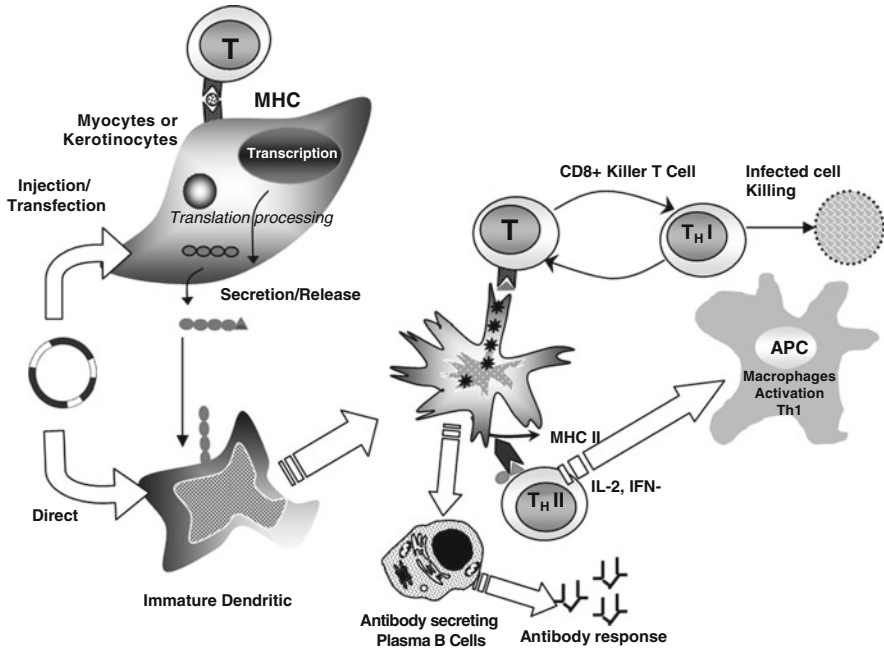


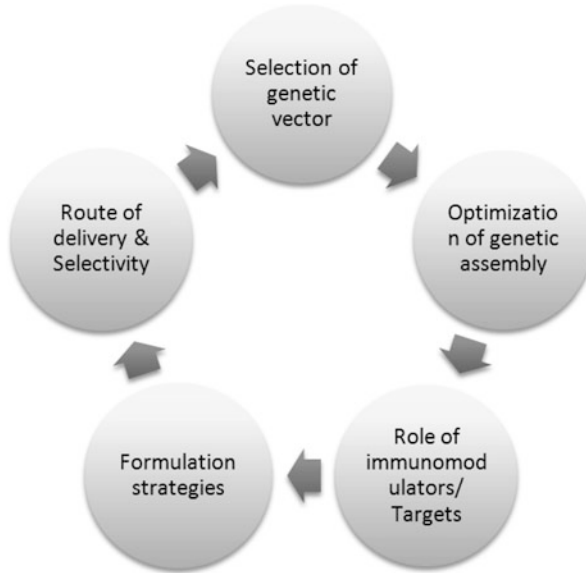
Fig. 2 Induction of cellular and humoral immunity by DNA vaccines

plasmid DNA and transfers it to the tissue resident APCs for T-cell stimulation (Kutzler and Weiner 2008).

## 2.2 Factor Influencing the Immunization of DNA Vaccines

The success of DNA vaccines concerns improving their immunogenicity and safety. Therefore, there is an urgent need for the development of potent and safe adjuvants and delivery systems that can be used with new generation of vaccines. As shown in Fig. 3, there are several ways in which antigen expression and immunogenicity can be improved for the DNA vaccine platform.

There are lot of steps undertaken to modify immunogenicity and safety of DNA vaccines (Fig. 3). Promoter is an important component of the plasmid that drives high levels of expression of the gene of interest. Various promoters have been utilized to improve the expression of vaccine genes. The human cytomegalovirus (CMV) promoter has been extensively used for high levels of protein expression in mammalian cells (Boshart et al. 1985). However, there are some drawbacks associated with CMV promoters like chromatin condensation by histone deacetylase. Recently, histone deacetylase inhibitors have been supplemented with CMV promoter-based plasmid that has shown increased expression of DNA vaccine antigens (Lai et al. 2010). Further, white spot syndrome virus (WSSV)



**Fig. 3** Factors affecting the immunogenicity of DNA vaccines

immediate-early promoter one (ie1) have also demonstrated better gene expression in insect cells compared with CMV promoter (He et al. 2008). The porcine circovirus type 1 capsid gene promoter has enhanced the antigen expression and immunogenicity in a HIV-1 plasmid vaccine (Tanzer et al. 2011).

Regulation of transcriptional termination is a key element in control of gene expression within the framework of a single transcriptional promoter (Barr et al. 2002). So one of the most effective ways to increase protein production is through the use of codon optimization or by adopting species-specific codon changes (Gustafsson et al. 2004). Plasmid backbone optimization has also been important contributory factor for DNA vaccine. Replacement of SV40t polyadenylation and splicing signals of the pAEC plasmid vectors by synthetic intron and synthetic rabbit beta globin-based termination/polyadenylation sequences and CpG motif have enhanced the cell-mediated IFN-gamma-secreting activity. The RNA polymerase II dependent cytomegalovirus immediate early (CMV IE) enhancer/promoter and T7 promoter in pSMCTA and pSHCTA has been utilized to enhance the expression of antigenic substances (Yu et al. 2005).

### 2.3 Viral-Vectored Vaccines

Viral vectors are a tool commonly used by molecular biologists to deliver genetic material into cells. Viral vector vaccines use live viruses to carry DNA into human cells. It consists of a non-replicating virus that contains some defined genetic

material from the pathogen to which immunity is desired. **Viruses** have evolved specialized molecular mechanisms to efficiently transport their **genomes** inside the cells they infect. Viral vector vaccines carry DNA into a host cell for production of antigenic proteins that can be tailored to stimulate a range of immune responses, including antibody, T helper cell (CD4<sup>+</sup> T cell), and cytotoxic T lymphocyte (CTL, CD8<sup>+</sup> T cell) mediated immunity (Draper and Heeney 2010). Retroviruses, parvoviruses, adenoviruses, lentiviruses, adeno-associated viruses, and the herpes simplex virus are being investigated for their ability to transfer DNA. Gene expression with high transfection efficiencies in tissues, such as kidney, heart, muscle, eye, and ovary, has been achieved by using viral vectors. Advantages of viral-vectored vaccines include their ease of production, a good safety profile, ability to potentiate strong immune responses, infect a broad spectrum of cell types, triggering T-lymphocyte activation, potential for nasal or epicutaneous delivery and mucosal immunization (Chamberlain 2002; Galimi and Verma 2002; Lien and Lai 2002; Martin et al. 2002; McTaggart and Al-Rubeai 2002; Wolf and Jenkins 2002).

### 2.3.1 Types of Viral Vectors

#### Retroviruses

The recombinant retroviruses have the ability to integrate into the host genome in a stable fashion because it contains a reverse transcriptase that allows integration into the host genome. 8–10 kB is the typical maximum length of an allowable DNA insert in a replication-defective viral vector.

#### Lentiviruses

Lentiviruses are a subclass of retroviruses. The unique feature of lentiviruses is to their ability to integrate into the genome of nondividing cells, whereas retroviruses can infect only dividing cells. When the virus enters the cell, the viral genome in the form of RNA is reverse transcribed and produce DNA, which is then inserted into the genome by the viral integrase (Cattoglio et al. 2007).

#### Adenoviruses

Their primary applications are in **gene therapy** and **vaccination** but their limits use in basic research due to it does not integrate into the genome and is not replicated during cell division. Respiratory, gastrointestinal and eye infections were commonly caused in humans after the contact with **adenoviruses**.



## Adeno-Associated Viruses

Adeno-associated virus (AAV) is a small virus that infects both dividing and nondividing cells of humans and some other primate species and may incorporate its genome into that of the host cell with causes a very mild immune response. These features make AAV a very attractive candidate for creating viral vectors for gene therapy (Goff and Berg 1976).

### 3 Improving Immunogenicity by Including Immune Modulatory Adjuvants

Nowadays, incorporation of molecular adjuvants has been the main strategy for melioration of vaccines. Co-injection of plasmids encoding cytokines, chemokines, or co-stimulatory molecules like death receptors, growth factors, adhesion molecules, toll-receptor ligands can be used individually or in combination to maximize substantial effect on the immune response in the clinic, in both prophylactic and therapeutic studies to plasmid-encoded antigen. For example, boost the humoral and cellular response when antigen co-administered with synthetic oligodeoxynucleotides containing unmethylated CpG motifs in mice (Higgins et al. 2007). Recently, immunomodulation is based on targeting antigen-presenting cells (APC) “majorly macrophages” by using macrosialin promoter. The immune response of the constructed plasmids expressing JEV envelope (E) protein under the control of aforesaid promoter and cytomegalovirus (CMV) immediate early promoter against JEV have induces comparable immunity in comparison to ubiquitous promoter construct (Ahsan and Gore 2011). NK group 2, member D (NKG2D) is also reported as potent-activating receptor expressed by cells of the innate and adaptive immune systems. Recombinant mouse CMV expressing the high-affinity NKG2D ligand RAE-1 $\gamma$  has shown better expression and profound virus attenuation in vivo and could be a powerful to develop immunogenic HCMV vaccine (Slavuljica et al. 2010). In vivo dendritic cells (DC) targeting is an attractive approach with potential advantages in vaccine efficacy, cost, and availability. Genetic targeting of the DC-specific CD11c-driven active transcription factor XBP1s to DC (XBP1s/DC) has potentiated vaccine-induced prophylactic and therapeutic antitumor immunity in multiple tumor models (Tian et al. 2012). Recently, heterodimeric antigen-presenting cells targeted multireceptor ligand approaches have been implemented to access the potential of more than one APC-specific targeting unit in the antigenic molecule. Results revealed that Heterodimeric Barnase-Barstar Vaccine Molecules were potent and provide a flexible platform for development of novel DNA vaccines with increased potency (Spang et al. 2012).

### ***3.1 Mechanisms of Adjuvant Action***

Some mechanisms of adjuvant action are discussed below:

1. Vaccine adjuvants can increase the potency and immune response of small, antigenically weak synthetic or recombinant peptides in immunologically immature, immunosuppressed, or senescent individuals.
2. They can improve the immune response to stronger antigens in respect of speed, vigor, and persistence. For example, aluminum adjuvants adsorbed DTP elicit early and higher antibody response after primary immunization than do unadjuvanted preparations.
3. Vaccine adjuvants can modulate antibody avidity, specificity, quantity, isotype, and subclass against epitopes on complex immunogens.
4. They target antigen to a cell-surface receptor on APCs by formation of multi-molecular aggregates.
5. They can direct antigen presentations by direct peptide exchange on surface MHC molecules or by MHC class I or MHC class II pathways by means of fusion or disruption of cell membranes (Newman and Powell 1995).

### ***3.2 Real and Theoretical Risks of Vaccine Adjuvant***

The most important characteristic of any adjuvanted vaccine is that it is more efficacious than the aqueous vaccine but unfortunately, the absolute safety of adjuvanted vaccines, or any vaccine, cannot be guaranteed. The real or theoretical risks of administering vaccine adjuvants are local acute or chronic inflammation, painful abscess, persistent nodules, ulcers, fever, hypersensitivity, anaphylaxis, chemical toxicity to tissues or organs, autoimmune arthritis, amyloidosis, anterior uveitis, glomerulonephritis or meningoencephalitis, immune suppression or oral tolerance, carcinogenesis, teratogenesis or abortogenesis and spread of a live vectored vaccine to the environment (Edelman and Tacket 1990; Bussiere et al. 1995; Goldenthal et al. 1998).

### ***3.3 Characteristics of the Ideal Adjuvant***

1. It must be safe, including freedom from side effects.
2. It should be affordable and stable.
3. It should be biodegradable or easily removed from the body after its adjuvant effect.
4. Efficacy and immunogenicity should be achieved using fewer doses and/or lower concentrations of the antigen.

5. It should elicit a more vigorous protective or therapeutic immune response combined with the antigen than when the antigen is administered alone.
6. It must be defined chemically and biologically, so that there is no lot-to-lot variation in the manufactured product.

