Chapter 1

Cationic Nanostructures for Vaccines

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Additional information is available at the end of the chapter

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1. Introduction

Cationic lipid bilayers, particles, polysaccharides and a variety of hybrid nanostructures provide adequate matrixes for supporting antigens such as peptides, proteins, DNA or oligonucleotides on model surfaces (latex, silica, silicon wafers, self-assembled monolayers, metals, polymers, insoluble drugs, biological cells and viruses).Particulate vaccines are currently an area receiving a high level of attention [1-3]. Particles deliver both antigen and adjuvant into the same population of antigen presenting cells limiting both the systemic distribution of the adjuvant and its potential toxicity [1]. Biological particlesrepresented by live or attenuated bacterial vacines, engineered biological vectors and virus-like particles are often less safe than synthetic particulates for which quality control and validation in vaccine development and production are more rapid [2]. While developing novel particulate vaccines, particle size and charge do matter [2]. Virus-sized particles (20–200 nm) are usually taken up by endocytosis via clathrin-coated vesicles, caveolae or their independent receptors [4], and preferentially ingested by dendritic cells (DC). Larger sized particles such as bacteria (500–5000 nm) are predominantly taken up by phagocytosis, and primarily ingested by macrophages. All particles used in vaccine formulations are consequently internalized efficiently by antigen presenting cells by one or a combination of the quoted mechanisms [5, 6]. Particles with diameters smaller than 500 nm, in particular the nanometric ones with sizes in the 40–100 nm range are more eficient to promote CD8 and CD4 type 1 T cell responses than those with diameters above 500 nm, although the latter could induce good type 2 and antibody responses [5]. Cationic microparticles are effectively taken up both by macrophages and dendritic cells since electrostatic attraction promotes particle binding and subsequent internalization. Cationized polymeric particles carrying antigen significantly enhanced both antibody production and cytotoxic T cells at low antigen dose [7] and induced maturation of dendritic cells [8, 9]. As a consequence particles for vaccines should be positive and available over a range of sizes. Cationic particles of aluminium compounds, identified as having

immunostimulatory properties more than 70 years ago, remain the only type of adjuvant licensed world-wide [3]. Oil-in-water emulsions or virosomes were licensed in some countries in compositions for influenza [10] or hepatitis B vaccines [11]. However, both of these adjuvants are characterised by inducing humoral immune response and are thus effective in elevating serum antibody titers whereas their ability to elicit cell-mediated immune response is limited [12]. For vaccines against influenza, malaria and HIV, the induction of a humoral response is insufficient and a substantial complementary cell-mediated immune response is necessary for adequate protection. For vaccines against tuberculosis, a cellular response seems to be the sole effector mechanism required for protection [13]. Among the cationic compounds used to produce cationic nanostructures to present antigens for vaccines formulations, some cationic compounds appear to be of special relevance.They are the cationic and synthetic lipid named dioctadecyldimethylammonium bromide (DODAB), the cationic polysaccharide named chitosan (CH) and its derivatives with pH-independent positive charge and the biocompatible cationic polymeric nanostructures.This chapter presents and critically evaluates these three types of cationic nanostructures.

2. Cationic nanostructures based on DODAB

DODAB is a cationic lipid which can be dispersed ultrasonically in aqueous solution using a macrotip probe [14]. Thereby, bilayer vesicles are simultaneously obtained and disrupted yielding nano-sized bilayer disks or cationic bilayer fragments (BF) stabilized by electrostatic repulsion at low ionic strength [14,15].DODAB BF have previously been used as antimicrobial agents [16] or in the production of lipid-covered particles such as bilayer-coated silica [17] or latex [18]. These cationic, bilayer-covered latex or silica particles where the bilayer is solely composed of DODAB were recently employed to present antigens to the immune system with better results than alum as adjuvants for induction of cellular immune responses [19, 20]. The open DODAB BF in aqueous dispersions are different from their mother vesicles presenting the following features: (i) osmotic non-responsiveness of the dispersion indicative of absence of inner vesicle compartment [21]; (ii) discoidal shape with disks exhibiting one bilayer thickness as visualised by means of transmission electronic microscopy (TEM) after electronic staining the nanostructures [22]; (iii) cryo-TEM micrographs performed without any staining [23]; (iv) fluid and solid state coexistence and complex formation with oppositely charged surfactant [24]; (v) solubilization of hydrophobic drugs at the borders of DODAB bilayer fragments, which does not occur for DODAB closed bilayer vesicles [25]. These bilayer fragments have more fluid environments at their edges that are absent in closed bilayer systems such as vesicles or liposomes. Due to its cylindrical molecular shape, DODAB molecules self-assemble in aqueous solutions as bilayers instead of micelles as shown in the seventies by transmission electron microscopy [26]. Supramolecular assemblies of DODAB bilayer fragments by themselves or after interaction with supporting particles have been combined with different model antigens in separate and tested as immunoadjuvants. The cationic nanoadjuvants with DODAB BF are either reduced to a single-component, nanosized system-DODAB

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BF-or are dispersions of cationic particles with controllable nature and size as obtained after covering silica or polystyrene sulfate latex (PSS) with a cationic DODAB bilayer (Figure 1).

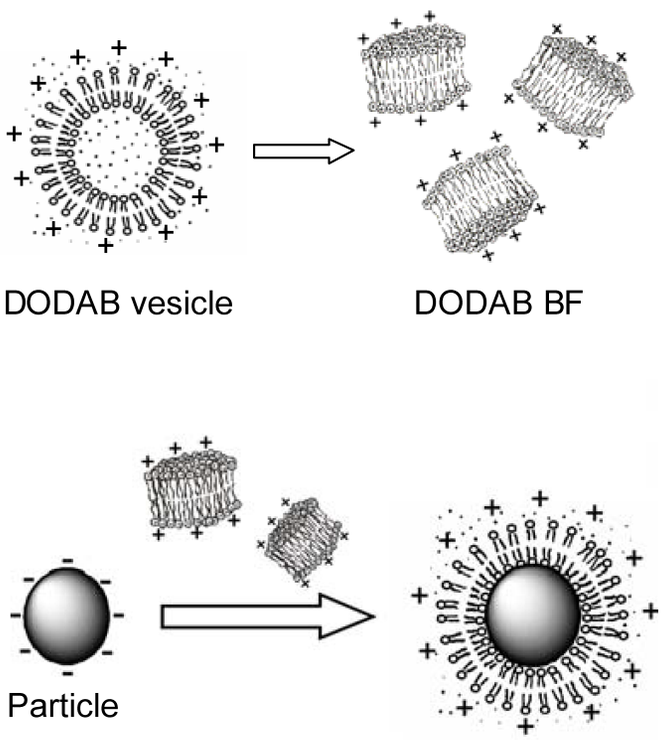

Figure 1. Cationic nanostructures and particles based on the cationic lipid DODAB useful to present antigens (reproduced with permission from [27]).The DODAB bilayer fragments are usually obtained at low ionic strength by sonication with a macrotip.Particles acting as supports for the cationic bilayer can be organic or inorganic. Reprinted from Vaccine, 27/42, Nilton Lincopan,Noelí M. Espíndola,Adelaide J. Vaz,Maria Helena B. da Costa,Eliana Faquim-Mauro,Ana M. Carmona-Ribeiro, Novel immunoadjuvants based on cationic lipid: Preparation, characterization and activity *in vivo*, 5760-5771.Copyright 2009, with permission from Elsevier.

DODAB BF may interact with proteins both via the hydrophobic effect and the electrostatic attraction at low ionic strength. This interaction has been properly characterized by means of dynamic light scattering for sizing, zeta-potential analysis and evaluation of immunoadjuvant activity *in vivo*. The model antigens employed with DODAB-based cationic nanostructures were bovine serum albumin (BSA), purified 18 kDa/14 kDa antigens from *Taenia crassiceps* cysticerci (18/14-*Tcra*) or a recombinant heat-shock protein from *Mycobacterium leprae* [27]. Antigen choices were due to different reasons. BSA adsorption at interfaces [28] and, specifically, onto large DODAB vesicles [29] is well described and has been useful to prevent nonspecific binding in immunoassays, biosensing and proteomics applications [28,30].The purified 18 kDa/14 kDa antigens from *Taenia crassiceps* cisticerci are proteins specific for this parasite found as circulating antigens and often employed in immunodiagnosis;they can be obtained from *in-vitro* cultures of *T. crassiceps* cysticerci in hybridoma media from vesicles budding from cysts contain the excretory/secretory (ES) antigens [31,32]. Human and pig infections by *T. solium* and *crassiceps*, respectively, represent an important health problem, with socio-economical repercussions affecting many countries in Latin America, Asia and Africa

[31]. The 18 kDa-hsp protein is a heat-shock protein of *M. leprae* displaying pronounced immunogenicity and considered able to induce proliferation of peripheral blood mononuclear cells and T-cell lines from *M. leprae* vaccinated subjects [33] andavailable at large amounts for studies on vaccine formulation; its overexpression and scaling-up in *Saccharomyces cerevisae* have already been described and the recombinant protein can be produced in large scale [34,35]. Nanostructured cationic adjuvant/antigen complexes based on DODAB were characterized over a range of adjuvant and antigen concentrations. Figure 2 shows the effect of antigen and adjuvant concentration on physical properties of the dispersions. Stable cationic nanostructures are available over a limited range of adjuvant and antigen concentrations which clearly depend on antigen nature and are different for different antigens.