### **3.4 Types of Immunoadjuvant**

#### **3.4.1 Freund's Adjuvants**

Freund's adjuvant is a solution of **antigen emulsified** in **mineral oil** and used as an **immunopotentiator** (booster). Freund's Complete Adjuvant (FCA) is composed of inactivated and dried **mycobacteria** whereas the incomplete form (FIA) lacks the mycobacterial components. Although, FCA has been proved as a potent inducer of cell-mediated immunity and ability to boost the humoral immune response, but associated adverse side effects like sterile abscesses, granulomas, muscle indurations, plasma cell neoplasia, ascites and amyloidosis has limits its utility. A modified version of FCA is known as Freund's incomplete adjuvant (FIA) in which antigen is administered in water-in-oil (W/O) emulsion but without mycobacterial components. It consists of a mixture of mineral oil (Drakeol 6VR, Bayol F, Marcol 52) (85 % v/v) and emulsifier (mannide monooleate) (15 % v/v) with an equal volume of aqueous solution of antigen. Mechanism of the Freund's adjuvants is allowing a gradual and continuous release of the antigen by establishment of a repository antigen-containing locus at the site of injection or interaction with mononuclear cells such as phagocytic cells, antigen presenting cells, etc. FIA has been included in veterinary vaccines (rabies, hog cholera, canine hepatitis) (Freund et al. 1948; Fastier and Hansen 1964; Ott 1966), as well as human vaccines (tetanus toxoid, influenza vaccines) (Salk et al. 1952). In general, both FIA and FCA are indeed very efficient in raising high antibody titers, induce cytotoxic T lymphocytes (CTL) and used in priming immunizations. Morozova et al. investigated that development of inflammatory response in the rat myocardium after immunization rats with single subcutaneous injection of cardiac myosin (800 ug/kg) with incomplete Freund's adjuvant (IFA) (Gjessing et al. 2012). There are very limited studies that have been conducted which signify their utility of Freund's adjuvant for DNA vaccination. It has been demonstrated that plasmid pv-16CpG suspended in IFA has significantly enhanced both type of cellular and humoral immune responses to HBsAg (Luo et al. 2012).

#### **3.4.2 Aluminum Compounds as Vaccine Adjuvants**

Aluminum compounds [aluminum phosphate ( $AlPO_4$ ), aluminum hydroxide ( $Al(OH)_3$ ), and alum] precipitated vaccines are currently the most commonly used adjuvants with human and veterinary vaccines owing to their good track record of

safety, low cost, and adjuvanticity with a variety of antigens (Gupta et al. 1993; Gupta and Siber 1995). However, aluminum adjuvants have certain limitations such as local reactions at the site of injection, IgE antibody responses augmentation, ineffectiveness for some antigens, and inability to supplement cell-mediated immune responses (Gupta et al. 1995). Two methods are used to prepare vaccines and toxoids with aluminum compounds—in situ precipitation of aluminum compounds in the presence of antigen and adsorption of antigen onto preformed aluminum gel (Aprile and Wardlaw 1966; Holt et al. 1994; Hem and White 1995; Gupta 1998). The mechanism of adjuvanticity of aluminum compounds includes formation of a depot, efficient uptake by antigen-presenting cells, stimulation of immune competent cells of the body through induction of eosinophilia, and activation of macrophages and complement. Recently, adjuvanticity of alum has been reported due to cell death and the subsequent release of host cell DNA, which acts as a potent endogenous immunostimulatory signal-mediating alum adjuvant activity (Marichal et al. 2011). Gupta et al. (1996) showed that diphtheria toxoid adsorbed aluminum phosphate induced significant antibody levels in rabbits. Previously, Manam et al. reported that aluminum phosphate adjuvant had shown no effect on the tissue distribution and integration frequency of delivery genetic materials (Manam et al. 2000). Similarly, Liang et al. (2004) showed the similar results indicated that there was not increase in HBsAg expression when plasmid pcDNA3.1-S mixed aluminum phosphate. However, they demonstrated the better antibody titer after intramuscular immunization of BALB/C mice with pcDNA3.1-S mixing aluminum phosphate adjuvant. This study revealed that aluminum phosphate has a potential for DNA vaccination (Liang et al. 2004). Recently, Yu et al. have demonstrated the role of aluminum adjuvant for DNA vaccines against botulinum neurotoxin (BoNTs) and shown induced protective humoral immune responses (Yu et al. 2010). Combined use of IL-12 with alum adjuvants for DNA immunization have also demonstrated the significant change in the survival rates of the vaccinated animals against *Toxoplasma gondii* (Khosroshahi et al. 2012).

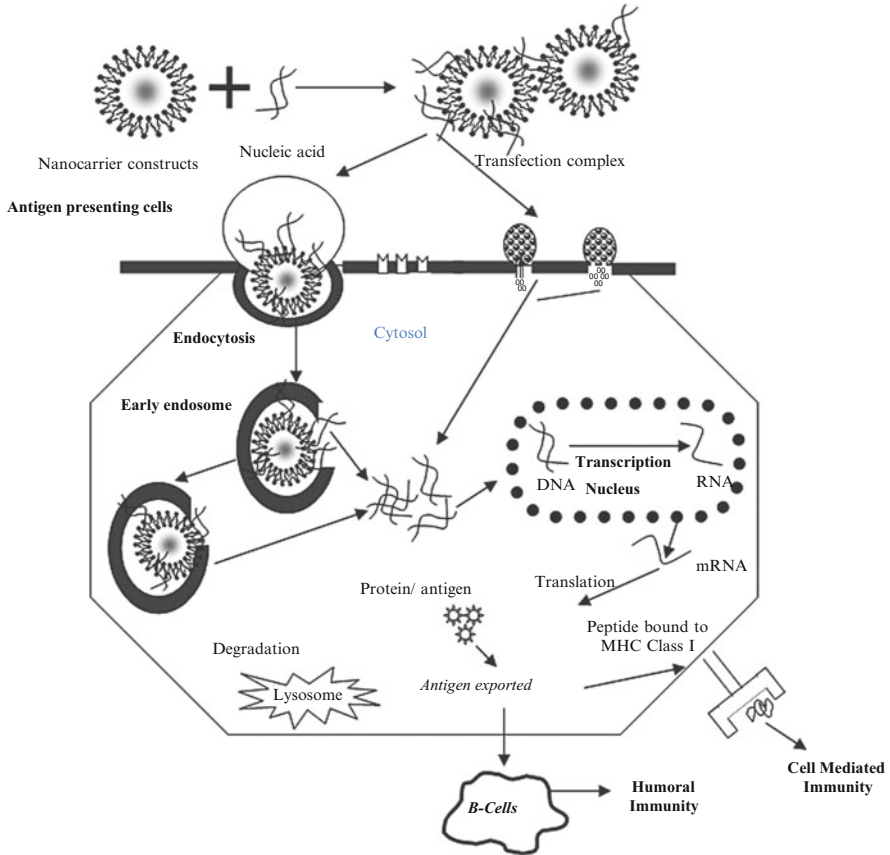
### 3.4.3 Cytokines as Vaccine Adjuvant

Cytokines are a group of secreted low-molecular weight proteins by the cells of the innate and adaptive immunity that have a major role in cell-to-cell communication. Cytokines play an important role in induction of immune responses during the processing and presentation of antigens. Numerous cytokines including interleukin-12 (IL-12), granulocyte-macrophage colony stimulating factor (GM-CSF), and interleukin-2 (IL-2) have been shown to significantly modulate the inflammatory process when given systemically. The local administration of IL-2 increases local expression of major histocompatibility (MHC) class II antigens and enhances skin antigen reactivity, but high bolus doses of IL-2 cause hypotension, exacerbation of underlying autoimmune disease, and induce vascular leak syndrome. This studies revealed that exogenous IL-2 could be a valuable adjunct in the treatment of

immunodeficiency virus (HIV) infected human by decreases the frequency of apoptotic peripheral blood mononuclear cells (PMBCs), which may contribute to the increase in circulating CD<sup>4+</sup> T cells. IL-2 also induces B-cell activation and antibody synthesis in vitro (Cordiali Fei et al. 1994). Among various improvement strategies, the incorporation of cytokine-expressing plasmids as molecular adjuvants has been widely studied in the past years, yet still without significant clinical application. This chapter reviews recent progress in the co-application of cytokine-encoding genes used for enhancement and direction of immunogenicity, as well as discusses their therapeutic potential for future applications. Co-administration of pro-inflammatory agents (such as various interleukins, tumor necrosis factor, and GM-CSF) plus TH2-inducing cytokines increase antibody responses, whereas pro-inflammatory agents and TH1-inducing cytokines decrease humoral responses and increase cytotoxic responses (which is more important in viral protection, for example). Co-stimulatory molecules like B7-1, B7-2 and CD40L are also sometimes used.

#### 3.4.4 MPL Immunostimulant

MPL (monophosphoryl lipid), a immunostimulant, is derived from the lipopolysaccharide (LPS) of *Salmonella minnesota*, R59. An important characteristic of MPL adjuvant activity is to enhance the generation of specific immunity without being directly associated with an antigen. The choice of an MPL adjuvant formulation will depend on several factors such as the nature of the antigen, desired immune response characteristics, and level of tolerable local reactogenicity. Aqueous dispersions of MPL in isotonic buffers when admixed with soluble protein antigens can provide a strong adjuvant effect. An advantage of these MPL plus antigen is that they tend to be well tolerated and induce little or no local tissue reaction at the injection site (Qureshi et al. 1985). MPL-A has been used to enhance immunity induced by DNA vaccination against human immunodeficiency virus type 1 (HIV-1). Results indicate that MPL performances as an effective adjuvant for immunogenic DNA injection despite reduced expression of encoding protein in muscle (Sasaki et al. 1997). Combination of MPL with antigen-encoded DNA has shown the enhanced protective neutralizing antibody response against glycoprotein of the CVS rabies virus (Lodmell et al. 2000). Lipid A has also been admixed with plasmid DNA (pDNA)-coated nanoparticles and studied for their immunological potential. Immunological results revealed that plasmid DNA with lipid A have shown significant higher immunological response, especially cellular response (Cui and Mumper 2003a, b). Studies indicated that LA is potential adjuvants to further enhance immune responses; however, limited studies have been utilized this adjuvant for DNA vaccination.



**Fig. 4** Schematic representation of immunological response greeted by novel DNA-loaded nanocarrier

## 4 Carriers, Vehicles, and Adjuvant Formulations

Several established methods have utilized for transferring plasmid DNA into cells, including calcium phosphate precipitation, electroporation, particle bombardment, liposomal delivery, polymeric delivery, viral-vector delivery, and receptor-mediated gene delivery. However, compared to viral vectors, nonviral vectors are easy to make and are less likely to produce immune reactions (Edelman and Tacket 1990). In addition, there is no replication reaction required. The engineered novel nano-construct may deliver immunogens safely, with the appropriate kinetics, to the appropriate location, and possibly together with the adequate recognition and maturation stimuli (Fig. 4). The use of nonviral particulate carriers for DNA-based vaccination could provide better and safe delivery of encapsulated genetic material, circumvent the need for muscle involvement and facilitate instead the uptake of the

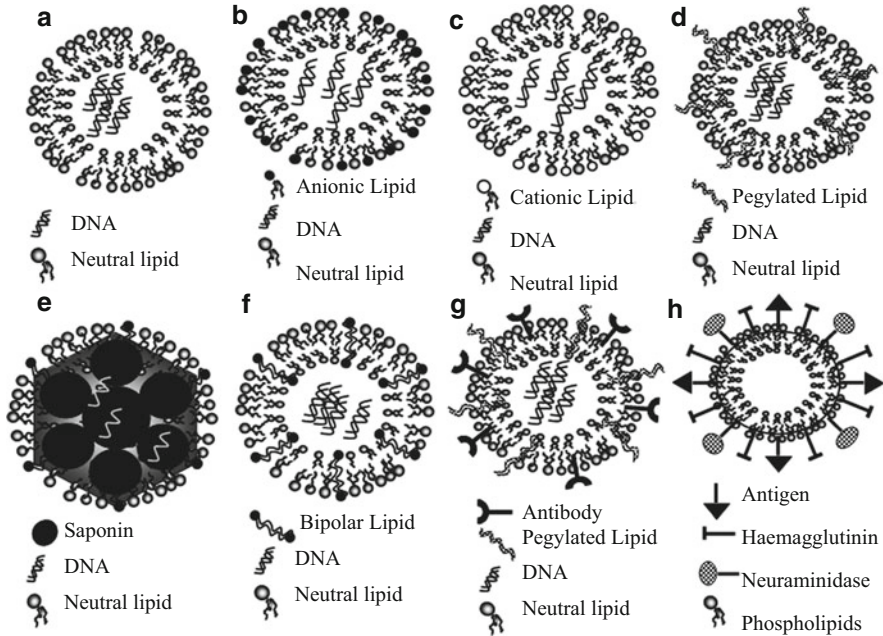
DNA by APCs. However, transfection of APCs with encapsulated DNA into particulate carrier systems will be dependent upon choice of carrier surface charge, size, and lipid/polymer composition, or presence of other biological [e.g., interleukin 2 and interferon- $\gamma$  (IFN- $\gamma$ )]. Toxicity, transfection efficiency, nucleic acid (NA) degradation and free NA release are challenging problems for all of the current nonviral gene delivery systems, including lipid and polymers carrier systems (Pouton et al. 1998; Cui and Mumper 2003a, b).

One current trend in DNA vaccine formulation is the use of biodegradable polymeric microparticles and liposomes delivery systems for DNA vaccines are excellent formulations for delivery and enhanced immunogenicity in several different hosts like mice, nonhuman primates and humans (Herrmann et al. 1999; Kaur et al. 2004). As noted earlier, genetic materials attached to a particulate carrier are more likely to bring about a successful immunological reaction and some, such as chitosan particles, can act as adjuvants in their own right. Natural polymers such as gelatin or albumin have been used as particulate drug delivery systems, although they are of uncertain purity and certainly have the potential for immunogenicity (Pouton et al. 1998; Cui and Mumper 2003a, b; Xiang et al. 2006; Pichichero 2008).

Plasmid DNA is trapped on the surface of the polymers like polylactide-co-glycolide, chitosan, polyethyleneimine, amine-functionalized polymethacrylates, cationic poly( $\beta$ -amino esters), poloxamers, and polyvinylpyrrolidone (Densmore 2003). Polymer-trapped plasmid DNA is delivered systemically or directly to mucosal surfaces (orally or via the respiratory tract), where the complex is taken up by dendritic cells (DCs) and results in upregulation of DC activation markers and further augments systemic and mucosal immune responses. Liposomes offer considerable flexibility towards vaccine optimization due to its structural versatility, including vesicle surface charge (both cationic and anionic liposomes can be made), size, and lipid content. Liposome with other suitable adjuvants can protect DNA from degradation by serum proteins during transfer of DNA across membranes and after the release of genetic material following fusion with endosome (Gao and Huang 1995; Nakanishi and Noguchi 2001).

## ***4.1 Lipid-Based Carrier Systems***

Among the different approaches to drug delivery, lipid vesicles for both hydrophobic and hydrophilic drugs have attracted much attention. Lipid-based gene delivery is the focus of several specialized high-technology companies, of which Vical (San Diego, CA, USA), Genzyme (Farmington, MA, USA), GeneMedicine (The Woodlands, TX, USA) and Megabios (Burlingame, CA, USA) have products in clinical trials. Some of the engineered liposomal and non-liposomal versions like pH-sensitive cationic and anionic liposomes, pH-sensitive immunoliposomes, fusogenic liposomes; genosomes (DNA-liposomes/lipid complexes), lipofection TM (lipid-DNA complex) and recently cochleates are investigated as the major



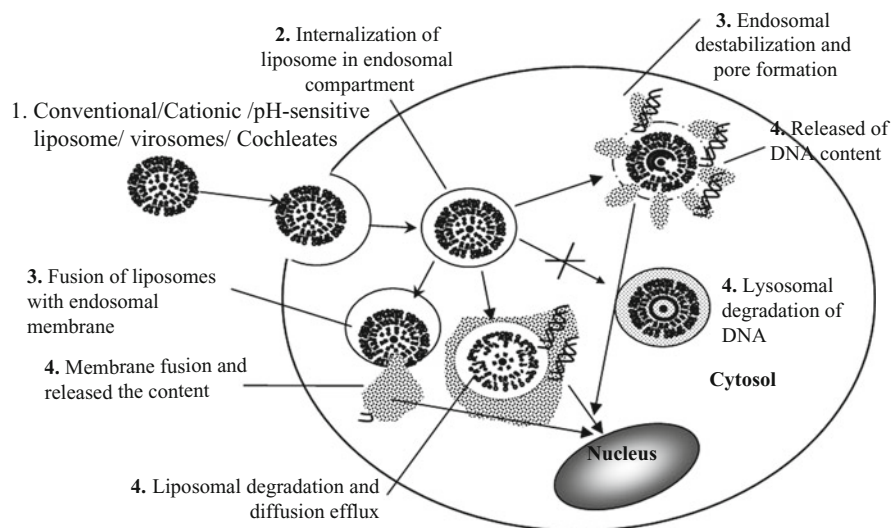
**Fig. 5** Scheme presentation of different lipid-based vesicular systems (a) conventional liposomes, (b) anionic liposomes, (c) cationic liposomes, (d) pegylated liposomes, (e) ISCOMS, (f) Archeosomes, (g) immunoliposomes and (h) Virosomes

gene vectors (Fig. 5). However, most of the commercially available nonviral gene vectors used for transfection is cationic liposome–DNA complexes (Fenske and Cullis 2008).

#### 4.1.1 Liposomes as Immunological Adjuvant and Vaccine Carriers

Liposomes are self-assembling structures comprising concentric amphipathic lipid (e.g., phospholipid) bilayers separated by aqueous compartments (Baca-Estrada et al. 2000; Saupé et al. 2006). In 1974, first humoral immune responses observed in mice after injection of liposome-entrapped diphtheria toxoid (Allison and Gregoriadis 1974; Manesis et al. 1978). Liposomal vaccines that have been investigated in human trials include malaria, HIV, hepatitis A, influenza, prostate cancer and colorectal cancer (Katre et al. 1998). In a liposome-based drug delivery system, genetic material is encapsulated in the liposome and then administered to the patient to be treated. Advantage of the use of liposomal DNA is that it may be taken up directly by APCs such as dendritic cells, which results in transfection and MHC classes I and II expression, which stimulates the CD4<sup>+</sup> and CD8<sup>+</sup> T cells by antigenic peptide and induces CTL responses and also B cells to produce antibodies, whereas vaccination with naked plasmid DNA, the plasmid is taken



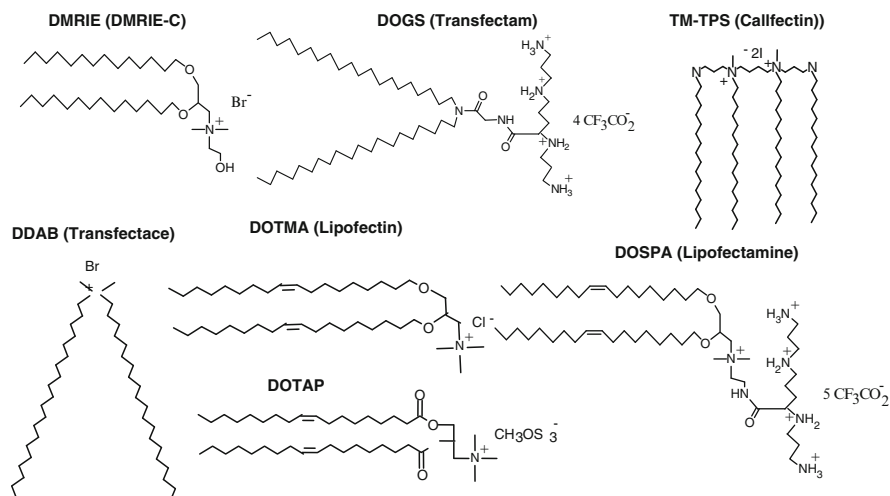


**Fig. 6** Overview of mechanistic aspects of different liposomal formulations

up by the myocytes, which are transfected. Unfortunately, there are a number of problems associated with the use of conventional liposomes as genetic vaccine delivery vehicles. The relatively low transfectivity of liposomes, particularly evident with insufficient quantities of polynucleotide within liposomal formulations, can be overcome by adding positively charged amphipathic lipid moieties to liposomal formulations. Several phospholipids may be used for the preparation of liposomes entrapped vaccines include phosphatidylcholine, phosphatidic acid, triolein, phosphatidylglycerol, phosphatidylserine, distearoyl phosphatidylcholine, dioleoylphosphatidylethanolamine, phosphatidylethanolamine, Polyethyleneglycol 6000 etc. Overall, by modification, these systems may provide high membrane fluidity, flexibility, endocytosis and fusiogenic behavior, that is making this system far better than other particulate carriers (Fig. 6).

### Cationic Liposomes

Cationic liposomes are widely explored nowadays for the delivery of DNA into eukaryotes. They are formed by simple mixing of positively charged lipid bilayers with negatively charged naked DNA. The resulting cationic liposomes–DNA complexes (lipoplexes) are taken up via endocytosis, followed by their release from an early endosomal compartment (Duzgunes et al. 2003). Cationic lipid–DNA complexes have been used successfully to deliver plasmid DNA to the lungs, brain, tumors and skin, by local administration, or to vascular endothelial cells after systemic, intravenous injection (Brigham et al. 1989). In addition to different cationic lipids (Fig. 7), zwitter ionic lipids or helper lipids (DOPE and cholesterol)



**Fig. 7** Structures of some of the commonly used cationic lipids. Abbreviations: DOTMA: *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium chloride; DOTAP: *N*-[1-(2,3-dioleoyloxy)propyl]-*N,N,N*-trimethylammonium methylsulphate; DOSPA: 2,3-dioleoyloxy-*N*-[2(sperminecarboxamide)ethyl]-*N,N*-dimethyl-1-propanammonium trifluoroacetate; DDAB: Dimethyldioctadecylammonium bromide; DOPE: Dioleoylphosphatidylethanolamine; DOGS: Dioctadecyl amido glycyl spermine; DC: Chol: dimethylaminoethane carbamoyl cholesterol

have also shown an important role in membrane perturbation and fusion for intracellular delivery of genetic material.

Liu et al. have shown that lipoplexes showed much higher transfection in the liver than naked DNA alone (Liu et al. 2003). Gregoriadis et al. for the first time showed that intramuscular immunization of mice with pRc/CMV HBS (encoding the S region of hepatitis B antigen; HBsAg) entrapped into positively charged (cationic) liposomes leads to greatly improved humoral and cell-mediated immunity (Gregoriadis et al. 1997). These cationic liposome-entrapped DNA vaccines generate titers of anti-HBsAg IgG1 antibody isotype in excess of 100-fold higher and increased levels of both IFN- $\gamma$  and IL-4 when compared with naked DNA or DNA complexed with preformed similar (cationic) liposomes. Further, modification of liposomal surface with polymer offers potential for oral administration of plasmid DNA and able to elicit markedly enhanced transgene-specific cytokine production following *in vitro* restimulation of splenocytes with recombinant antigen (Somavarapu et al. 2003). Modification of lipid/DNA complexes by the polymer poly(D,L-lactic acid) was found to be consistently and significantly more effective than either unmodified liposomal DNA or naked DNA in eliciting transgene-specific immune responses to plasmid-encoded antigen when administered by the s.c. route (Bramwell et al. 2002). Surface-modified mannosylated cationic liposomes were developed for targeted delivery of pDNA to APCs, and the results verified that Man lipoplex induces significantly higher pUb-M gene transfection into dendritic cells and macrophages than unmodified

lipoplex and naked DNA and it also strongly induces CTL activity against melanoma, inhibits its growth and prolongs the survival after tumor challenge compared with unmodified liposomes (Lu et al. 2007).

### Anionic Liposomes

An anionic lipid formulation called fluid liposomes was capable of delivering fluorescently labeled oligonucleotides into bacterial cells. It was composed of DPPC and 1,2-dimyristoyl-*sn*-glycero-3-[phospho-*rac*-(1-glycerol)] (DMPG). Lack of further progress of these systems may be attributed to the poor association between DNA molecules and anionic lipids by electrostatic repulsion between these negatively charged species (Perrie and Gregoriadis 2000). Liposomes have been prepared from mixtures of anionic and zwitter ionic lipids, 1DOPG and DOPE, respectively, at a molar ratio of 17:83 (DOPG:DOPE). Efficient and relatively safe DNA transfection using anionic lipoplexes makes them an alternative for gene delivery (Patil et al. 2004). Similarly, endosomolytic bacterial protein listeriolysin O (LLO) incorporated in an anionic liposome-entrapped polycation-condensed DNA delivery system (LPDII) has been developed that demonstrated better condensation of the DNA with improved transfection efficiency due to endosomolytic properties of LLO (Lorenzi and Lee 2005). Combination of cationic lipoplexes and PEGylated anionic liposomes has also been used to prepare anionic PEGylated lipoplexes. Studies demonstrated that the gene expression of the developed formulation was similar for the cationic formulation taken as a control and the anionic formulations prepared (Mignet et al. 2008). Overall, anionic lipoplex formulation shown promise as a nonviral vector with high-transfection efficiency and low cytotoxicity.

### pH-Sensitive Liposomes

A growing amount of literature describes the role of pH-sensitive liposomes for targeting and/or release encapsulated genetic material within cellular compartment. pH-sensitive liposomes are designed to release their contents in response to acidic pH within the endosomal system, while remaining stable in plasma thus improving the cytoplasmic delivery of biopharmaceuticals. They can be generated by the insertion of DOPE into acidic lipids liposomes such as cholesteryl hemisuccinate or oleic acid (Venugopalan et al. 2002). It is reported that detergent removal method is a superior method for preparing glycosaminoglycan-resistant and pH-sensitive lipid-coated DNA complexes. This method is produced stable, but acid activatable, lipid-coated DNA complexes (Lehtinen et al. 2008). At the neutral cellular pH 7, these lipids undergo protonation and collapse into a non-bilayer structure of endosomal compartmentalization which in turn helps in the rapid release of DNA into the cytoplasm. Recently, citraconyl-DOPE (a chemical derivative of DOPE), deliver DNA-based therapeutics to cancer cells, in this manner combining the targeting and the rapid endosome release (Reddy and Low 2000). Addition of

pH-sensitive fusogenic peptide, GALA (peptide composed of repeating sequences of Glu–Ala–Leu–Ala) in lipidic preparation is also promising method to enhance the expression of the desired proteins. Studies demonstrated that addition of 0.1  $\mu\text{M}$  GALA to the plasmid/liposome complex significantly increased the transfection efficiency, especially in the case of lipofectin, but higher concentration of GALA decreased transfection efficiency (Futaki et al. 2005; Nakase et al. 2011). Similarly, pH-sensitive histidine-modified galactosylated cholesterol derivative (Gal-His-C4-Chol) has also been synthesized that demonstrate much greater transfection activity than conventional liposomes in HepG2 hepatic cells (Shigeta et al. 2007). Further, pH-sensitive TAT-modified PEGylated liposomes are utilized for delivery of tumor-specific stimuli-sensitive drug and gene delivery systems (Kale and Torchilin 2007).