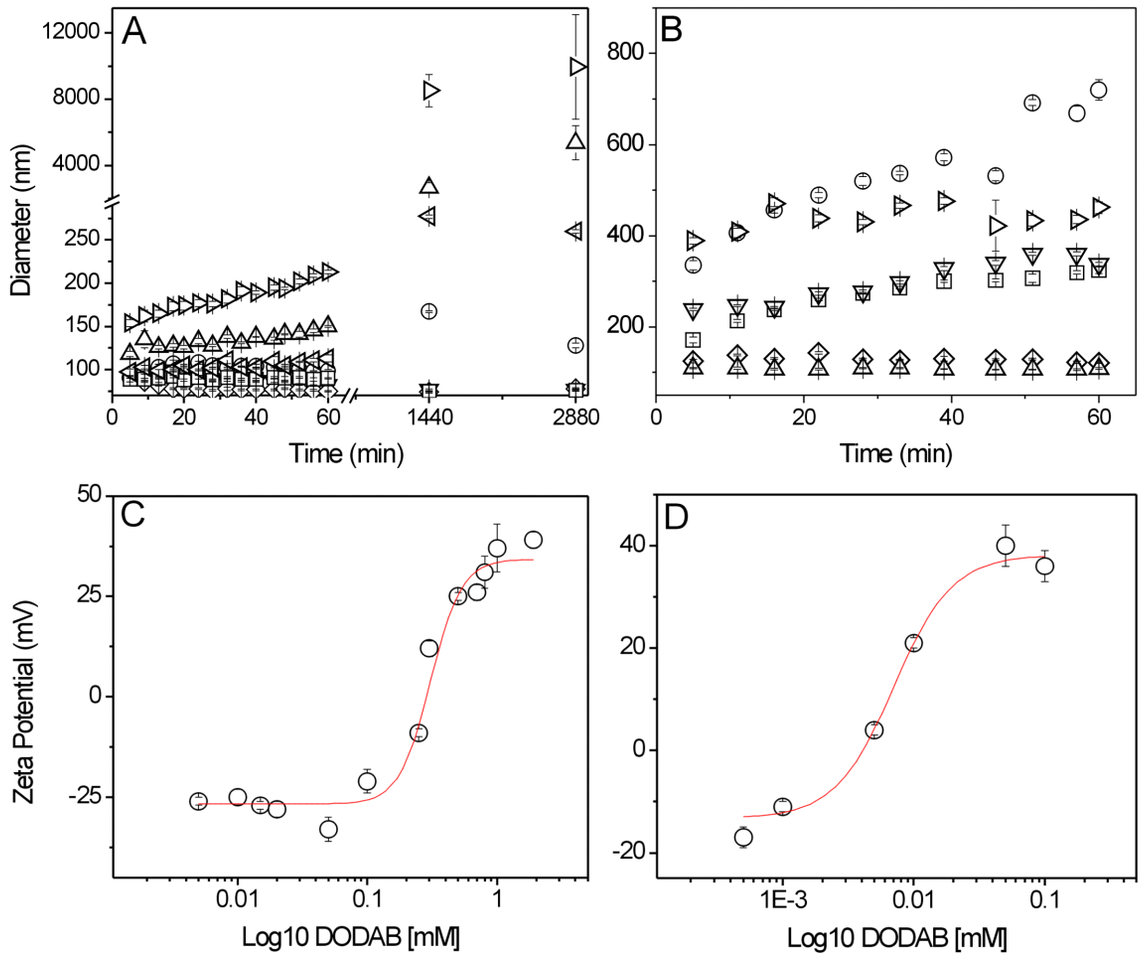

Figure 2. Effect of time and DODAB concentration on the zeta-average diameter of DODAB/BSA (A) or DODAB BF/18/14-Tcra complexes (B). In (A), the kinetics were obtained upon adding DODAB BF at a final concentration of 0.005 ((▷), 0.01 (△), 0.02 (◁), 0.05 (○), 0.5 (◇), 0.8 (□), and 1.0 mM DODAB (◁) to 0.5 mg/mL BSA. In (B), the kinetics were obtained upon adding DODAB BF at a final concentration of 0.0005 (□), 0.001 (○), 0.005 (▷), 0.01(▽), 0.05(◇), and 0.1 mM DODAB (△) to 0.05 mg/mL 18/14-Tcra. The effect of [DODAB] on zeta-potential of complexes with 0.5 mg/mL BSA (C) or 0.05 mg/mL 18/14-Tcra (D) were obtained after 1 h interaction at 25 °C in 1 mM NaCl.Reproduced with permission from reference [27].Reprinted from Vaccine, 27/42, Nilton Lincopan,Noelí M. Espíndola,Adelaide J. Vaz,Maria Helena B. da Costa,Eliana Faquim-Mauro,Ana M. Carmona-Ribeiro,Novel immunoadjuvants based on cationic lipid: Preparation, characterization and activity in vivo,5760-5771.Copyright 2009, with permission from Elsevier.

The humoral and cellular immune responses induced by stable cationic adjuvant/antigen complexes were evaluated in mice from determination of antigen-specific-IgG antibody in serum by ELISA, delayed type hypersensitivity (DH) reactions from footpad swelling tests and cytokines analysis. The results evidence the good colloid stability of the complexes, complete absence of toxicity in mice (i.e. local or general reactions) and their potential utility to induce Th 1 immune response at reduced doses of cationic and toxic DODAB lipid. Possibly due to its chemical stability and low cost when compared to other natural or synthetic lipids, DODAB use as an immunoadjuvant started more than forty years ago [36,37]. This was well before the bilayer nature of DODAB self-assembly in water solution had been described [26]. DODAB as an effective immunoadjuvant has been intensively investigated aiming at subunit vaccine design [38-41]. A major problem of liposomal formulations based on DODAB has been the usually high concentration employed, namely 1-10 mM DODAB [38-41]. DODAB is a cationic lipid and as such, cytotoxic [14-16] requiring dose minimization for administration in vivo. Using the DODAB bilayer fragments to present antigens only 0.1 mM DODAB was employed [27]. The large cellular imune response achieved might be related to the total surface area available for antigen association, which is much larger in the BF dispersion than in closed, large and sometimes multibilayered liposomes. Moreover, the hydrophobic interaction possibly available from BF edges could be an additional and powerful driving force for antigen adsorption and presentation. The second advantage of the bilayer fragments besides their large surfaces area is their size. Depending on sonication power and time plus composition of the dispersing medium, which determine colloid stability, DODAB BF/antigens complexes may have their sizes reduced to and stabilized at a few tenths of nanometers acting similarly to solid inert beads of nanometric size (40-50 nm). These beads turned out to be effective for antigen delivery to antigen-presenting cells (APC), generating potent and combined humoral and CD8+T cell immunity [3]. They are also expected to be optimal for dendritic cells uptake since their size is inside the range of particle diameters (500 nm and below) for optimal dendritic cells uptake of antigens and elicitation of an adequate cellular response [2,5,42]. In reconstituted pig gastric mucus, sub-200 nm particulates from poly (D, L-lactic-co-glycolic) acid and DODAB condensing DNA on particles surface exhibited improved transport rates, stability in mucus, and ability to transfect cells [43]. Silica/DODAB, latex/DODAB and DODAB BF (Figure 1) are available over the sub-200 nm range of sizes thus presenting potential also for design of mucosal vaccines. The third advantage of the bilayer fragments is the absence of depots at the injection site [27]. These depots have been reported for other DODAB formulations at concentrations much higher than 0.1 mM [37,41,44].

Consistently with electrostatic forces between negatively charged antigens and positively charged DODAB BF, cationic bilayer fragments readily adsorb BSA, 18-14/*Tcra* and the hsp-18 kDa recombinant protein.Rapid and extensive adsorption of BSA, anti-BSA or ovalbumin onto large DODAB liposomes was indeed reported previously [29,45,46].On the other hand, bacteria, fungus and eukaryotic cells were also found to adsorb DODAB vesicles and bilayer fragments with high affinity at low ionic strength [14-16], so that cationic DODAB liposomes readily adsorb antigen, such as ovalbumin, and bind avidly to dendritic cells [41], thereby enhancing antigen uptake. Delivery of antigen to cells by immediate contact with the cell surface via electrostatic interaction followed by the induction of active uptake seems to be the mechanism behind the ability of DODAB liposomes or bilayer fragments to act as immunoadjuvants. Sizes and zeta-potentials for assemblies of antigen and cationic lipid based adjuvants

depend on cationic lipid and antigen concentrations. Adjuvant-antigen stability around sizes that are close to the one of adjuvants themselves indicates that the proteins readily adsorb and stabilize them. The adjuvants also stabilized the proteins acting as important dispersing nanocarriers able to induce remarkable degree of protein disaggregation by attaching the proteins either electrostatically or hydrophobically to their structure. At [DODAB] ≤ 0.1 mM and 0.001-0.05 mg/mL of antigen concentration, DODAB based adjuvant /antigen assemblies are cationic, well-dispersed, colloidally stable and immunogenic combining the advantages of low DODAB dose, low cost, controllable sizes for optimal dendritic cells uptake, high chemical stability, ability to incorporate multiple antigens and minimization of toxicity. Their performance is remarkably superior to the one of alum as adjuvant regarding Th1 mediated responses. In contrast to alum or cationic liposomes at 1-10 mM of cationic lipid, local or systemic adverse effects in mice were completely absent at 0.1-0.01mM DODAB.

An important component of the early innate immune response to viruses and bacteria is the secretion of cytokines, which mediate many of the effector functions of innate immunity. IL-10 is an inhibitor of activated macrophages and dendritic cells and is an example of negative feedback regulation because it is produced by macrophages to inhibit their function. This cytokine also inhibits the production of IL-12 and expression of class II major histocompatibility (MHC) molecules. IL-12 is also secreted by macrophages and dendritic cells inducing T cells differentiation into Th1 and natural killer (NK) cells with increased IFN-gamma synthesis and cytotoxic activity. IL-12 and IFN-gamma are the most important cytokines in innate responses to intracellular bacteria such as *Mycobacterium leprae* or tuberculosis [13]. Figure 3 shows the high levels of IL-12 and IFN-gamma induced by the novel cationic adjuvants while presenting the hsp-18 kDa of *M. leprae* to lymphonode cells suggesting a possible application of the novel adjuvants for the design of subunit vaccines against intracelular bacteria. As in DH, adaptive immunity against intracellular bacteria is principally cell mediated and consists of activation of macrophages by CD4+T cells as well as killing of infected cells by CD8+cytotoxic T lymphocytes (CTL).

On basis of IL-12 enhancement of IFN-gamma production and development of Th1 cells, this interleukin itself has been used as a vaccine adjuvant for many infections that are combated by cell-mediated immunity, e.g. leishmaniasis [47]. Subunit vaccines against protozoa that survive within macrophages require as principal defense mechanism cell-mediated immunity, particularly directed to macrophage activation by Th1 cell-derived cytokines. Leishmaniasis mucocutaneous and disseminated is caused by *Leishmania donovani* and CD4+Th1 cells are required to activate macrophages to kill phagocytosed parasites. Resistance to the infection is associated with activation of Leishmania-specific Th1 CD4+T cells which produce IFN-gamma and thereby activate macrophages to destroy intracellular parasites. Conversely, activation of Th2 cells by the protozoan results in increased parasite survival and exarcerbation of lesions because of the macrophage-suppressive actions of Th2 cytokines [47]. Other significant example is the protective role played by CD8+T cells in immunity to the hepatic stages of malaria. These effects may be mediated by direct killing of sporozoite-infected hepatocytes or indirectly by the secretion of IFN-gamma and activation of hepatocytes to produce nitric oxide and other agents that kill parasites. IL-12 induces resistance to sporozoite challenge in rodents and nonhuman primates, presumably by stimulating IFN-gamma production [48].