### Immunoliposomes

Immunoliposomes are sophisticated gene delivery systems in which incorporation of functionalized antibodies attached to lipid bilayers used for cell targeting (Maclean et al. 1997). Using immunoliposomes, tissue-specific gene delivery has been achieved in the brain, embryonic and breast cancer tissue. Recently, immunoliposomes containing an antibody fragment were successfully used in targeted delivery of tumor-suppressing genes into tumors in vivo (Xu et al. 2002). Chloramphenicol acetyltransferase (CAT) gene-encoded plasmid was entrapped in pH-sensitive immunoliposomes comprising of H-2Kk antibody-coated liposomes with DOPE, cholesterol, and oleic acid. Studies revealed that approximately 20 % of the injected immunoliposomes were taken up by the target RDM-4 cells. Uptake was much less when liposomes without antibody were used (Wang and Huang 1987). Similarly, these authors have also reported that compositions of liposomes have altered the distribution for targeted drugs. Delivery was also dependent on the lipid composition of the liposome. The pH-sensitive lipid composition gave eight-fold higher efficiency than the corresponding pH-insensitive composition (Wang and Huang 1989). Ligand-modified immunoliposomes has been used to efficiently deliver plasmid DNA expressing NS3-NS5B (HCV-specific antigenic sequence) to antigen-presenting cells. Results confirm that this is as a more efficient delivery system than direct intramuscular inoculations with naked DNA (Zubkova et al. 2009). Overall, studies have shown that immunoliposomes are efficiently used for targeted delivery of genetic material, especially in treatment of genetic disorders; however, very limited work has been done for delivery of DNA vaccines.

### Stealth Liposomes

Stealth liposomes (polyethylene glycol(PEG)-conjugated lipids) are sterically stabilized liposomal formulations. PEGylation prevents the liposomal vesicles by opsonization and recognition from the reticuloendothelial system and conjunction

with other polymeric delivery systems such as PLL to achieve longer circulation half-lives (Mannisto et al. 2002). PEG grafted liposomes carrying antigenic epitope of gp41, a transmembrane protein of HIV-1 has shown higher immune response and prolonged persistence of antibodies than plain liposome-based antigenic formulations (Singh and Bisen 2006). Further, it is also reported that grafting of PEG on cationic liposomes have resulted in enhanced lymphatic drainage, but there is no improvement in immune responses, when compared to non-PEGylated liposomes (Carstens et al. 2011). Similarly, immune cell-specific ligand anchored PEGylated liposomes have been developed to provide selective uptake at immunological cell. Ultrasound (US)-responsive and mannose-modified gene carriers, Man-PEG(2000) bubble lipoplexes, have been utilized for transfer of ovalbumin (OVA)-expressing plasmid DNA to selectively and efficiently into antigen-presenting cells. Developed systems have demonstrated 500–800-fold higher gene expressions in the antigen-presenting cells (APCs) selectively in vivo compared with the conventional lipofection method (Un et al. 2010).

### Virosomes

Virosomes are lipidic envelope devoid of genetic information, which retain the antigenic profile and fusogenic properties from their viral origin. Reconstituted lipid vesicles equipped with viral glycoproteins seems to possess many ideal properties for delivery of immunogens such as no limitation of size of encapsulated immunogens, high efficiency for cytosolic delivery, simplicity in handling and brevity of incubation time (Okamoto et al. 1997). Virosome-mediated delivery has low toxicity and high immunogenicity with various prospective applications for the treatment and prevention of cancer, neurodegenerative disorders, and infectious diseases. The use of immunopotentiating reconstituted influenza virosomes (IRIV) as delivery system of DNA appear to be a promising tool in vaccinology and gene therapy. IRIVs are spherical, unilamellar vesicles with a mean diameter of ~150 nm, short surface projections of 10–15 nm. IRIVs are prepared by a mixture of natural and synthetic phospholipids containing 70 % egg yolk phosphatidylcholine (for enhancement of immune responses), 20 % synthetic phosphatidylethanolamine (able to directly stimulate B cells to produce antibodies), and 10 % envelope phospholipids originating from H1N1 influenza virus. IRIVs were first utilized in the manufacture of hepatitis A vaccine. The adjuvant function of virosomes is based on their virus-like particle structure providing repetitive antigen presentation to B cells, partial protection from extracellular degradation, and a depot effect (Gluck et al. 1992).

### Proteasomes

This immunogenic delivery system generally uses a noncovalent interaction between the proteasomes and antigen to form the appropriate complexes for

delivering apolar or amphiphilic antigens. In most cases, these trials have involved intranasal administration of the vaccine and qualified as safe and well-tolerated materials through various human clinical trials. Proteasome-conjugated *Shigella flexneri* 2a LPS vaccine shows an immune response similar to that observed after immunization with the live pathogen (Fries et al. 2001). Intranasal delivery of proteasome-based vaccines may be able to produce both systemic and mucosal immunity. Another very similar category of vaccines is the conjugate vaccine. These vaccines consist of a relatively non-immunogenic (especially in infants) antigen linked to a more immunogenic carrier such as a protein or toxoid. The conjugate vaccines for *H. influenzae* type B (Hib) were developed using Hib polysaccharide conjugated to either diphtheria toxoid (PRP-D), OMP of *Neisseria meningitidis* (PRP-OMP), mutant diphtheria toxoid CRM197 (HbOC) or tetanus toxoid (PRP-T) to provide the Hib antigen immunogenic (Heath 1998).

### Cochleates

Cochleates are phospholipid calcium precipitates with a unique structure consisting of a large continuous solid lipid bilayer sheet rolled up into a “Jelly roll-like” structure (Papahadjopoulos et al. 1975). Cochleate delivery vehicles composed of simple, natural materials (phosphatidylserine and calcium) are unique vaccine carrier and delivery formulations (Mannino and Gould-Fogerite 1995). They are nontoxic, noninflammatory, and biodegradable. Cochleates are prepared through the calcium-induced fusion of negatively charged phospholipid liposomes to collapse into solid sheets that roll up or stack, excluding water. The entire cochleate structure is a series of solid layers, components within the interior of the cochleate structure remain intact provides protection from degradation when exposed to harmful environmental conditions or enzymes. The protection of encochleated materials and structural stability of the cochleate allows for efficient delivery of DNA by various routes like mucosal (oral, intragastric, intranasal, and intraocular) and parenteral (intramuscular, subcutaneous, intraperitoneal, and intradermal). Strong, long-lasting, mucosal and circulating, antibody and cell-mediated responses are generated. Protection from challenge with live viruses following oral or intramuscular administration has been achieved (Mannino et al. 1998). Cochleates efficiency can be improved by attachment of surface glycoproteins of enveloped viruses and can be integrated into the lipid bilayers. DNA cochleates can be formed by trapping oligonucleotides or high molecular weight plasmids within or between the lipid bilayers (Papahadjopoulos et al. 1975).

### Virus-Like Particles

Virus-like particles (VLPs) are small particles consisting of one or more viral coat proteins can act as an adjuvant by carrying peptide sequences inside the APC and feeding into the endogenous processing pathway (Schirmbeck et al. 1995). These

are safe, highly immunogenic, no additional adjuvant is needed, well tolerated, noninfective, and can easily be handled in the laboratory. It uses nature's own mechanism and structural principles to trigger the immune system for protective effects by stimulating both cellular immunity by effectively stimulating CD4 proliferative responses and cytotoxic T lymphocyte (CTL) responses and humoral immunity by efficiently cross-linking the membrane-associated immunoglobulin molecules that constitute the B-cell receptor (Chackerian 2007; Jennings and Bachmann 2008; Buonaguro et al. 2010).

### Immune-Stimulating Complex

The immune-stimulating complex (ISCOM) is a highly versatile and effective particulate antigen delivery system that has been extensively studied as an adjuvant system for a range of viral, bacterial, parasite, and other antigens. ISCOMs are three-dimensional "cage-like" structures, which have been shown to form upon detergent removal from mixtures of saponins, detergents, and cholesterol. The ISCOM (immunostimulating complex) is a complex consisting of protein antigen, cholesterol, phospholipid, and the saponin adjuvant Quil A. A similar vaccine delivery vehicle and adjuvant has also been developed that uses the same material minus the antigen and is referred to as ISCOMATRIX<sup>®</sup>. The antigen can be added later to the ISCOMATRIX<sup>®</sup> during formulation of the vaccine. This material seems to work similarly to ISCOMs but provides for more general applications by removing the requirement for hydrophobic antigens (Pearse and Drane 2005). ISCOMs potentiate both humoral and cellular immune responses to incorporated antigens (Cox et al. 1998). ISCOMs stimulate APCs to produce IL-1, IL-6, and IL-12 and induce T-helper cells of both Th1 and Th2 type and the cell-mediated immune response includes CD8<sup>+</sup> class I restricted cytotoxic T cells in a variety of experimental animal models and have now progressed to phase I and II human trials (Claassen and Osterhaus 1992; Barr and Mitchell 1996). Oral administration of ISCOM vaccines has been shown effectiveness and immune-potentiating effect, but this route requires the use of high and frequent dosing. A study in which ISCOM vaccines may be able to elicit strong mucosal immune responses when administered in the pelvic pre-sacral space of sheep, which could be useful for immunization against viral infections of the female genital tract (Thapar et al. 1991). A Quil A-containing ISCOM with modified cholera toxin A1 (CTA1-DD) used as a mucosal vaccine carrier system for the influenza virus PR8 antigen (Helgeby et al. 2006). Dong-Ji et al. have utilized combinational approach by priming with *C. trachomatis* mouse pneumonitis (MoPn) major outer membrane protein (MOMP) DNA and boosting with ISCOM of MOMP protein and shown the potential for protection of BALB/c mice against MoPn lung infection (Dong-Ji et al. 2000). Nasal vaccinations with P6 DNA vaccine and Matrix-M (immunostimulatory complex adjuvant) have shown significant higher IgA-producing cells in addition to Th1 and Th2 cytokine expression. This strategies may provide a new way for the induction of specific immunity at mucosal sites (Kodama et al. 2011).

## Archaeosomes

Archaeosomes are nanometric size liposomes made from the polar ether lipids of archaea found in eukaryotes and bacteria. Polar ether lipids of archaeosomes are providing excellent physicochemical stability and self-adjuvanting properties for delivery of vaccine preparations. Archaeosomes have demonstrated relatively higher stabilities to oxidative stress, high temperature, alkaline pH, action of phospholipases, bile salts, and serum proteins (Patel and Chen 2005; Benvegna et al. 2009). Archaeosomes facilitated a strong antibody (Th2) response to entrapped protein antigens. The antibody humoral response was superior to that obtained with conventional liposomes and was in some instances comparable to that obtained with the potent but toxic Freund's adjuvant (Patel and Sprott 1999; Patel and Chen 2005). Sprott et al. have also been described the role of co-enzyme Q10 into archaeosome-based antigen formulation. Incorporation of CoQ10 into archaeosomes and conventional liposomes can enhance the phagocytosis of the resultant vesicles by macrophage cells that allow the alteration in targeting profiles to specific tissues when the vesicles are administered to an animal via different routes and further enhance the immune response to coadministered immunogens. Recently, "cationic archaeosomes," based on mixtures of neutral/cationic bilayer-forming lipids and archaeobacterial synthetic tetraether-type bipolar lipids, have shown better transfection efficiency and can be utilized for DNA vaccination (Rethore et al. 2007).

## ***4.2 Polymeric Particulate(s) for Administration of Vaccines***

Among the variety of lipid delivery systems, polymeric delivery systems have emerged as a promising alternative because of their ease of preparation, purification and chemical modification as well as their enormous stability. Polymeric nonviral carriers (polyplexes) are one of the effective means of delivering a therapeutic or other biologically active substance in controlled and sustained manner. Polymeric particulate delivery system induces adjuvant effect on the incorporated antigen and reduces the frequency of vaccination required to establish long-term protection. Both natural and synthetic polymers have been considered to encapsulate antigenic materials for vaccination (Table 3). Various polymeric delivery systems have been developed using these polymers like micellar systems, emulsions, polymerosomes, nanoparticles, microspheres, nanocapsules, dendrimers, and dendrosomes (Fig. 8). However, there are several associated concerns for the use of polymers as vaccines delivery systems such as toxicity, irritancy, allergenicity, and biodegradability. The advantages of using natural polymers include their low cost, biocompatibility and aqueous solubility. However, the natural polymers may also be limited in their use due to the presence of extraneous contaminants, variability from lot to lot and low hydrophobicity. In contrast, synthetic polymers are more reproducible and can be prepared with desired degradation rate, molecular weight and copolymer



composition. Nevertheless, synthetic polymers may be disadvantageous due to their limited solubility, they are often soluble only in organic solvents and consequently may not release biologically active antigen (Rice-Ficht et al. 2010).

Polymeric vaccines may offer improved stability and activity of encapsulated antigen materials by avoiding exposure to organic solvents used during formulation and acidic pH conditions caused by degradation of the polymer (Duncan et al. 2005). Effective application of a polymeric nanoparticulate delivery system is greatly dependent on the specific polymer used, as this will dictate the properties of the nanoparticle in vivo (Hanson et al. 2008). For example, polycationic polymers can interact with negatively charged DNA, resulting in a improved intracellular DNA delivery to occur.

Whereas noncondensing polymers are neutral or slightly negatively charged polymers that physically encapsulate materials and can be used to target APCs and M-cells in the mucosa (Bhavsar and Amiji 2007). There are a number of factors that affects the physicochemical properties of polymeric delivery vehicles like molecular weight, degree of branching, cationic charge density buffer capacity, polyplex properties and the experimental conditions like the polyplex concentration, the presence or absence of serum during transfection, the incubation time and the transfection model chosen for the gene delivery experiment. To reduce its cytotoxicity and improve transfection efficiency, polyplexes have been modified by conjugating with polyethylene glycol (PEG), histidine, and targeting ligands including polysaccharides, transferrin, and galactose.

Various biodegradable polymers like aliphatic polyesters such as poly(lactic acid) (PLA), poly(glycolic acid) (PGA), poly( $\epsilon$ -caprolactone) (PCL), poly(hydroxybutyrate) (PHB), and their copolymers being evaluated for their uses as vaccine adjuvants and delivery systems (Panyam and Labhasetwar 2003). Recently, poly(amino acid)s-based copolymers have also been employed for the delivery of protein, vaccine, and genetic materials such as poly-L-glutamic acid, poly-L-aspartic acid, poly-L-lysine, poly-L-arginine, poly-L-proline, poly-L-asparagine, and poly-L-histidine. Polyamino acids have properties that mimic proteins, making them ideal for vaccines delivery. They provide better adjuvanticity, low toxicity, biodegradability and targeting into intracellular compartments (Chiang and Yeh 2003). Various type of polysaccharides, such as agarose, alginate, carrageenan, hyaluronic acid, dextran, chitosan, cyclodextrins, mannan, and pullulan, have been used for delivery of vaccines (Table 3).

#### 4.2.1 Block Copolymer-Based Particulate Systems

At specific concentrations and temperatures, when amphiphilic molecules, or molecules containing hydrophobic and hydrophilic regions, are maintained, naturally form association colloids known as amphiphilic micelles as a result of hydrophobic interactions. Poly (ethylene glycol) (PEG) is commonly incorporated as the hydrophilic segment in both amphiphilic micelles (Gaucher et al. 2005).

**Table 3** Overview of different polymers used in vaccines

Polymer with their property	Advantages	Limitations	Applications	Results
<i>Collagen</i> : Major protein component of the extracellular matrix. It interacts with cells in connective tissues and transduces essential signals for the cell regulation				
Reissmann et al. (2012)	Good biocompatibility, high mechanical strength	Contamination, high cost	<i>Streptococcus pyogenes</i> , <i>S. dysgalactiae</i>	Facilitate acute infection
<i>Gelatin</i> : A denatured protein obtained by acid and alkaline processing of collagen. Insoluble in water to prepare hydrogel through chemical cross-linking, with water-soluble carbodiimides and glutaraldehyde				
Lou et al. (1995)	Easy processability, good biodegradability	Poor mechanical properties, Brittle	<i>Mycobacterium bovis</i>	Capable of targeting fibronectin-bearing surfaces associated with some tumors
<i>Silk fibroin</i> : Silkworm <i>Bombyx mori</i> produces silk to weave its cocoon, and its major components are fibroin and sericin. This is light weight, extremely strong and elastic and exhibits mechanical properties comparable to the best synthetic fibers produced by modern technology				
Zhang et al. (2012)	Environmentally safe, biocompatibility, excellent mechanical properties	Less production, high brittleness	Model antigen	Enhance the stability, up to 60 °C over more than 6 months
<i>Fibrin</i> : Fibrin is a protein matrix produced from fibrinogen, providing an immune-compatible carrier for delivery of active biomolecules, antigens. Fibrin naturally contains sites for cell binding and has been investigated as a substrate for cell linkage, distribution, relocation, and propagation				
Khan et al. (2012)	Induce improved cellular interaction, used as a cell carrier as well as antigen carrier	Rapid degradation, instable, low mechanical stiffness	<i>Cryptococcus neoformans</i>	Elicited high immune globulin (Ig G(I) and (2a) isotype response
<i>Elastin</i> : Elastin is synthesized by vascular smooth muscle cells and secreted as a tropo-elastin monomer that is soluble, hydrophobic and non-glycosylated.				
Elastin is a potent regulator of vascular smooth muscle cells activity, regulations important for preventing fibro-cellular pathology				
Gaudreau et al. (2007)	Conferring elasticity, precise molecular weight, low polydispersity	Become insoluble and aggregate at a critical temperature	<i>Staphylococcus aureus</i>	Produces a combined Th1 and Th2 response

<i>Soybean</i> : The most cultivated plant in the world is rich in proteins (40–50 %), carbohydrates (26–30 %), and lipids (20–30 %). It is a species of legume native that can be processed into protein-rich products					
Moravec et al. (2007)	Abundant, renewable, inexpensive, environment friendly biodegradable	Application of soy-based polymers in this field is still very narrow	<i>Escherichia coli</i>	Induced both systemic IgG and IgA, mucosal IgA antibody response after administered orally to mice	
<i>Chitosan</i> : Fully/partially deacetylated form of chitin. Degree of deacetylation of commercial chitosan is usually between 70 and 95 %, and the molecular weight between 10 and 1,000 kDa. Chitosan exhibits a pH-sensitive behavior as a weak polybase due to the large quantities of amino groups on its chain					
Verheul et al. (2011)	Enhanced immune response, mucoadhesive property	Contamination, long degradation period	Ovalbumin (OVA)	Enhances the immune genicity of OVA after nasal and intradermal vaccination	
<i>Starch</i> : Stored as insoluble granules composed of $\alpha$ amylase (20–30 %) and amylopectin (70–80 %). Physical properties of starch are greatly influenced by the amount of water present. Degradation products are oligosaccharides that can be readily metabolized to produce energy					
Strindelius et al. (2004)	Inherent biodegradability, overwhelming, abundance	Hard processing, brittle	Recombinant cholera B	The mucosal response significantly greater via nasal administration	
<i>Alginate</i> : Originates from sea-weed. Structurally similar to natural glycosaminoglycans (GAG). It is an anionic polymer with carboxyl end groups is a good mucoadhesive agent. High degree of swelling and shrinking during cationic cross-linking					
Borges et al. (2008)	Biocompatible, resistance to acid	Poor mechanical properties, uncontrolled degradation	Hepatitis B surface antigen (HBsAg)	Enhancement of the immune response after subcutaneous injection	
<i>Hyaluronic acid</i> : Major macromolecular components of the (ECM). Hyaluronan is a naturally occurring non-sulfated glycosaminoglycans and a major macromolecular component of the intercellular matrix of most connective tissues such as cartilage, vitreous of human eye, umbilical cord, and synovial fluid					
Verheul et al. (2011)	Biocompatible, easily functionalized, good cell recognition	Poor mechanical properties, expensive	Ovalbumin (OVA)	Enhances the immune genicity of OVA after nasal and intradermal vaccination	
<i>Dextran</i> : Branched, high molecular weight polymer of D-glucose, produced by different bacterial strains by dextran sucrose enzyme from sucrose					
Shu et al. (2000)	Induces strong humoral responses, biodegradable and biocompatible	Not desirable for tuberculosis, high cost, anaphylaxis	<i>Streptococcus bovis</i> , Lactobacillus	Induced the highest serum IgG responses	

(continued)

Table 3 (continued)

Polymer with their property	Advantages	Limitations	Applications	Results
<i>Carrageenans</i> : Extracted from red marine algae, can form thermo reversible gel at room temperature. Due to the strong ionic nature, Carrageenans exhibit high degree protein reactivity				
Garg et al. (2012)	Thisotropic nature, highly flexible molecules	High melting temperature, poor degradation	Model protein and plasmid DNA	Highest antibody titers of mouse blood sera were got via parenteral immunization
<i>Gellan gum</i> : Produce by <i>Pseudomonas elodea</i> . Its ability to form transparent gels, in its native or high acyl form, two acyl substituent's D-acetate and D-glycerate are present				
Bacon et al. (2000)	Heat resistant, acid resistant	Low acyl form produces firm, non-elastic brittle	Influenza virus	Enhanced the local and serum antibody responses via intranasally administration
<i>Cellulose</i> : Most abundant organic polymer in the world. Highly cohesive, hydrogen-bonded structure, gives cellulose fibers exceptional strength and makes them water insoluble despite their hydrophilicity				
Sakoda et al. (2012)	Readily available, low cost, biocompatible	Poor degradation in vivo, need more time to regenerate	Influenza A and Influenza B	Removing chicken egg-derived impurities from allantoic fluids contain influenza viruses
<i>Galactose</i> : Recognized by mammalian hepatocytes through asialoglycoprotein receptor leading to regulation of a degradative pathway in glycoprotein homeostasis				
Al-Mariri et al. (2002)	Improved cell attachment, viability and metabolic functions	Less stability	<i>Yersinia enterocolitica</i>	Trigger Th1-type responses, induced a significant protective immunity against <i>B. Abortus</i> 544 infection
<i>Heparin</i> : Heparin is a highly sulfated GAG constituting the extracellular matrix. It preserves the stability and biological activity of the growth factors.				
Temmerman et al. (2004)	High stability, induce immunity	Poor degradation, expensive	<i>Mycobacterium tuberculosis</i>	Essential for effective T cell immunity to this antigen in infected healthy humans and in mice
<i>Poly (lactic acid) (PLA)</i> : The lactic and glycolic acid polymer are the most widely used synthetic polyesters for absorbable implants, antigen delivery and tissue engineering. Mechanical, degradation properties can be tuned by varying polymer segments				

Basarkar et al. (2007)	Good Biocompatibility, excellent biodegradation rate, good bioresorbable	Poor stiffness, poor compression strength	Model DNA	Achieve prolonged release of pDNA, and transfection efficiency
<i>Poly(ethylene glycol) (PEG)</i> : Used as an injectable gel, mechanical, degradation properties can be tuned by varying polymer segments	Target antigen-presenting cells (APC), biocompatible, non-toxic	Poor mechanical properties, poor stiffness	Hepatitis B, Vibrio cholera antigens, Influenza virus antigens	Induce both serum antibodies, mucosal IgA, cell-mediated responses, secondary immune responses (isotype switching)
<i>Poly(propylene fumarates) (PPF)</i> : Linear polyester whose repeating unit contains two ester bonds and one unsaturated carbon-carbon double bond. Hydrolysis of the ester bond allows PPF to degrade and degradation products of PPF have been shown to be primarily fumaric acid and propylene glycol	Biocompatible, biodegradable, conquer hydrophobicity	Slower degradation, least encapsulation, lack mechanical strength	Model plasmid DNA	Excellent control on the plasmid release
<i>Polyhydroxyalkanoates (PHAs)</i> : Physical properties include non-linear optical activity and piezoelectricity, i.e., the capacity of a material to suffer electric polarization due to mechanical stress	Biodegradable, highly biocompatible, thermoplastic materials	High brittleness, progressive erosion	Staphylococcal vaccine	Protection provide from high temperature
<i>Poly(amido-amines)s (PAA)</i> : Amine group containing tertiary amino and amido groups, which regularly arranged along their polymer chain	Highly versatile, nontoxic, incorporate peptide or protein structure	High degradation rate	Model DNA	Provide controlled arrangements of DNA in the polymer chain
<i>Pluronic F-127 (PEO-PPO-PEO)</i> : Copolymer of polyethylene oxide and polypropylene oxide. Pluronic F-127 is biocompatible hydrogel with surfactant properties	Stimulated cells proliferation, improved cartilage matrix deposition in terms of histology, biochemistry	Difficult to maintain its stability	<i>Bordetella bronchiseptica</i>	Significantly higher immune-stimulating activities following nasal administration to mice
<i>Polyphosphazenes</i> : Due to the flexible P-N backbone of polyphosphazenes, researchers have assessed the scope of this polymer with regard to both hard and soft tissue engineering as well as vaccine development				