In viral infections, Il-12 enhances the cytotoxic activity of natural killer cells so that NK cell-mediated killing of virus-infected cells eliminates the reservoir of infection. In this respect, vaccination against the dengue virus is urgently needed in tropical or neotropical regions of the planet and some recombinant DNA vaccines expressing membrane and envelope of viral proteins have been proposed [49]. Possibly the cationic adjuvants available from our group would properly enhance the required Th-1 response for a more effective vaccination against dengue.

Another possible application for the novel adjuvants might be in immunotherapy for tumors. This approach is based in augmentation of host immunity to tumors with tumor vaccines. Immune responses that are able of killing tumor cells consist of CTLs, NK cells, and activated macrophages and these may be actively enhanced by vaccination with tumor cells or antigens, administration of tumors modified to express high levels of cytokines that stimulate T cell proliferation and differentiation, and systemic administration of cytokines [49]. The induction of T cell responses in tumors depends on processing and presentation of tumor antigens to T cells by professional antigen-presenting cells (APCs) which might internalize the tumor antigen adsorbed onto the novel cationic adjuvants. These APCs may stimulate CD8+T cells and CD4+helper T lymphocytes to differentiate for recognition and killing of tumor cells.

Naïve CD4+T cells may differentiate into distinct subsets, such as Th1 and Th2 cells in response to different antigens. For example, the enhancement in production of IL-10 and Il-13 by lymphonode cells elicited by the antigens of *Taenia crassiceps* presented by the DODAB BF adjuvant can be appreciated in Figure 3 [27]. These cytokines are typically associated with responses to allergens and parasites such as helminths and mediate differentiation of CD4+-T cells into Th2 cells [50]. Consistently, low levels of these cytokines were elicited by the *M. leprae* antigen presented by the novel adjuvants (Figure 3). Responses were indeed different for the helminthes and the bacteria antigens and very antigen-specific as they should be [27].

The size, charge and hydrophobic features of DODAB BF led to novel applications in solubilization of hydrophobic drugs [25,51,52], production of biomimetic particles from bilayer coverage of silica [17] or polystyrene particles [53] and design of vaccines [27].Recently, BF was also combined with oligonucleotides [54]. Since synthetic oligonucleotides can inhibit the replication of the Rous sarcoma virus [55], antisense oligonucleotides have been considered a great promise as therapeutic agents and several oligonucleotide-based formulations have reached the clinical trial phase [56,57]. Antisense oligonucleotides have also been extensively used in research on gene expression and function [58-60], vaccine formulation [61], allergy [62] and cancer therapeutics [63].Major obstacles as their degradation by nucleases and poor delivery to the target cells [60,64] suggest the essential role of suitable carriers able to protect oligonucleotides in the biological milieu [60,63–65].There are peculiar features for the interaction between BF and oligonucleotides in comparison to other electrolytes.Effects of salt, dAMP or poly (dA) concentration on BF size and zeta-potential are shown on Figure 4 taken from reference [54]. From 0 to 0.25 mM salt, Dz and zeta-potentials decreased with salt concentration possibly due to massive phosphate anion binding. From 0.25 to 2.5 mM of divalent salt, Dz increased but zeta-potential remained approximately constant and low (Fig. 4A and D).Dz and zeta-potential decreased with dAMP concentration (0– 2.5 mM) (Fig. 4B and E). At 0.05 mM poly (dA) and 0.5 mM DODAB, extensive BF aggregation and/or fusion took place as depicted from large Dz (N 500 nm) (Fig. 4C) and zero of zeta-potential (Fig. 4F). The screening of DODAB

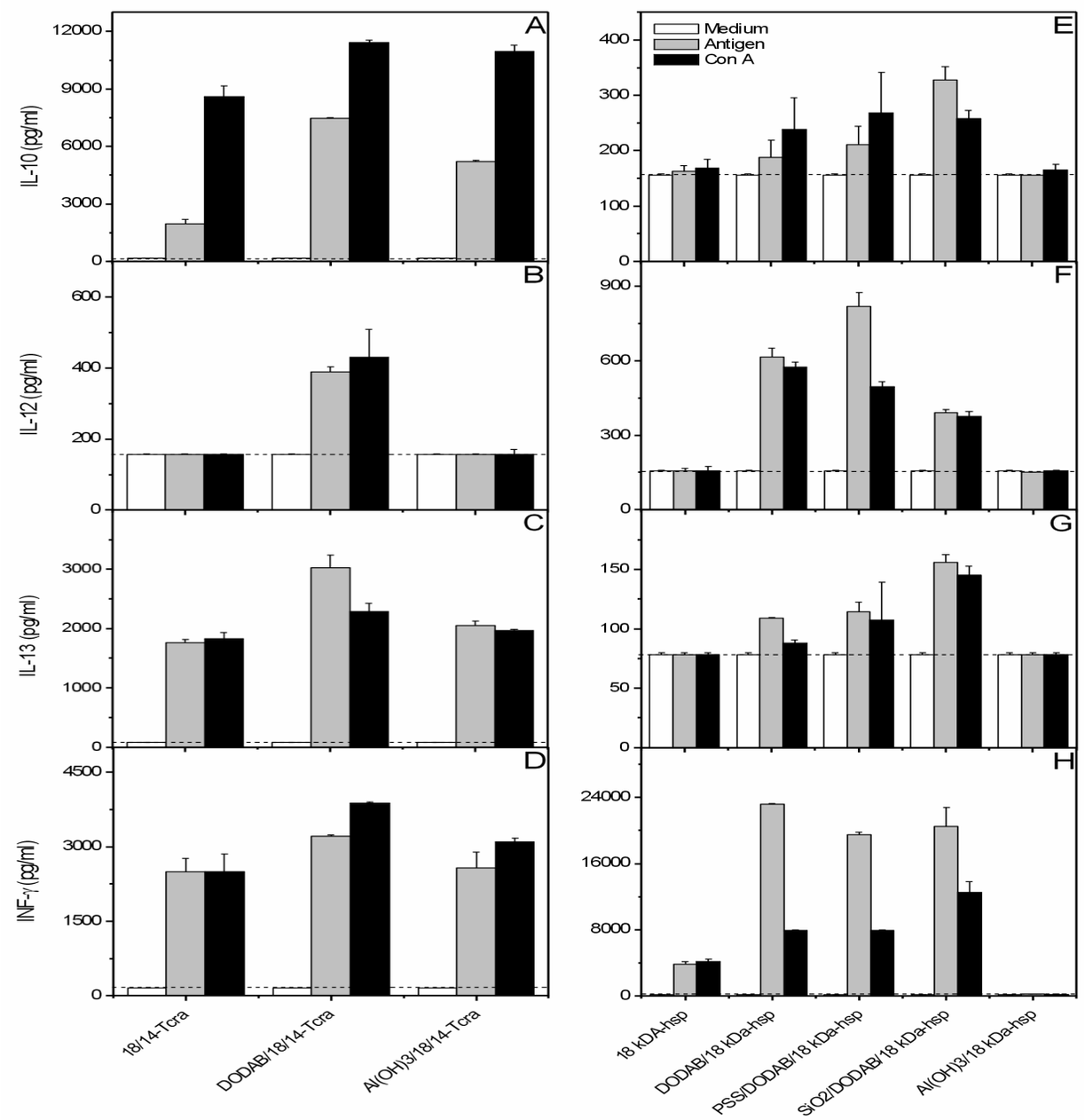

Figure 3. Quantitative analysis of cytokines secreted by lymph node cells from DODAB/18/14-Tcra and Al(OH)3/18/14-Tcra immunized mice (on the left column, A-D) or from 18kDa-hsp, DODAB/18kDa-hsp, PSS/DODAB/18kDa-hsp, SiO_2/DODAB/18 kDa-hsp and $Al(OH)_3$/18kDa-hsp immunized mice (on the right column, E-F). Cells from lymph nodes of BALB/c mice previously immunized with 10 µg of 18/14-*Tcra* administered alone, in DODAB BF or in $Al(OH)_3$ were *in vitro* stimulated with medium, 160 µg/ml of 18/14-*Tcra* or 2.5 µg/mL of ConA for 48 hours and the supernatants collected for cytokine analysis by sandwich kit enzyme-linked immunosorbent assay (ELISA). The results were expressed as mean of the cytokine concentration of two distinct assays ± standard deviation. Limits of detection are shown as horizontal dashed lines. Similarly, cells from lymph nodes of BALB-c mice previously immunized with 15 µg of 18 kDa-hsp from *M. leprae* administered alone or in DODAB BF, PSS/DODAB, silica/DODAB or Al $(OH)_3$ were *in vitro* stimulated with medium, 250 µg/ml of 18 kDa-hsp or 2.5 µg/mL of ConA for 48 hours before following cytokines analysis as above (reproduced with permission from reference [27]). Reprinted from Vaccine, 27/42, Nilton Lincopan, Noelí M. Espíndola, Adelaide J. Vaz,Maria Helena B. da Costa, Eliana Faquim-Mauro, Ana M. Carmona-Ribeiro, Novel immunoadjuvants based on cationic lipid: Preparation, characterization and activity *in vivo*, 5760-5771.Copyright 2009, with permission from Elsevier.

charges by Na_2HPO_4, followed by a decrease in electrostatic repulsion between fragments, could be responsible for DODAB BFs aggregation and/or fusion. DLS data also showed that diameters increase upon addition of Na_2HPO_4 concentrations above 0.5mM (Figure 4A), and this diameter increase up to 400 nm is related to a decrease in the zeta-potential of the fragments (Figure 4D). The charge screening of DODAB charged heads by Na_2HPO_4 explains the decrease in zeta-potential (Figure 4D) as well as the tighter bilayer packing represented by higher mean phase transition temperature for the bilayer [54]. Addition of dAMP leads to a decrease in diameter and zeta-potential of the assemblies (Figures 4B and E). It was shown that DODAB bilayer fragments are able to order dAMP molecules on their surface, causing the dAMP bases to stack [68]. In this case, bulky moieties of dAMP would be exposed at the fragments surface representing a steric hindrance to fragments aggregation and/or fusion which would also contribute to colloid stabilization in dispersion. For polynucleotides such as poly(dA), charge neutralization leads to flocculation whereas charge overcompensation upon increasing poly(dA) concentration leads to colloidal restabilization due to electrostatic repulsion (Figures 4C and F) [54]. This behavior has been often described for polyelectrolytes interacting with particles of opposite charge [67,68]. Beyond the neutralization point, the system regains stability due to charge overcompensation. This phenomenon was observed only for the electrolyte poly (dA) and not for the electrolytes Na_2HPO_4 and dAMP, which are unable to completely neutralize the bilayer [54], as shown in Figure 4. Colloid instability induced by oligonucleotide or salt could be associated with bilayer fusion [54]. In contrast, mononucleotide neither reduced colloid stability over the low range of concentrations tested nor caused BF fusion [54].