(continued)

Table 3 (continued)

Polymer with their property	Advantages	Limitations	Applications	Results
Gilbert et al. (2003)	Biocompatible	Vary crystallinity and hydrophobicity	Recombinant HIV-1 submit vaccines	Safety and stability will be increased
<i>Polyanhydrides</i> : Due to its surface erosion properties, their polymers have been developed into various antigen delivery systems	Biocompatible, non-mutagenic, non-cytotoxic, enhanced protein stability	Complex processing and storage	<i>Salmonella enteritidis</i>	Induce sustain innate immunity to provide nonspecific protection
<i>Polyvinyl alcohol (PVA)</i> : Hydrophilic polymer, produced by hydrolysis of polyvinyl acetate. The PVA with high degree of hydrolysis is not soluble in water at room temperature but is soluble at elevated temperatures (usually above 70 °C)				
Fundueanu et al. (2007)	Good mechanical stability and flexibility	Limited durability, degradation rate not controllable	Model plasmid DNA	Provide protection from acidic media and exhibit high mucosal immunity
<i>Polyhydroxyethylmethacrylate (PHEMA)</i> : Polyhydroxyethylmethacrylate (PHEMA) is a nonbiodegradable polymer that forms a hydrogel in water				
Garg et al. (2012)	Biocompatible, high purity	Hypersensitivity, brittle	HIV <i>gag</i> gene, DNA vaccine	Significantly improved the prime effect of DNA vaccine through intranasal admin
<i>Poly(N-isopropylacrylamide) (PNIPAAm)</i> : Temperature-sensitive, which has a simultaneously hydrophilic and hydrophobic structure demonstrates a low critical solution temperature at about 32 °C				
Twaites et al. (2005)	Mechanical stability and flexibility, biocompatible	Difficult to maintain stability for longer duration	Model DNA	Excellent control on DNA transport and transgene expression
<i>Poly(ester-amide)s</i> : This polymer is made up of a soft PEG segment, connected to a hard diester-diamide segment through an ether bond. It is a high performance thermoplastic elastomer. It is used to replace common elastomers—thermoplastic polyurethanes, polyester elastomers, and silicones—for these characteristics: lower density among TPE, superior mechanical and dynamic properties (flexibility, <b>impact resistance</b> , <b>energy return</b> , <b>fatigue resistance</b> ) and keeping these properties at low temperature (lower than -40 °C), and good resistance against a wide range of chemicals. It is sensitive to UV degradation				
Li and Hu (2002)	Enhanced cell mediated immunity, superior mechanical and thermal properties	Enzymatic degradation	Melanoma antigen derived peptides	Enhanced cellular immunity, MHC I- and MHC II-restricted T-cell responses

*Poly(methyl methacrylate) (PMMA)*: Poly(methyl methacrylate) (PMMA) is a transparent thermoplastic, often used as a light weight or shatter-resistant alternative to glass. It is sometimes called acrylic glass. Chemically, it is the synthetic polymer of methyl methacrylate. PMMA is an economical alternative to polycarbonate (PC) when extreme strength is not necessary. Additionally, PMMA does not contain the potentially harmful bisphenol-A subunits found in polycarbonate

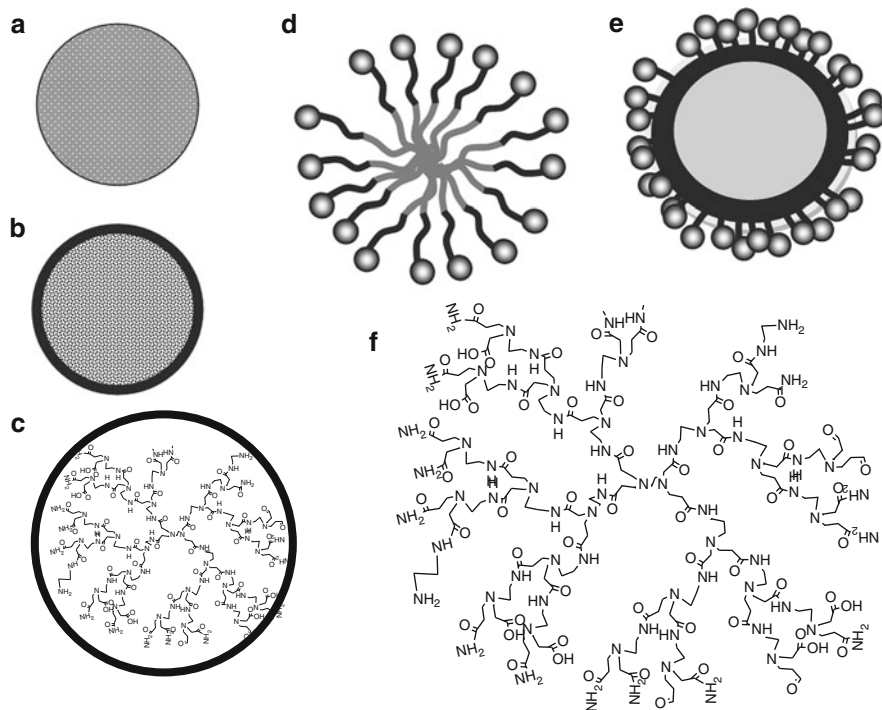
Lou et al. (2009) Easy handling and processing, low cost Brittle, stability problem PMMA particle-mediated DNA vaccine Initiate strong immune responses by stimulating production of inflammatory cytokines

*Polyesters*: Polyesters include naturally occurring chemicals, such as in the cutin of plant cuticles, as well as synthetics through step-growth polymerization such as polycarbonate and polybutyrate. Depending on the chemical structure, polyester can be a thermoplastic or thermoset; however, the most common polyesters are thermoplastics

Lau (2011) Biocompatible, easily metabolizable, enhance uptake by APC, increase both humoral and cellular response Poor adhesive property, no protective immunity in humans Measles virus Enhanced cellular immunity, MHC I- and MHC II-restricted T-cell responses

*Poly(lactide-co-glycolide)(PLGA)*: PLGA or poly(lactic-co-glycolic acid) is copolymer, is synthesized by means of random ring-opening copolymerization of two different monomers, the cyclic dimers (1,4-dioxane-2,5-diones) of glycolic acid and lactic acid. During polymerization, successive monomeric units (of glycolic or lactic acid) are linked together in PLGA by ester linkages, thus yielding a linear, aliphatic polyester as a product

Moore et al. (1995) Degradation products are naturally occurring metabolites and readily absorbed by neighboring cells Generate acidic environment and effect the stability *Yersinia pestis*, HIV gp140 Dominant Th1 response



**Fig. 8** Scheme presentation of different polymeric carrier systems (a) nanoparticles, (b) nanocapsules, (c) dendrosomes, (d) micelles, (e) polymersomes and (f) dendrimers

Block copolymer micelles are colloidal particles with a size around 5–100 nm, which are currently under investigation as carriers for delivery of biopharmaceuticals. In contrast to cationic polymeric systems, nonionic polymers enhance gene expression through mechanisms, which most likely do not involve DNA condensation and facilitated transport within cells. Adjuvant-active nonionic block copolymers that are flexible, linear structures, flanked on both ends by hydrophilic polyoxyethylene (POE) with a core of hydrophobic polyoxypropylene (POP) with variable ratios (Newman et al. 1998). The block copolymers are useful as general surfactants and display enhanced biological efficacy as vaccine adjuvants. Osmolarity, pH and buffer salts mainly affected the size and morphology of the particles. Molecular weight and formulation mainly affected titer and isotype of antibody. Jain et al. evaluated a system of combined poly(lactic acid) (PLA) and poly(ethylene glycol) (PEG) for the delivery of a recombinant hepatitis B surface antigen (HBsAg). PLA forms the hydrophobic core in an aqueous medium, which controlling the release of the antigen as it degrades into lactic acid. An outer shell form by PEG allows for prolonged release patterns and enhanced mucosal uptake to occur (Jain et al. 2009). Hunter et al. (1991) showed that the adjuvant activity of block copolymers varies with the lengths of the chains of polyoxypropylene (POP) and polyoxyethylene (POE). Pluronic block copolymers have been used



extensively in a variety of pharmaceutical formulations like low molecular mass drugs and polypeptides. Kabanov et al. (2002) described that these molecules can modify the biological response during gene therapy in the skeletal muscle, resulting in an enhancement of the transgene expression and therapeutic effect of the transgene. Block copolymers were recently used to promote gene delivery of plasmid encoding a food allergen, bovine beta-lactoglobulin. Tetronic 304 based block copolymers have decreased BLG-specific IgE concentrations and reduced local inflammatory response (Adel-Patient et al. 2010). Similarly, triblock copolymers consisting of three alternating hydrophobic and hydrophilic segments are also used to delivery genetic materials. Biodegradable and nontoxic triblock copolymers of PLA-PEG-PLA and PLGA-PEG-PLGA were also utilized micellar carriers for delivery of encapsulated plasmid pcDNA3.1(+)-MA against HCV. Developed carrier system has provided long-term better adjuvant effect with no side effects (Yang et al. 2011). Similarly, copolymers of a hydrophilic poly(ethylene glycol) block and a cationic poly(aminoethyl methacrylate) (PAEM) block have been used for DNA vaccine delivery. Synthesized polyplexes based carrier systems have induced a modest up-regulation of surface markers for DC maturation and better uptake by DCs in the draining lymph nodes (Tang et al. 2010). Further, cationic block copolymers poly(ethylene glycol) (PEG) with a positively charged poly(dimethylamino)ethyl methacrylate have been synthesized and utilized for HIV-1 TAT DNA molecules. Results indicated that synthesized cationic block copolymers was safe and ability to deliver genetic material for cell machinery and promising candidate for DNA vaccination (Caputo et al. 2002). Similar to cationic block polymers, nonionic block copolymers of poly(ethyleneoxide)-poly(propyleneoxide) (PEO-PPO) have also been utilized DNA vaccination using a beta-galactosidase (betaGal) encoding plasmid (McIlroy et al. 2009). Herpes simplex virus type-1 genes specifying glycoproteins gB and gD have been also delivered by nonionic block copolymers. Plasmid-encapsulated block polymers have protected the mice against lethal HSV-1 challenge when immunization was performed via the i.m. route (Baghian et al. 2002).

#### 4.2.2 Dendrimer

Dendrimers are a unique class of polymeric nanoconstructs having highly branched, three-dimensional, nanoscale architecture with very low polydispersity and high functionality. First discovered in the early 1980s by Donald Tomalia and co-workers, these hyperbranched molecules were called dendrimers. Dendrimers are highly branched, synthetic spherical macromolecules with layered architectures that can be considered analogous to a globular protein. They have the potential for high loading capacities due to small diameters (1.5–14.5 nm) through mechanisms such as complexation or formation of chemical bonds at terminal branch points or other active sites (Wiwattanapatapee et al. 2000). In addition, the low polydispersity of dendrimers should provide reproducible pharmacokinetic behavior in contrast to that of some polymers containing fractions with vastly

different molecular weight within a given sample (Parekh 2007). Several dendrimer-based products have been approved by the FDA and successfully commercialized for treatment and diagnosis of diseases, including VivaGel™ (Starpharma) designed as a topical microbicide, SuperFect®, (Qiagen Pvt Ltd.) used for gene transfection, and Alert Ticket™ (US Army Research Lab) for anthrax detection (Merdan et al. 2002). In the past decade, research has increased on polyamidoamine, polyethylenimines, polylysine, polypropyleneimine, polyaryl ether, polyester, polyglycerol and their derivatives for the design and synthesis of biocompatible dendrimers.