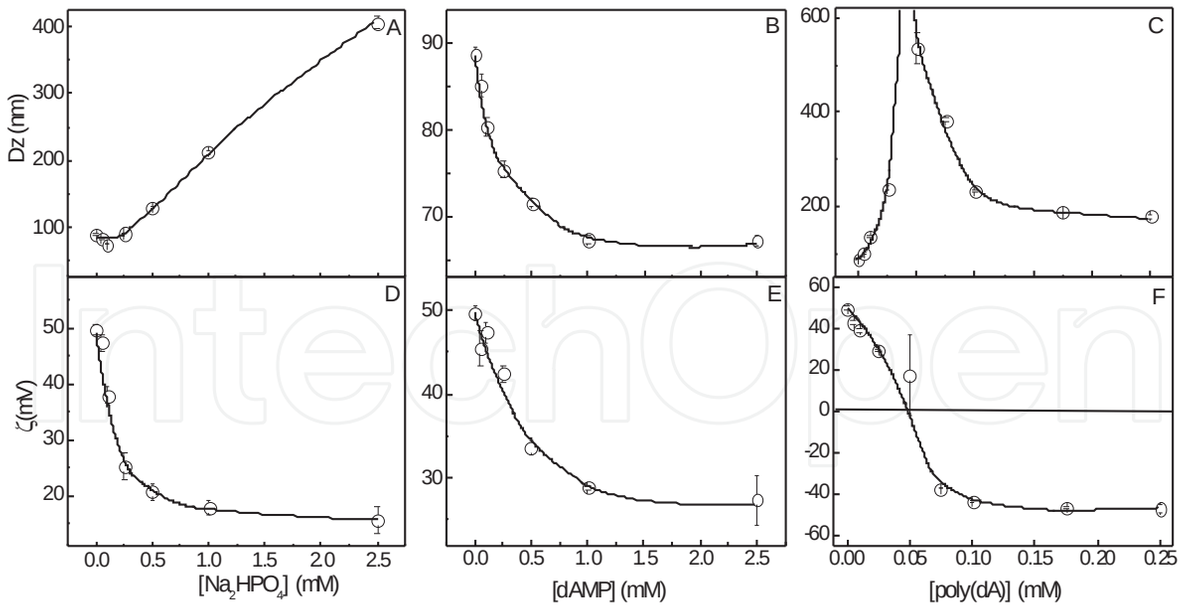

Figure 4. Effect of [Na_2HPO_4], [dAMP] or [poly (dA)] concentrations on the zeta-average diameter (A, B and C) and zeta-potential of DODAB BF at 0.5 mM DODAB (D, E and F).Reprinted from reference [54].Reprinted from Biochimica et Biophysica Acta (BBA)-Biomembranes, 1808/3, Julio H.K. Rozenfeld,Tiago R. Oliveira,M. Teresa Lamy,Ana M. Carmona-Ribeiro, Interaction of cationic bilayer fragments with a model oligonucleotide, 649-655.Copyright 2011, with permission from Elsevier.

Particles are finding a large variety of biomedical and pharmaceutical applications since their size scale can be similar to that of biomacromolecules (e.g., proteins, DNA) and structures (e.g., bacteria and viruses). Their utility for imaging, gene and drug delivery, and vaccine design is undeniable [69,70].Particulate systems are naturally targeted to antigen presenting cells (APC) so that particles deliver antigens to APC more efficiently than soluble antigen [71,72].Positively charged particles with diameters of 500 nm and below were shown to be optimal for dendritic cells uptake [42].DODAB bilayers electrostatically combine with a vast variety of negatively charged biomolecules or biological structures [14]. Silica [17], latex [21,73,74] or hydrophobic drug particles [51,75] have been coated with DODAB with optimal bilayer deposition on particles achieved by coalescence of bilayer fragments at an adequate ionic strength [17,76]. Figure 5 shows how DODAB can cover oppositely charged polystyrene nanoparticles modifying their charge as shown in reference [19]. These cationic nanoparticles contrast with alum regarding their small size and very low polydispersity as shown in Table 1 taken from reference [19]. The optimal bilayer coverage of polystyrene sulfate (PSS) nanoparticles with a DODAB bilayer produces homodisperse particles that successfully present a mixture of purified 18/14 *Taenia crassiceps* proteins (18/14-Tcra) to the immunological system [19].

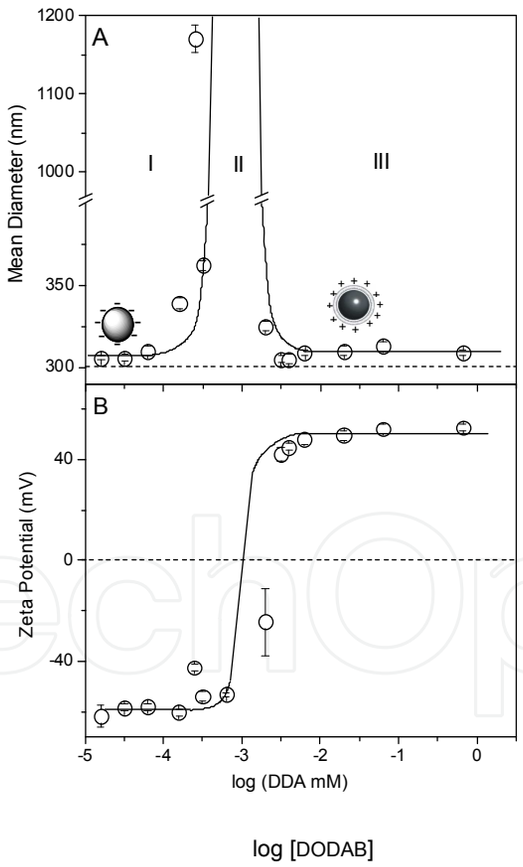

Figure 5. Effect of [DODAB] (in mM) on mean z-average diameter (A) and zeta-potential (B) of PSS particles at 5 x 10^9 particles/mL, 25 °C, in 1 mM NaCl. Bare particle diameter is 301 ± 2 nm. Regions I, II and III define particle charge, which is negative, zero and positive, respectively, from reference [19]. Reprinted from International Journal of Pharmaceutics, 340 / 1–2, N. Lincopan, N.M. Espíndola, A.J. Vaz, A.M. Carmona-Ribeiro, Cationic supported lipid bilayers for antigen presentation, 216-222.Copyright 2007, with permission from Elsevier.

Dispersion	[DODAB] (mM)	[Ag] (μg/mL)	Mean diameter (nm)	Zeta-Potential (mV)	Polydispersity
PSS	-	-	301 ± 2	-60 ± 1	0.064 ± 0.020
DODAB	2.00	-	81 ± 1	45 ± 2	0.230 ± 0.006
PSS/DODAB	0.01	-	309 ± 2	48 ± 2	0.040 ± 0.010
18/14-*Tcra*	-	25	310 ± 5	-52 ± 1	0.214 ± 0.030
DODAB/18/14-*Tcra*	0.01	25	295 ± 3	6 ± 6	0.167 ± 0.023
PSS/DODAB/18/14-*Tcra*	0.01	25	328 ± 3	11 ± 8	0.060 ± 0.020
$Al(OH)_3$	-	-	883 ± 29	28 ± 3	0.381 ± 0.013
$Al(OH)_3$/18/14-*Tcra*	-	25	9574 ± 2361	-23 ± 1	0.525 ± 0.030

Table 1. Physical properties of particles, DODAB dispersion, DODAB-covered particles, proteins and proteins/DODAB-covered particles at 1 mM NaCl.Particles concentration is 5 x 10^9 particles /mL; PSS particle diameter from transmission electron microscopy is 301±2 nm.The $Al(OH)_3$ and PSS were tested at final concentration of 0.05 and 0.075 mg/mL, respectively,from reference [19]. Reprinted from International Journal of Pharmaceutics, 340 / 1–2, N. Lincopan, N.M. Espíndola, A.J. Vaz, A.M. Carmona-Ribeiro, Cationic supported lipid bilayers for antigen presentation, 216-222. Copyright 2007, with permission from Elsevier.

Several cationic agents have been employed for DNA compaction such as cationic peptides and proteins [77], cationic lipids [78], cationic polyelectrolytes, cationic surfactants, or iron (III) [79].DNA compaction has also been used to model chromatin structure and its influence on gene expression. The self-assembled complex of basic histone proteins wrapped by approximately two turns of DNA is a nucleosome, which is the building block in the chromatin structure where DNA of lengths on the order of meters suffers compaction into an~10 μm diameter cell nucleous. Phage DNA, for example, is remarkable for its density of packing. In solution the 40 kbp T7 genome with its contour length of 13.6 μm might span a space several micrometers across and in an infected bacterium, ~1 μm across. Thus, confinement to a 55 nm capsid represents a compaction marked by a density increase by a factor of ~10^4 [80]. Systematic studies on the physical chemistry of the association between cationic nanoparticles and DNA yield rather complex phase diagrams as a function of particle size and concentration [81,82].The way in which positively charged nanoparticles tie up DNA is not obvious, and mechanisms change dramatically with particle size [83]. Only the smallest (10 nm) particles allowed transcription to occur at intermediate loading densities. Larger particles shut transcription down rather abruptly [81]. Cationic nanoparticles have found many uses such as efficient cell transfection agents *in vitro* [84-86] and complexation with long-chained DNA as a simple model of chromatin for transcription studies [81, 87].The compaction of long duplex DNA by cationic nanoparticles (NP) used as a primary model of histone core particles has been systematically studied regarding the effect of salt concentration, particle size, and particle charge by means of single-molecule observations from fluorescence and transmission electron microscopy [87]. DNA compaction proceeds through the formation of beads-on-a-string structures of various morphologies with DNA adsorbed amount per particle depending weakly on NP concentration but increasing with particle size and being optimal at an inter-

mediate salt concentration [87]. Three different complexation mechanisms were proposed: free DNA adsorption onto NP surface, DNA wrapping around NP, and NP collection on DNA chain [87]. On the other hand, particle size has been recognized as an important parameter that determines the mechanism of particle entry into cells. Particles with a diameter of 200 nm or less enter cells almost exclusively via the clathrin-coated pathway whereas particles with a larger diameter penetrate cells via caveolae-mediated endocytosis [88,89].Cationic biomimetic particles produced from adsorption of dioctadecyldimethylammonium bromide (DODAB) bilayers onto polystyrene sulfate (PSS) microspheres have been described by our group since 1992 [18,73,76,90].These cationic bilayer-covered particles exhibit a narrow size distribution and can be produced at any desired size ranging from 70-500 nm of mean hydrodynamic diameter [90]. Polystyrene sulfate (PSS) particles with different sizes were covered by a dioctadecyldimethylammonium bromide (DODAB) bilayer yielding the so-called cationic biomimetic particles (PSS/DODAB). These cationic particles are highly organized, present a narrow size distribution and were obtained over a range of particle sizes [53,90].Thereafter, upon adding λ, T5 or T2-DNA to PSS/DODAB particles, supramolecular assemblies PSS/DODAB/DNA were obtained and characterized over a range of DNA concentrations and particle sizes (80-700 nm). Over the low DNA concentration range, PSS/DODAB/DNA assemblies were cationic, colloidally stable with moderate polydispersity and high cytotoxicity against *E. coli*. From the DNA concentration corresponding to charge neutralization, neutral or anionic supramolecular assemblies PSS/DODAB/DNA exhibited low colloid stability, high polydispersity and moderate cytotoxicity [53]. Some nucleosome mimetic assemblies were observed by atomic force microscopy (AFM) at charge neutralization (zeta-potential equal to zero) [55].Figure 6 shows how cationic nanoparticles can induce DNA compaction [53].

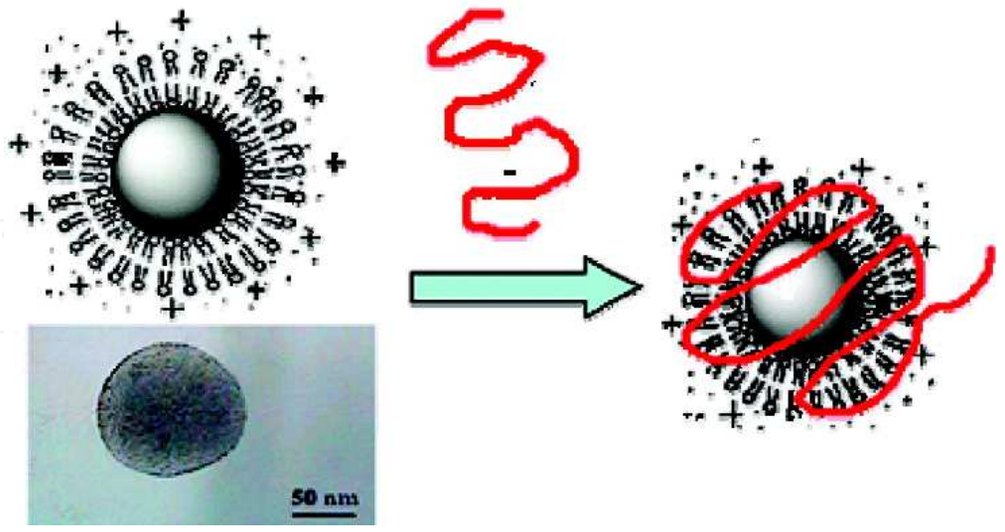

Figure 6. DNA compaction by biomimetic cationic particles of polystyrene microspheres covered by a DODAB cationic bilayer from reference [53].Adapted with permission from Rosa H, Petri DF, Carmona-Ribeiro AM.Interactions between bacteriophage DNA and cationic biomimetic particles. J Phys Chem B. 2008; 112(51):16422-30.Copyright (2008) American Chemical Society.

DNA sequences containing unmethylated CpG dinucleotide are recognized as danger signals by the immune system since they are typical of bacteria and viruses but rare in vertebrates [91, 92]. Natural or synthetic sequences containing unmethylated CpG motifs activate cells that express Toll-like receptor 9 to induce an innate immune response characterized by the production of Th1 and proinflammatory cytokines [92]. Hence, CpG has been extensively used in the induction of cellular immune responses against cancer [63, 93], intracellular infections by pathogens [94, 95] and allergies [62,96]. Both CpG [92] and DODAB BF [27] were reported to improve Th1 responses against antigens when used separately. Recently, DODAB BF and CpG were combined in a single assembly aiming at the comparison between the small, stable and cationic DODAB BF carrying ovalbumin (OVA) and the small, stable and anionic DODAB BF/OVA/CpG assemblies of very similar sizes but opposite charges [97]. Both adjuvants produced similar enhanced Th1 immune responses despite their opposite charges emphasizing the novel concept that particle charge does not matter. In comparison with the traditional alum, the size minimization for the cationic assemblies elicited different responses: alum drove the Th2 whereas DODAB BF/OVA/CpG and DODAB BF/OVA drove the Th1 response. The effects of DODAB BF or DODAB BF/CpG adjuvants with opposite charges but very similar sizes showed that the charge is not important but the size is [97]. Table 2 shows the physical properties of the assemblies. A comparison between mean hydrodynamic diameter (Dz), zeta-potentials and polydispersities for DODAB BF/OVA/CpG and $Al(OH)_3$/OVA/CpG as reproduced from reference [97]. At 20 μM CpG and 0.1 mg/mL $Al(OH)_3$, the dispersion was characterized by a low colloidal stability (large Dz, low zeta-potential (ζ) and high polydispersity). OVA stabilized both $Al(OH)_3$ or $Al(OH)_3$/CpG to a certain extent (Table 2). However, sizes were still larger than those determined for assemblies based on DODAB BF (Table 2). In particular, polydispersity for DODAB BF/OVA/CpG was notably low (0.150) and well below the one obtained for DODAB BF only (0.251), suggesting that OVA/CpG induced stabilization of the DODAB BF dispersion (Table 2).

Dispersion	Dz ± δ (nm)	ζ ± δ (mV)	Polydispersity ± δ
DODAB BF	67 ± 0	47 ± 1	0.251 ± 0.006
DODAB BF/ OVA	274 ± 2	21 ± 0	0.291 ± 0.008
DODAB BF/ OVA/ CpG	245 ± 1	-26 ± 1	0.150 ± 0.020
$Al(OH)_3$	3147 ± 197	16 ± 2	0.415 ± 0.018
$Al(OH)_3$/ OVA	916 ± 17	-29 ± 1	0.231 ± 0.020
$Al(OH)_3$/ CpG	3584 ± 74	9 ± 1	0.407 ± 0.026
$Al(OH)_3$/ OVA/ CpG	570 ± 19	-35 ± 2	0.210 ± 0.020

Table 2. Physical properties of alum or DODAB BF dispersions combined with ovalbumin (OVA) and/or CpG oligonucleotide. Concentrations are 0.1mg/mL OVA, 0.1mM DODAB BF, 0.1mg/mL $Al(OH)_3$ and 20μM CpG. Reprinted from reference [97]. Reprinted from Journal of Controlled Release, 160/2, Julio H.K. Rozenfeld, Sandriana R. Silva, Priscila A. Ranéia, Eliana Faquim-Mauro, Ana M. Carmona-Ribeiro, Stable assemblies of cationic bilayer fragments and CpG oligonucleotide with enhanced immunoadjuvant activity *in vivo*, 367-373.Copyright 2012, with permission from Elsevier.

At 0.1 mg/mL OVA, the dependence of DODAB BF/OVA size and zeta-potential on time and [DODAB] established 0.1 mM DODAB as suitable for obtaining stable and cationic DODAB BF/ OVA assemblies [97]. At 0.1 mM DODAB, 0.1 mg/mL OVA and 0.006 mM CpG, the zeta-potential is zero showing charge neutralization [97]. At [CpG]> 0.006 mM, good colloidal stability for the anionic assemblies due to charge overcompensation was observed whereas at 0.020 mM CpG, these DODAB BF/OVA/CpG assemblies turned out to be highly effective *in vivo* generating responses similar to those elicited by the stable and cationic DODAB BF/OVA. The anti-OVA delayed-type hypersensitivity (DTH) reaction and the secretion of IFN-gamma and IL-12 resulted 6, 42 and 9 times larger for the DODAB BF/OVA/CpG-immunized mice than the same responses by OVA-immunized mice, respectively [97].Figure 7 A and B illustrate the colloidal stability of the assemblies over a range of DODAB concentrations.

The delayed-type hypersensitivity reaction (DTH) is an important *in vivo* response mediated by cells that can be quantified from the footpad sweelling test [44]. Mice immunized with OVA alone or with DODAB BF/CpG or with OVA / $Al(OH)_3$ exhibited a footpad swelling equal to the one observed for naive mice [97]. The largest increase in footpad swelling was observed for DODAB BF/OVA/CpG mice immunization, which was about 6 times larger than the one observed for naive mice and 1.3 times larger than the one observed for DODAB BF/OVA [97]. Figure 7 C shows the improved DTH response in mice induced by the assemblies as adapted from reference [97].