Dendrimers form complexes by electrostatic interaction with all forms of nucleic acids such as DNA, RNA, and antisense oligonucleotides. The nature of the dendrimer–nucleic acid complexes (“dendriplexes”) is dependent on the stoichiometry and concentration of the DNA-phosphates, dendrimer amines, bulk solvent properties (e.g., pH, salt concentration, buffer strength), and even the dynamics of mixing. High ionic strength interferes with the binding process and affects the nature of complexes formed by the different generations, for example, higher-generation PPI dendrimers in higher concentrations form water-soluble dendriplexes, whereas the G1 and G2 PPI dendrimers lead to the formation of electroneutral complexes (Tang and Szoka 1997). Dendrimer–DNA complex is formed by simply mixing the components in an aqueous solution. Transfection property can be improved by the use of an excess of cationic dendrimer because the negatively charged phosphate groups on the DNA neutralize the positively charged amine groups on the dendrimer through electrostatic interaction and an overall positively charged system is important in cell uptake (Bielinska et al. 1999). Immunogenicity and efficacy of DNA vaccines can be improved by physical conjugation of the PAMAM dendrimer to the MHC class II-targeting peptide. Therefore, dendrimers can be further explored for DNA-based vaccine development against malaria parasite (Pietersz et al. 2006). In a recent study, dendriplexes, complexes of dendrons and condensed plasmids containing the gene for protective antigen (PA) of *Bacillus anthracis*, were encapsulated in poly(lactide-co-glycolide) (PLG) particles using the double emulsion method. Studies indicated that the PLG-dendriplex particles produced superior levels of anti-PA IgG antibodies when compared to animals immunized with the PLG particles (Ribeiro et al. 2007). Conjugation of fifth-generation polyamidoamine (G5-PAMAM) dendrimers, a DNA-loading surface, with MHC class II-targeting peptides that can selectively deliver these dendrimers to APCs under conditions that enhance their immune stimulatory potency. DNA conjugated with this platform efficiently transfected murine and human APCs in vitro. Subcutaneous administration of DNA–peptide–dendrimer complexes in vivo preferentially transfected dendritic cells (DC) in the draining lymph nodes, promoted generation of high affinity T cells, and elicited rejection of established tumors. Taken together, our findings show how PAMAM–dendrimer complexes can be used for high transfection efficiency and effective targeting of APCs in vivo, conferring properties essential to generate effective DNA vaccines. Multiple antigenic peptide (MAP) dendrimer system is being used for vaccine and immunization purposes. MAP-based delivery

can prepare by addition of multiple immune-functional components, like B/T-cell epitopes, cell-penetrating peptides, and lipophilic moieties or by controlled synthesis of nanomaterials like micelles, dendrimers, and nanoparticles (Fujita and Taguchi 2011). A tetravalent multiple antigen peptide (MAP) dendrimer with four identical branches of a C-terminal peptide sequence of the rat GH-BP (GH-BP263-279) was synthesized and used as an immunogen in rabbits. The tetravalent rat GH-BP263-279 MAP dendrimer served as an effective immunogenic antigen in eliciting specific antibodies (Aguilar et al. 2009). Similar to MAP dendrimers, glycopeptide dendrimers containing both carbohydrates and peptides can be also used in delivery of vaccine components (Niederhafner et al. 2008; Sebestik et al. 2011).

### 4.2.3 Dendrosomes

The encapsulated **dendrimer–nucleic acid** complex within a **lipophilic** shell known as dendrosomes. These are novel vesicular, spherical, supramolecular entities and possess negligible hemolytic toxicity and higher **transfection** efficiency. Dendrosome are reported to be completely nontoxic both **in vitro** as well as **in vivo**. Poly (propyleneimine) dendrosome-based genetic immunization found to be highly effective against **hepatitis B** when compared to dendrimer–**plasmid DNA** complex, and the results indicate that dendrosomes hold great potential in DNA vaccination. In dendrosomes, the poly(propyleneimine) dendrimer–DNA complex is largely protected by multilamellarity of the vesicles. It has been reported that polyamidoamine dendrimer-based dendrosomes are efficient systems for the delivery of **s10siRNA** targeting **E6/E7 oncogenes** in **cervical cancer** (Pourasgari et al. 2009). **In vitro** superior transfection efficiency displayed by PAMAM dendrosomes as comparison to other nonviral gene delivery vectors. Nontoxic self-assembled dendritic spheroidal nanoparticles (Den123) have been used for the delivery of **pCMV-Betv1** loaded dendritic spheroidal nanoparticles (Den123) have shown low toxicity, enhanced transfection efficiency, and improved the immune response against birch pollen allergy (Balenga et al. 2006). Similarly, efficiency of dendrosome (a gene porter) is assessed in transferring recombinant human rotavirus VP2 cDNA. Studies revealed that dendrosome has lower cytotoxicity and better transfectivity in A549, a human lung cell line (Pourasgari et al. 2009). Dendrosome has been used to deliver the DNA vaccines encoding HIV-1 p24-gp41 gene. Studies have proved the efficacy of this carrier for the delivery of recombinant plasmids construct (Roodbari et al. 2012).

### 4.2.4 Polymersomes

Polymersomes are self-assembled polymeric colloidal vesicular systems containing aqueous inner core. Polymersomes are made up from amphiphilic block copolymers that allow polymersomes to stably encapsulate or integrate a broad

range of active molecules. The aqueous core can be utilized for the encapsulation of therapeutic hydrophilic molecules and the membrane can integrate hydrophobic drugs within its hydrophobic part. Further, the brush-like surface properties of the polymersome can provide better biocompatibility and blood circulation times. These systems have better loading efficiency, stabilities and provide sustained, controlled release of encapsulated therapeutics. Further, these systems have also been used to deliver biotherapeutics, especially peptides, proteins, and nucleic acids to site-specific cellular environment due to escape from endolysosomes (Levine et al. 2008; Christian et al. 2009). Amphiphilic diblock copolymer of poly (oligoethylene glycol methacrylate)-block-poly(2-(diisopropylamino)ethyl methacrylate) in association with tannic acid forms DNA-loaded polymersomes. Developed systems have demonstrated better cytosolic release of encapsulated nucleic acid materials (Lomas et al. 2011). Further, calcein-loaded polymersomes have also observed for their cytosolic delivery within dendritic cell (Scott et al. 2012). Similarly, poly(g-benzyl-L-glutamate)-K (PBLG50-K) polymersomes have been used for delivery of influenza hemagglutinin antigen. The immunogenicity and adjuvanticity of developed polymerosomes was better for administered the influenza antigen. In future, this nanostructured polymeric vesicular system may have huge potential for delivery of protein and DNA vaccines.

#### 4.2.5 Multiple-Emulsion Delivery Systems

Emulsions can be manufactured as water-in-oil (W/O) or oil-in-water (O/W) particulate carrier systems. Emulsion carrier systems are similar in size to pathogens and taken up by epithelial or M cells in the mucosal surfaces for successive delivery of the vaccine component to APCs and lymphoid tissue. A nanoemulsion formulation of intranasal hepatitis B vaccine showed improved vaccine efficacy, stability and ease of distribution (Makidon et al. 2008). Multiple emulsion formulations can also be used as vaccine carrier systems due to its longer stability and high entrapment efficiency of protein antigens without damage during emulsification procedures. Types of surfactants, processing methods and stabilizers is requisite for making stable multiple-emulsions (Hanson et al. 2008). The emulsion adjuvant MF59 immunostimulator has been shown to result in the recruitment of antigen-presenting cells (APCs) to the site of injection and to increased uptake of soluble antigen by the APCs. It has been formulated by a simple mixing of the antigen with the adjuvant and has shown excellent compatibility with a variety of subunit antigens. MF59 shows strong immunogenicity as comparison to other adjuvant is clearly seen in pre-clinical data published by Ott et al. They reported that when immunized guinea pigs and goat with glycoprotein D of herpes simplex virus (HSV) type 2 in the presence of MF59 showed a 34-fold and ninefold increases in antibody titers compared to aluminum hydroxide, respectively (Ott et al. 1995).

An oil-in-water (o/w) emulsion, syntex adjuvant formulation (SAF) is an effective adjuvant composed of a muramyl dipeptide derivative (threonyl-MDP). Threonyl-MDP demonstrated a lack of side effects (pyrogenicity, uveitis, adjuvant-induced arthritis) and increased adjuvant activity. SAF adjuvant used with a variety of antigens, such as influenza and malaria, and showed both cell-mediated and humoral immune responses. SAF, or a suitable equivalent, provides an excellent tool for vaccine research (Lidgate et al. 1989; Lidgate et al. 1992). There are several different types of Montanide™, including ISA 50 V, 51, 720 (water-in-oil emulsions), and ISA 206 (water-in-oil-in-water emulsion). ISA 51 and 720 have been used in human's vaccine formulations, while ISA 206 and 50 V have been used only in veterinary vaccine formulations. They are composed of metabolizable squalene-based oil with mannide monooleate emulsifier and permit antigens to be released more rapidly. The Montanide emulsions induce high antibody titers and CTL responses due to the formation of a depot at the site of injection. These emulsions have been used as vaccines against malaria, HIV and various cancers and found to be safe and fairly well tolerated (Lawrence et al. 2000; Toledo et al. 2001).

## 5 Improving Immunogenicity by Using Next-Generation Delivery Strategies

Various physical delivery methods are being heavily investigated because of direct transfection of APCs with the DNA vaccine (Porgador et al. 1998). The transcutaneous microneedle has the ability to bypass the stratum corneum layer of the skin, thus reaching Langerhans cells—the APCs of the skin. Jet-injection mechanical devices deliver DNA vaccines into the viable epidermis and increased efficacy in the prevention and/or therapy of infectious diseases, allergic disorders and cancer (Chen et al. 2002; Imoto and Konishi 2005; Roberts et al. 2005). The tattoo-perforating needle device has been used to puncture the skin and transfer DNA into skin-associated cells. The bundles of fine metal needles that oscillate at a constant high frequency have shown better expression of reporter genes in mice and induction of immune responses. Electroporation has been extensively studied to deliver therapeutic genes that encode a variety of hormones, cytokines, enzymes or antigens in large animal species such as dogs, pigs, cattle and nonhuman primates. Several different strategies of this technology are being pursued. However, too little is currently known about several of these devices and much additional research in this area is warranted (van Drunen Littel-van den Hurk et al. 2004; Roos et al. 2006; Hirao et al. 2008).

## 5.1 Routes of Administration

Nanotechnology is the development of engineered devices due to their small size at the micromolecular level in the nanometer range and large surface area, which enhances their action for early diagnosis of cancer and infectious diseases. Advances in nanotechnology have also proved to be beneficial in therapeutic fields such as drug discovery, drug delivery and gene/protein delivery. This concept has been found to be useful in developing nanovaccines using different routes of administration like oral, nasal and parenteral.

### 5.1.1 Oral Route

The oral route is the most popular and convenient route of administration. Oral delivery refers to absorption from the buccal through the rectal mucosa. Several barriers associated with genetic vaccination through the oral are generally attributed to (a) low permeability across biological membranes, (b) harsh gastric environment, (c) hepatic first-pass metabolism, and (d) chemical instability. The major drawback with oral route of administration is a higher concentration and is required for the vaccine to be effective due to dilution during the transport of the vaccine through the gastrointestinal tract. To date, most gene delivery strategies have concentrated on the parenteral route of delivery and oral administration has been largely ignored. Different nano- and microparticulate delivery systems using natural and synthetic lipid and polymers have been utilized to improve the stability and immunogenicity of oral DNA vaccines (Bhavsar and Amiji 2007). Oral vaccination with DNA–chitosan nanoparticles has appeared interesting because of their great stability and the ease of target accessibility, besides chitosan immunostimulatory properties. Studies demonstrated that 47 % of protection against parasite infection after delivery chitosan nanoparticles loaded with DNA encoding Rho1-GTPase protein of *Schistosoma mansoni* (Oliveira et al. 2012). Similarly, chitosan nanoparticles are used for DNA vaccine against *Vibrio anguillarum* through oral route. Studies revealed that chitosan–DNA (pVAOMP38) complex showed moderate protection against experimental *V. anguillarum* infection after oral vaccination in Asian sea bass (Rajesh Kumar et al. 2008). The orally administered tresylmonomethoxypolyethylene glycol (TMPEG) grafted liposome complexes with modified vaccinia virus Ankara (MVA(IIIIB/beta-gal) is also capable of delivering the transgenes to mucosal tissues and enhances the Env-specific cellular and humoral immune responses after repeated oral immunization of BALB/c mice (Naito et al. 2007). Mannosylated niosomes loaded with hepatitis DNA have shown humoral (both systemic and mucosal) and cellular immune response upon oral administration (Jain et al. 2005). Chitosan-coated and polyplex-loaded liposomes (PLLs) containing plasmid pRc/CMV-HBs are developed for oral delivery of vaccines specifically for targeting to Peyer's patch. Chitosan-coated PLL

demonstrated better uptake of encapsulated DNA to the distal intestine and provide better stability from enzymatic degradation (Channarong et al. 2011).