Since OVA isoelectric point is 4.5 [98], this protein is negatively charged at 6.3, the pH of water. Thus, OVA adsorption onto DODAB BF is initially electrostatically driven.The OVA titration with DODAB BF determined ranges of DODAB concentration for occurrence of stable DODAB BF/OVA assemblies as illustrated in Figure 7 A and B. Similar results had been previously described also for DODAB BF/ bovin serum albumin or DODAB BF/ purified antigens from *Taenia crassiceps* [27].When the net charge of the assemblies is zero (at charge neutralization), maximal aggregation was observed for the assemblies. Further increasing DODAB concentration, stabilized them to a certain extent (Figure 7 B). However, sizes and polydispersities were still higher than those of DODAB BF in absence of OVA. Optimal colloidal stability was only achieved upon CpG addition to the system yielding high and negative zeta-potentials plus remarkably small polydispersity [98]. Oligonucleotides with less than 20 nucleotide residues usually behave as rigid charged rods in solution [99]. CpG would also adsorb as rigid charged rods on vacant positive sites of DODAB BF/ OVA assemblies inducing charge overcompensation and recovery of colloidal stability as previously described for a model oligonucleotide above charge neutralization [56].

Figure 7 also illustrates the improvement in the cellular OVA-specific response from the analysis of cytokines secreted by lymph node cells of mice as adapted from reference [97]. IFN-γ and IL-12 production is associated to the Th1 response whereas IL-10 and IL-13 production reflects the Th2 response. Levels of IFN-γ and IL-12 secretion observed in cell cultures of mice immunized with OVA or $Al(OH)_3$/ OVA were low and close to the detection limit for these cytokine assays (Figure 7). For mice immunized with OVA/CpG or DODAB BF/OVA assemblies, the secretion of IFN-γ and IL-12 substantially increased in comparison to secretion from cultured cells of OVA-immunized mice [97]. For the DODAB BF/OVA/CpG immunized mice,

the secretion of IFN-γ and IL-12 increased by 33 and 49 %, respectively when compared to DODAB BF/OVA-immunized mice group and 52% and 35% when compared to OVA/CpG-group. In contrast, the highest secretion of IL-10 and IL-13 was observed in cultures of cells from mice that were immunized with Al(OH)3 /OVA whereas all other assemblies resulted in poor production of these cytokines [97]. Immune responses were similar for anionic DODAB BF/OVA/CpG and cationic DODAB BF/OVA of similar sizes showing that the charge is not important but the size is. The adsorption of antigen on the surface of DODAB large vesicles was shown to stimulate active antigen capture and presentation by dendritic cells (DCs) [41]. Administration of antigen adsorbed on DODAB large vesicles (LV) resulted in formation of an antigen depot at the site of injection which hampered the rapid clearance of antigen that takes place in absence of a carrier [100]. In contrast to DODAB LV, the small DODAB BF/antigen assemblies did not result in any observable depot effect [27, 97]. Furthermore, since the depot was absent for DODAB BF/CpG/OVA the immunostimulatory effect must have occurred via direct effect of the assemblies on the lymphonode antigen presenting cells [97]. Only nanoparticles can specifically target lymph node-resident cells [101]. CpG combined with DODAB BF yielded improved cellular Th1 response [97] possibly due to the appropriate targeting of DODAB BF/CpG/antigen to DCs in charge of expressing endosomal toll like receptor 9 (TLR9) in the lymph nodes [102]. The enhanced Th1 response by anionic DODAB BF/OVA/CpG relative to OVA alone was evidenced by the 6 times increase in DTH reaction, the 42 times increase in IFN-γ secretion by lymph node cells in culture and the 9 times increase in IL-12 secretion also by lymph node cells in culture (Figure 7) [97].

Other delivery systems based on anionic [103] or cationic lipid bilayer [41,44,100,104,105] have also been successful for improving Th1 response against important antigens such as those of influenza [103], hepatitis A and B [103,104], and fungal infections [105]. In general, cationic lipids are known for the production of a large inflammatory response [41, 44].However, for small cationic bilayer fragments as immunoadjuvants, this adverse reaction is absent [27, 97]. The small and anionic DODAB BF/OVA/CpG assemblies [97] also did not elicit adverse reactions similarly to other anionic assemblies [103]. There was no depot effect for these small assemblies [97] and their net negative charge ensured the absence of the adverse reactions observed previously for the large cationic liposomes and vesicles [100]. Recently, based on cross reactivity with *Neisseria lactamica* outer membrane vesicles (OMV) antigens, DODAB BF were combined with OMV to develop a vaccine against *Neisseria meningitidis* in young children [106]. Complexes of 25 μg of OMV in 0.1 mM of DODAB BF were colloidally stable, exhibiting optimal physical properties for efficient antigen presentation [106].

3. Cationic nanostructures based on chitosan and other biocompatible polymers

Chitin is a long-chain polymer of N-acetylglucosamine, a derivative of glucose which can be deacetylated to yield chitosan as shown in Figure 8.

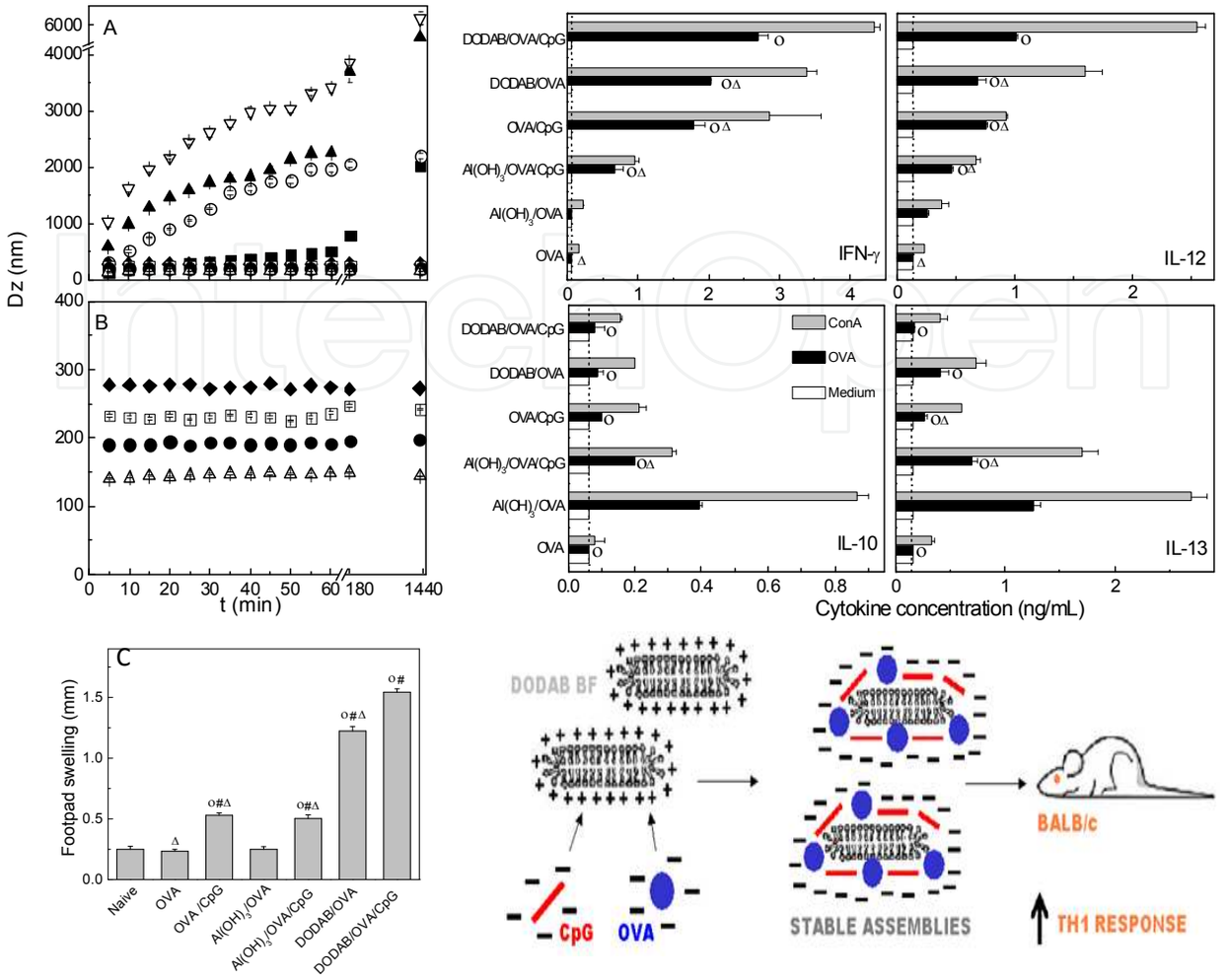

Figure 7. Nanostructures of cationic bilayer fragments and CpG oligonucleotide with enhanced immunoadjuvant activity *in vivo*. In (A), the effect of time and DODAB concentration on zeta-average diameter of DODAB BF/OVA assemblies is seen from the kinetics obtained after adding DODAB BF at a final concentration of 0.005 (■); 0.01 (○); 0.02 (▲); 0.05 (▽); 0.1 (◆); 0.2 (□); 0.5 (●) and 1mM DODAB (△) to 0.1mg/mL OVA. In (B), kinetical data are detailed for the larger DODAB BF concentrations.Assemblies were prepared in 1mM NaCl.In (C), delayed-type hypersensitivity response for BALB/c mice immunized with OVA in different adjuvant formulations determined from the footpad swelling (nm) ± standard error of the mean. Final concentrations are 0.1mM DODAB BF, 20μM CpG and 0.1mg/mL $Al(OH)_3$ and 0.1mg/mL OVA. $p< 0.05$ compared to naive (○), $p< 0.05$ compared to OVA/$Al(OH)_3$ (#) and $p< 0.05$ compared to OVA/DODAB/CpG (△) from reference [97].Adapted from Journal of Controlled Release, 160/2, Julio H.K. Rozenfeld, Sandriana R. Silva, Priscila A. Ranéia, Eliana Faquim-Mauro, Ana M. Carmona-Ribeiro, Stable assemblies of cationic bilayer fragments and CpG oligonucleotide with enhanced immunoadjuvant activity *in vivo*, 367-373.Copyright 2012, with permission from Elsevier.

Since they are biocompatible, biodegradable by deacetylases, mucoadhesive, and nontoxic, with antimicrobial, antiviral, and adjuvant properties, chitin, chitosan and their derivatives have been widely applied in medicine, pharmacy and vaccine design [107, 108]. Chitosan is soluble in diluted acids but is insoluble in water due to deprotonation of its amino moiety [109]. The poor solubility of chitosan at the pH of water represents a serious limitation for its applications as an immunoadjuvant in the clinics [107]. Several chitosan derivatives have been obtained to circumvent this limitation.For example, by attaching galactose moieties to the

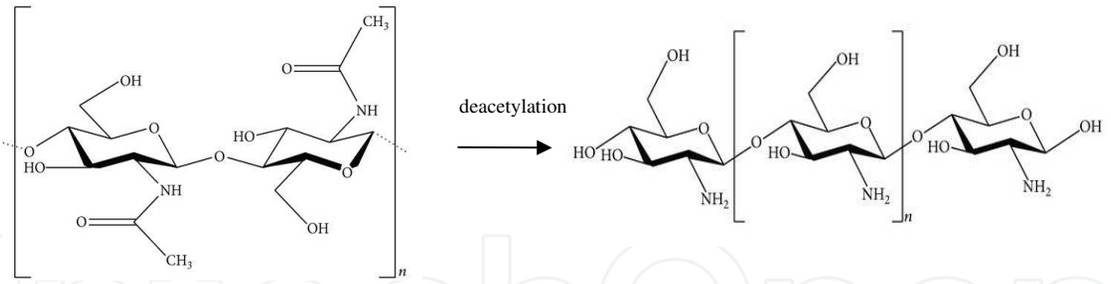

Figure 8. Chitin yielding chitosan by deacetylation.

chitosan molecules, a new water-soluble compound, glycated chitosan (GC), was synthesized [109,110]. GC is a non-toxic biodegradable product used in laser immunotherapy (LIT) which combines local laser irradiation and local administration of GC at primary or metastatic cancers where the tumoral antigens of the irradiated tumor cells combined with GC elicit a potent immune response against the cancer [111,112]. After the first step involving tumor irradiation with a laser beam that causes swelling and disruption of tumor cells by thermal effect, the local injection of GC as immunoadjuvant would lead to the capture of the tumoral antigens by dendritic cells and migration to the lymph nodes where the antigens would be presented to T cells, thus activating cytotoxic T-lymphocytes [113-115]. LIT using GC induced regression of primary and secondary tumours in rats and caused resistance to repeated challenges with tumours of the same type [116]. Furthermore, rats developed immunity could be adoptively transferred [116].

Chitosan nanoparticles have been obtained by ionotropic gelation, complex coacervation, emulsion and microemulsion techniques, and self-assembly of hydrophobically modified chitosan [117]. Ionotropic gelation consists of the ionic crosslinking of chitosan with multivalent counter-ions such as sodium tripolyphosphate (TPP) by adding a dilute chitosan acid solution to a solution of TPP or vice versa, with stirring [118]. Chitosan particles of nanometric size were obtained for chitosan concentrations up to 2.8 g L^{-1} and TPP concentrations from 0.21 to 0.43 g L^{-1}. The size and surface charge of particles can be modified by varying the ratio of chitosan and stabilizer. The main problematic aspects of the technique are the poor colloidal stability of the dispersion which may require the addition of stabilizers, and the need of using very dilute solutions which may be inconvenient when large amounts of nanoparticles are required [117]. Complex coacervation is achieved by mixing two oppositely charged polyelectrolytes. The polyelectrolyte or coacervate complex is structured as nanoparticles. Chitosan–poly (acrylic acid) (PAA) nanoparticles carrying a positive charge with sizes from 50 to 400 nm were obtained by the dropwise addition of dilute chitosan solutions [119]. Carboxymethylcellulose and alginate have also been complexed with chitosan to prepare nanoparticles. Chitosan–carboxymethylcellulose nanoparticles were subsequently coated with plasmid DNA (pDNA) [120]. Chitosan–alginate nanoparticles were loaded with insulin [121]. Nanoparticles have also been obtained in which the polyanion is the active principle itself as for example heparin or DNA or even siRNA. Chitosan–heparin nanoparticles crosslinked with TPP were described [122]. Figure 9 shows on the left a scanning electron micrograph of chitosan– heparin nanoparticles adapted from reference [117] and, on the right, an atomic force micrograph of

chitosan-carboxymethylcellulose nanoparticles [123] prepared by complex coacervation without using without using any organic solvent or crosslinker. These coacervates have the interesting property of combining with cisplatin [123] but can also be combined with peptides, proteins and DNA and therefore are potentially useful for vaccine design.

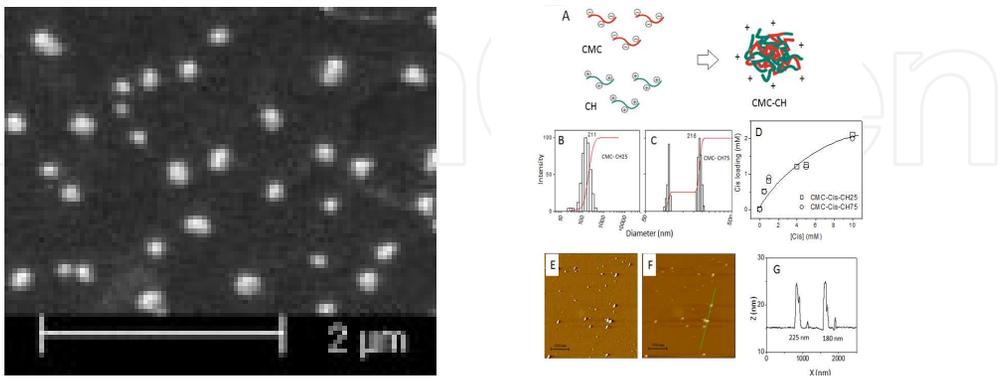

Figure 9. Nanoparticles obtained by complex coacervation of oppositely charged polyelectrolytes: chitosan-heparin on the left [117] and chitosan-carboxymethylcellulose on the right [123].On the left, adapted with permission from Peniche H, Peniche C. Polym Int. Chitosan nanoparticles: a contribution to nanomedicine.2011; 60:883–889. DOI: 10.1002/pi.3056, http://onlinelibrary.wiley.com/doi/10.1002/pi.3056/abstract. Copyright 2011, John Wiley and Sons.On the right, adapted with permission from Vieira DB, Kim V, Petri DFS, Menck CFM, Carmona-Ribeiro AM. Supramolecular assemblies of cisplatin and polyelectrolytes: preparation, characterization and activity against cancer cells. In: Laudon M, Romanowicz B. (eds.) Nanotech Conference & Expo 2012: An Interdisciplinary Integrative Forum on Nanotechnology, Microtechnology, Biotechnology and Cleantechnology, Santa Clara, CA, United States, June 18-21, 2012 (2012), 3, 182-185. Publisher: (CRC Press, Boca Raton, FLA) ISBN 978-1-4665-6287-5.

Other interesting chitosan derivatives have been synthesized and used for the preparation of nanoparticles such as N-trimethylchitosan (TMC) and mono-N-carboxymethyl chitosan (MCC) [124]. In general, mucosal applications of antigens result in poor immune responses. Therefore, mucoadhesive adjuvants are required to enhance the immune response by improving both antigen protection and its cellular uptake. Nanoparticles of TMC or MCC were prepared using the ionic gelation method and loaded with tetanus toxoid (TT) exhibiting high loading efficacy (>90% m/m), sizes within the range of 40-400nm and negative or positive surface charge for MCC and TMC, respectively [124]. The structural integrity of the TT in the formulations was confirmed by SDS-PAGE electrophoresis and the intranasal application in mice elicited high serum IgG titres [124].Figure 10 illustrates the excellent uptake by macrophages of these nanoparticles labeled with a green fluorescent marker as adapted from [124].

Due to the positive charge of chitosan and TMC, and the negative charge of TT, the antigen loading is driven by the electrostatic attraction. There is also a superior association and internalization of chitosan or chitosan derivatives nanoparticles with cells due to their mucoadhesive character [125-127]. CS nanoparticles have a higher association and internalisation with gastrointestinal tissue cells due to electrostatic interactions compared to polystyrene nanoparticles [125].More recently, hybrid nanoparticles based on the complexation between oppositely charged chitosan derivatives (positively charged TMC and negatively

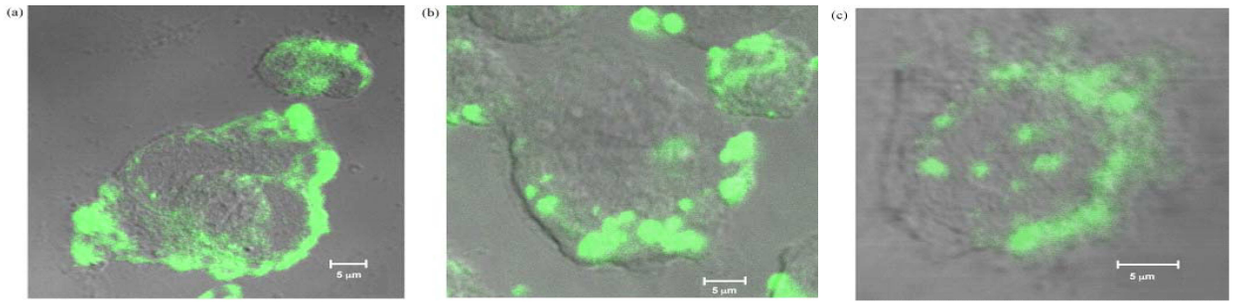

Figure 10. Uptake of chitosan (a), TMC (b) and MCC nanoparticles loaded with bovine serum albumin and labeled with a green fluorescent marker adapted from reference [124].Reprinted from International Journal of Pharmaceutics, 363/1–2, B. Sayın, S. Somavarapu, X.W. Li, M. Thanou, D. Sesardic, H.O.Alpar, S. Şenel, Mono-N-carboxymethyl chitosan (MCC) andN-trimethyl chitosan (TMC) nanoparticles for non-invasive vaccine delivery, 139-148. Copyright 2008, with permission from Elsevier.

charged MCC) without using any organic solvent or crosslinker yielded results similar to those described for the non hybrid nanoparticles [128].