### 5.1.2 Nasal Route

The nasal route has been chiefly employed for producing local action on the mucosa. This route has a number of advantages, such as the high permeability of the nasal epithelium, which allows a higher molecular mass cut-off for permeation of approximately 1,000 Da, as well as the rapid drug absorption rate. Accurate and repeated dispensing of vaccine, mucociliary clearance, presence of peptidases, proteases and nuclease enzymes in the mucus or associated with nasal membrane, variation in extent of absorption with the mucus secretion and mucus turnover and deposition of the formulated vaccine to all areas of the nasal mucosa (especially lymphoid tissues), potential of uptake of vaccine formulations by the primary olfactory nerves in the nasal cavity, local irritation and unpleasant taste from concentrated drug reaching the mouth are major challenges associated with intra-nasal delivery of vaccines (Oliveira et al. 2007; Sharma et al. 2009). These problems can be overcome by design of appropriate antigen carriers. Nanocarriers for nasal vaccines are able to facilitate the transport of the associated antigen across the nasal epithelium, thus leading to efficient antigen presentation to the immune system and provide the protection and stability of encapsulated genetic materials (Koping-Hoggard et al. 2005). Further, use of mucoadhesive agents offers a strategy for the facilitation of increased residence time and increased vaccine efficacy (Alpar et al. 2005). Polycarbophil (PC) or polyethylene oxide (PEO)-based in-situ mucoadhesive polymers have demonstrated better nasal absorption of plasmid DNA (Park et al. 2002). Several studies have proven that wide applicability of chitosan nanoparticles for the nasal delivery of DNA vaccines like severe acute respiratory syndrome coronavirus (SARS-CoV) (Raghuwanshi et al. 2012), pneumococcal surface antigen A (PsaA) (Xu et al. 2011), hepatitis B antigen-encoding plasmid (Khatri et al. 2008), and DNA plasmid-expressing epitopes of respiratory syncytial virus (Iqbal et al. 2003). Further, several modifications on the chitosan polymers have also been made to improve the potential of chitosan nanoparticles for nasal administration of DNA vaccines like preparation of low molecular weight chitosan, development of water soluble chitosan (*N*-trimethyl chitosan), etc. Blends of poly(lactic-co-glycolic acid) (PLGA) and polyethylene oxide (PEO) have exhibited the capacity to associate and release plasmid DNA in a controlled manner. Results showed that DNA-loaded nanoparticles elicit significantly pronounced immune response compared to the naked plasmid DNA for up to 6 weeks (Csaba et al. 2006). Dry-powder influenza virosomes-based vaccines have also been advantageous for mucosal immunization (de Jonge et al. 2007). Needle-free nasal immunization, using nanoemulsion is made of soya bean oil, alcohol, water and detergents emulsified into droplets of 40 nm, has been reported to be a safe and effective hepatitis B vaccine (Makidon et al. 2008). The release of liquid or particles into the airflow enters one nostril via a sealing nozzle and exits through

the other nostril and minimizes the risk and problems related to deposition of particles in the lung, which occurs during conventional inhalation from a nebulizer and increases the delivery of particles to the posterior part of the nasal mucosa. Encapsulation of the antigen into bioactive nanoparticles is a promising approach to nasal vaccine delivery (Slutter et al. 2008).

### 5.1.3 Ocular Route

The ocular route holds immense potential for peptides/proteins intended for pathological ophthalmologic conditions. The eye mucosa is a possible route for mucosal vaccine because it is an important entry point for environmental antigens and infectious materials occupying most of the external ocular surface (Streilein et al. 1997). Lymphoid follicles are found in close association with the epithelium of the conjunctival mucosa in humans, rabbits, guinea pigs, dogs, pigs, and many other mammals (Chodosh et al. 1998). However, this certain drawbacks associate with this route are poor drainage of instilled solutions, tear turnover, poor corneal permeability, metabolism (enzymatic degradation) and low capacity for transport, nasolacrimal drainage, and systemic absorption. Eye drop vaccination of influenza A/PR/8 virus (H1N1) induced both influenza virus-specific systemic and mucosal Ab responses and protected mice completely against respiratory infection with influenza A/PR/8 virus (Seo et al. 2010). Ocular mucosal delivery of peptide epitopes of herpes simplex virus (HSV-1) glycoprotein D (gD) has mixed with oligodeoxy nucleotides containing unmethylated CpG motifs (CpG2007). Results suggested enhanced local and systemic immune response after multi-instillation of gD peptide epitopes with CpG2007 adjuvants (Nesburn et al. 2005). Ocular mucosal administration of iron nanoparticles with glutamic acid containing DNA vaccine herpes stromal keratitis (pRSC-gD-IL-21) have confers protection against mucosal challenge with herpes simplex virus type 1 in mice (Hu et al. 2011).

### 5.1.4 Vaginal Route

Vaginal mucosa is a portal of entry to many viral and bacterial pathogens. Vaginal route serves as a potential site of drug administration for local and systemic absorption of therapeutically important molecules, proteins, peptides, small interfering RNAs, oligonucleotides, antigens, vaccines and hormones (Hussain and Ahsan 2005). It is one of alternative site for the systemic delivery of protein drugs because of the relatively high permeability of the vaginal epithelium, by passage of the hepatic first-pass metabolism, large surface area and rich blood supply (Gupta et al. 2011). Quadrivalent human papilloma virus (HPV) [types 6, 11, 16, 18] recombinant vaccine (Gardasil<sup>®</sup>; Silgard<sup>®</sup>) is composed of virus-like particles (VLPs) formed by self-assembly of recombinant L1 capsid protein from each of HPV types 6, 11, 16, and 18. The VLPs are highly immunogenic, inducing high levels of neutralizing antibodies against the particular HPV types when



administered to animals or humans (McCormack and Joura 2011). Human papillomavirus (HPV)-based gene transfer vectors (pseudovirions; PsVs) have been used to deliver SIV genes to the vaginal epithelium. Studies revealed that intravaginal vaccination with HPV-based PsVs vector delivering SIV Gag DNA for HIV have induced mucosal, humoral, and cellular immune response in serum and the vaginal tract (Gordon et al. 2012). Thermo-sensitive mucoadhesive vaginal vaccine delivery systems have also been tested for the local and systemic antibody responses to HPV 16 L1 virus-like particles (Park et al. 2003). Vaginal delivery of vaccines which is associated with vaginal infection could be better alternative to induce an immune response in the genital mucosa capable of controlling the entry of the pathogen.

### 5.1.5 Topical Route

Noninvasive gene delivery approaches could be able to deliver and express naked plasmid DNA to tissue-specific localized delivery to skin. There are several advantages of needle-free noninvasive gene administration such as limited toxicity, potential cell receptor-independent uptake, minimal DNA size restrictions, and the potential for multiple treatments via a relatively uncomplicated administration modality, thus improving patient compliance. Topically applied formulation, especially nanosystems have been shown to enter skin, accumulate in hair follicles, diffuse via dendritic cells to draining lymph nodes, and elicit antigen-specific humoral and cell-mediated immunity (Nasir 2009). A number of methods have been developed to perform noninvasive topical gene delivery, which includes passive diffusion of genetic materials between a skin patch and skin, as well as active processes such as iontophoresis, sonophoresis, electroporation, and chemically enhanced diffusion (Mehier-Humbert and Guy 2005). Topical vaccination has been achieved using topical application of naked DNA with or without tape stripping and DNA/lipid-based complex such as liposomes, niosomes, Transfersomes, or microemulsion (Cui and Sloat 2006). Ethanol-in-fluorocarbon-based microemulsion has been for topically delivery of anthrax protective antigen (PA) protein-encoding DNA vaccine (pGPA). pGPA-loaded microemulsion has significantly enhanced the anti-PA antibody responses (Cui and Sloat 2006). Similarly, DNA delivery by novel lipid-based biphasic delivery system has significant deliver plasmid DNA into the “viable” layers of skin (Foldvari et al. 2006). Plasmid DNA-encoding hepatitis B surface antigen (HBsAg)-loaded cationic transfersomes are also utilized for topical immunization. Results revealed that DNA-loaded cationic transfersomes elicited significantly higher anti-HBsAg antibody titer and cytokines level as compared to naked DNA. It was also observed that topical application of DNA-loaded cationic transfersomes elicited a comparable serum antibody titer and endogenous cytokines levels as produced after intramuscular recombinant HBsAg administration (Mahor et al. 2007). 40- or 200-nm sized polystyrene nanoparticles have been studied to target active compounds to the hair follicle and may result in a better penetration and higher efficiency of

compound uptake by skin resident cells. Studies demonstrated that 40 and 200 nm NPs and modified vaccinia Ankara (MVA) expressing the green-fluorescent protein penetrated deeply into hair follicles and uptake by APCs and transport to the draining lymph nodes (Mahe et al. 2009). Nanoengineered genetic vaccine formulation has been developed for topical immunization comprising of emulsifying wax (oil phase), CTAB (cationic surfactant), Mannan (DC ligand), dioleoylphosphatidylethanolamine (endosomolytic agents), and cholesterol. All pDNA-coated nanoparticles, especially the mannan-coated pDNA-nanoparticles with DOPE, have shown significant immune response (IgG titers; 16-fold over “naked” pDNA alone) (Cui and Mumper 2002).

Diffusion patches and tape stripping techniques are used for delivery of small (<500 Da) and large molecules, respectively. Liquid jet injector is an approach in which DNA vaccine is delivered around the Langerhans cells by a high-speed injector. (Chen et al. 2002) reported that particle-mediated gene-gun DNA immunization use similar mechanical devices to deliver DNA vaccines into viable epidermis (Chen et al. 2002). Microneedle arrays is a set of needles of microscale length with their nanoscale tips coated with DNA and can accurately, efficiently and safely deliver biomolecules to the viable cells of the epidermis. Recently, Tran et al. (2008) developed a unique nanoliposomal ultrasound-mediated device for delivering small interfering RNA (siRNA) specifically targeting melanocytic tumors present in the skin and they observed that decrease early melanocytic lesion development in the skin and prevent the spread of cutaneous metastases of melanoma (Tran et al. 2008). These results suggested that skin may provide an appealing, noninvasive route of delivery for DNA vaccines and other therapeutic genes. Table 4 represents positive and negative aspects of various routes of administration, which are very helpful for selection of particular route.

## 6 Summary, Conclusions, and Future Challenges

Novel vaccine carriers, adjuvant, vehicles, and particle-based delivery strategies are being evaluated in a variety of vaccines, including those against diseases such as cancer, malaria, AIDS, hepatitis, etc., in which a cellular and/or mucosal immune response is desired. Various immunity responses were generated by different adjuvant like MF59 and MPL<sup>®</sup> generated Th1 responses, VLPs, virosomes, nondegradable nanoparticles, and liposomes generated cellular immune responses in humans. Viral vectors, ISCOMs and Montanide<sup>™</sup> ISA51, 720 and various nanoparticulate immunopotentiators and antigen delivery vehicles have shown CTL responses. The desirable responses can be achieved by using combination of various adjuvants. Systemic antibodies produced in humans when viral-vectored vaccines as well as proteasomes given IN. The clinical trials required for vaccine approval are often very long and difficult. Furthermore, since many vaccines are often administered to healthy individuals, and frequently to infants, it is critical that they are proven safe and well tolerated in nonhuman primates before entering

**Table 4** Various routes of administration with their positive and negative aspects.

Routes of administration	Positive aspects (+)	Negative aspects (–)
Oral	<ul style="list-style-type: none"> <li>• Cheap mass vaccination</li> <li>• Mucosal immunity</li> <li>• Simple ingestion</li> <li>• Minimal delivery risk</li> <li>• Mucosal antibody and modest systemic antibody, can induce tolerance</li> <li>• Safest route</li> </ul>	<ul style="list-style-type: none"> <li>• Stability in the GI tract</li> <li>• Requires potent mucosal adjuvant</li> <li>• High antigen dose required</li> <li>• Limited number of clinical trials</li> <li>• Small number of animal models of viral and bacterial diseases</li> </ul>
Intranasal	<ul style="list-style-type: none"> <li>• Mucosal and lung immunity</li> <li>• Minimal delivery risk</li> <li>• Medium antigen dose required</li> <li>• Mucosal, systemic antibody and T cell, can induce tolerance</li> <li>• Many animal models of viral and bacterial diseases</li> </ul>	<ul style="list-style-type: none"> <li>• Safety issues in humans</li> <li>• Requires mucosal adjuvant</li> <li>• Limited number of clinical trials</li> </ul>
Pulmonary	<ul style="list-style-type: none"> <li>• Mucosal and lung immunity</li> <li>• Mass vaccination with nebulizer or inhaler or by spraying animals</li> </ul>	<ul style="list-style-type: none"> <li>• Safety issues in humans</li> </ul>
Parenteral	<ul style="list-style-type: none"> <li>• Clinically relevant</li> <li>• Systemic immunity</li> <li>• Low antigen dose required</li> <li>• Potent systemic antibody and T cell</li> <li>• Many viral, bacterial and parasitic disease models in animals and humans</li> <li>• Extensive number of clinical trials</li> </ul>	<ul style="list-style-type: none"> <li>• Needle required (painful)</li> <li>• Requires medically trained personnel</li> <li>• Higher delivery risk due to possible transmission of infection by contaminated needles and syringes</li> <li>• Require alum and variety of systems effective adjuvant</li> </ul>

human trials. While the development of novel vaccine delivery systems and adjuvant has been aided by nanotechnology, it must be necessary to perceived potential problems such as their high surface area and reactivity, the ability to cross biological membranes, slow biodegradability of some materials, its safety and tolerability before its approval. Many challenges must be met before new classes of vaccines become available like ability to stimulate humoral, cellular and mucosal immune responses, longer duration response, easily metabolized of vaccine components in body, cost-effective production, and lesser risk and less invasive approaches for the administration of vaccinations. As these challenges are met, the prevention and therapy of many previously untreatable diseases should become increasingly possible.

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