A very promising approach is the development of vaccines based on the initiation of immune response by transfecting dendritic cells (DC) with DNA encoding tumor-associated antigens or immunostimulatory molecules such as cytokines or chemokines [129].Although unmodified chitosan may not be a good gene delivery carrier for DCs because of its low transfection efficiency, some modified chitosans showed improved behavior in delivering genes into DCs. IL-12 gene was delivered to DCs *in vivo* using mannosylated chitosan (MC) via mannose receptor-mediated endocytosis [130,131]. MC not only has good physicochemical properties and low cytotoxicity, but also transfect DCs with much higher efficiency than do the unmodified chitosan particles *in vitro* [130]. *In vivo*, intratumoral injection of MC /plasmid encoding murine IL-12 complex into BALB/c mice bearing CT-26 carcinoma cells clearly suppressed tumor growth and angiogenesis, and significantly induced cell cycle arrest and apoptosis [131]. Mannose-bearing chitosan nanoparticles were also synthesized to entrap complexes of DNA with polyethyleneimine (PEI) and improve the delivery of DNA into antigen-presenting cells (APCs) after intramuscular (i.m.) injection [132]. Compared with the traditional chitosan microspheres, these nanoparticles targeted the DCs (which express a high density of mannose receptors when they are immature) and released their PEI/DNA cargo inside them. After i.m. immunization, the MC/PEI/DNA nanospheres induced significantly enhanced serum antibody and cytotoxic T lymphocyte (CTL) responses in comparison to naked DNA [132]. Figure 11 shows the MC/PEI/DNA nanoparticles and superior cumulative DNA release *in vitro* of these nanoparticles in PBS buffer as adapted from reference [132].

In recent years, DC vaccines, especially DNA-based DC vaccines, have been the focus of attention in immunotherapy against cancer [129]. Genetically engineered DCs previously modified *in vitro* can then be implanted *ex vivo* and activate the tumor-specific CTL response for the killing of cancer cells [129]. Most of these immunotherapy approaches based on DCs genetic modification for the treatment of melanoma [133,134], renal carcinoma [135] and other malignant diseases [136] are in phase I/II clinical trials. However, in many of these clinical trials adenovirus vector is the carrier still employed for DCs modification [137].Since the major

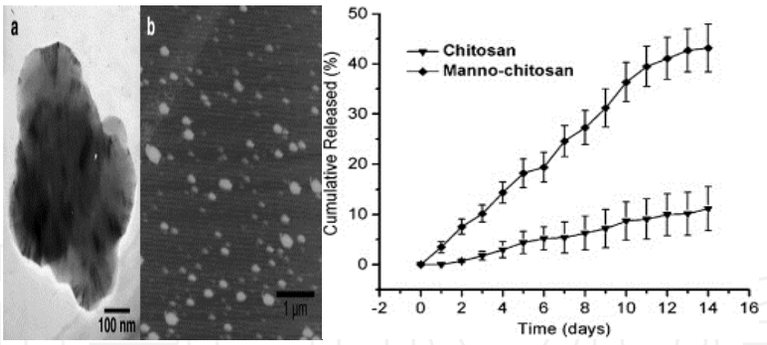

Figure 11. Atomic force micrograph of MC nanoparticles. Release profiles of chitosan and MC nanoparticles as determined from 10 mg of nanoparticles suspended in 1 ml of PBS at pH 7.4 (120mM NaCl, 2.7mM KCl, 10mM PBS) at 37 °C under stirring. At predetermined time intervals, the suspension was centrifuged and replaced with the same volume of fresh medium. The DNA concentration in the supernatant was determined by absorbance at 260nm.Adapted with permission from reference [132]. Reprinted from Journal of Controlled Release, 121 /3, Xianfeng Zhou, Bin Liu, Xianghui Yu, Xizhen Zhang, Yu Chen, Xueyun Wang, Yinghuan Jin, Yongge Wu, Yue Chen, Yaming Shan, Yan Chen, Junqiu Liu, Wei Kong, Jiacong Shen, Controlled release of PEI/DNA complex from mannose-bearing chitosan microspheres as a potent delivery system to enhance immune response to HBV DNA vaccine, 200-207. Copyright 2007, with permission from Elsevier.

barrier for the use of nonviral carriers is their low transfer efficiency compared with viral vectors, the design of novel and efficient nonviral carriers remains a most warranted area of research. Recently, CpG was combined with chitosan in order to enhance the Th1 and Th17-cell polarizing cytokines representing a convenient strategy to elicit cell-mediated immunity [138].Alum, aluminium phosphate and calcium phosphate adjuvants, as well as biodegradable PLG microparticles, inhibited the secretion of bioactive IL-12 by DCs.Thus many of the most widely used adjuvants in preclinical and clinical use exert a specific inhibitory effect on the secretion of IL-12 by DCs. In contrast, chitosan does not inhibit IL-12 secretion and CpG-chitosan is a potent stimulus for the induction of pro-inflammatory T-cell responses [138]. CpG-chitosan [138] behaved similarly to DODAB BF/CpG [98].

The design of DC-based vaccines depends strongly on the generation of effective DCs, so that technologies are needed that produce high antigen expression as a result of delivering DNA encoding antigen into the nucleus of DCs. Barriers to gene delivery into cells start with cell adhesion and uptake, escape from endosomes to the cytoplasm prior to fusion with the lysosomes, trafficking to the nucleus and uptake by the nucleus [139].In particular, large foreign molecules such as proteins and genes are mostly unable to enter the nucleus of non-dividing cells. An interesting strategy has been the combination of cationic nanoparticles with a nuclear localization signal (NLS) which enhanced pDNA delivery to the nucleus of DCs when combined with electroporation [140].

There is a consensus in the literature that additional immunostimulation provided by LPS, CpG and other molecules are required to improve the Th1 response of cationic chitosan or quaternized trimethylchitosan (TMC) [138,141].These nanoparticles containing ovalbumin as a model antigen (TMC/OVA nanoparticles) and an immunopotentiator (TMC/OVA/immunopotentiator nanoparticles) were recently evaluated [141]. The selection of immunopotentiators included Tolllike receptor (TLR) ligands lipopolysaccharide (LPS), PAM3CSK4 (PAM), CpG

DNA, the NOD-like receptor 2 ligand muramyl dipeptide (MDP) and the GM1 ganglioside receptor ligand, cholera toxin B subunit (CTB). The TMC/OVA/immunopotentiator nanoparticles were characterised regarding their immunogenicity by determining the serum IgG, IgG1, IgG2a titres and secretory IgA levels in nasal washes after intradermal and nasal vaccination in mice. After nasal vaccination, TMC/OVA nanoparticles containing LPS or MDP elicited higher IgG, IgG1 and sIgA levels than non-adjuvanted TMC/OVA particles, whereas nanoparticles containing CTB, PAM or CpG did not. After intradermal vaccination, the TMC/OVA/CpG and TMC/OVA/LPS nanoparticles provoked higher IgG titres than plain TMC/OVA particles [141]. Co-encapsulation of the antigen and an additional immunopotentiator into TMC nanoparticles improved the immunogenicity of the vaccine [141].

Nanoparticles of Fe_3O_4 coated with glutamic acid plus PEI were used to encapsulate DNA encoding a potent antigen of *Mycobacterium tuberculosis* (Ag85A-ESAT-6) plus IL-21 generating a strong immune response and marked growth inhibition of *M. tuberculosis* in mice [142]. More importantly, compared with using DNA vaccine Ag85A-ESAT-6-IL-21 alone, the nanoparticle-based DNA vaccine Ag85A-ESAT-6-IL-21 showed a statistically significant increase in the protective efficacy against *M. tuberculosis* infection in the immunized mice [142]. The expression of both the fusion protein of Ag85A-ESAT-6 and secreted IL-21 protein was confirmed by western blot [142]. Cationic poly(lactide-co-glycolide) (PLGA) nanoparticles were obtained by coating the PLGA nanoparticles with chitosan and used as an intranasal delivery vehicle as a means of administering foot and mouth disease virus (FMDV) DNA vaccine encoding the FMDV capsid protein and the bovine IL-6 gene as a means of enhancing mucosal and systemic immune responses in animals [143]. Guinea pigs and rats were intranasally vaccinated with the respective chitosan-coated PLGA nano/microparticles-loaded FMDV DNA vaccine formulations; the IL-6 gene was important as an additional immunoestimulator [143].

Cationized gelatin nanoparticles (GNPs) were used as carriers to improve delivery of immunostimulatory CpG oligonucleotides (CpG ODN) both *in vitro* and *in vivo* [144]. Gelatin nanoparticles were prepared according to the two step desolvation method including further optimization [145].Briefly, 1.25 g gelatin was dissolved in 25 ml highly purified water (5%w/w) at 50oC under constant stirring (700 rpm). The first desolvation step was initiated by quick addition of 25 ml acetone. After discarding the supernatant containing low molecular weight fractions of gelatin, the sediment was redissolved in 25 ml water under constant stirring at 50 oC. Depending on the desired particle size, the pH was then adjusted to a value between 2.3 (approx.resulting particle size: 150 nm) and 3.0 (approx. resulting particle size: 300 nm).Subsequently, the second desolvation step was initiated by drop-wise addition of 50 ml acetone (during constant stirring at 700 rpm) and resulted in the formation of nanoparticles. The nanoparticles were stabilized by cross-linking with glutaraldehyde (150 ml of a 25% solution) under stirring. After 12 h the particles were purified from non-reacted glutaraldehyde by centrifugation and redispersion in water. Cationization of the nanoparticles was achieved through introduction of a quaternary amino group by covalent coupling of cholaminechloride hydrochloride onto the particles surface.The delivery by cationic gelatin nanoparticles strongly increased the imunostimulatory effects of CpG oligonucleotides [144].

The effects of CpG are not restricted to modulation of immune responses in vaccine adjuvants. CpG itself has been considered for the treatment of asthma and other allergic diseases [62]. CpG oligonucleotides (CpG–ODN, resembling bacterial DNA) engage TLR-9 on B-cells, dendritic cells and other cell types, resulting in a cascade that includes induction of Th1-type and T-regulatory-type immune responses. Preclinical models of asthma have demonstrated that CpG–ODN are potent inhibitors of atopic responses, suppressing Th2 cytokine and, reducing airway eosinophilia, systemic levels of IgE, and bronchial hyperreactivity-in short the critical attributes of the asthmatic phenotype. In models of chronic allergen exposure, CpG–ODN are also effective at preventing the development of airway remodeling. In established asthma, CpG–ODN can reverse manifestations of disease, both when used alone or in combination with allergen immunotherapy. Early clinical trials have had mixed results, including a significant benefit when CpG–ODN were conjugated to ragweed allergen in an allergic rhinitis immunotherapy study, but only limited efficacy seen when administered prior to allergen challenge in asthmatics [145]. Further study of CpG–ODNs for the treatment of asthma and other atopic disorders is warranted by existing data.Figure 12 summarizes the cellular trafficking of CpG [62].

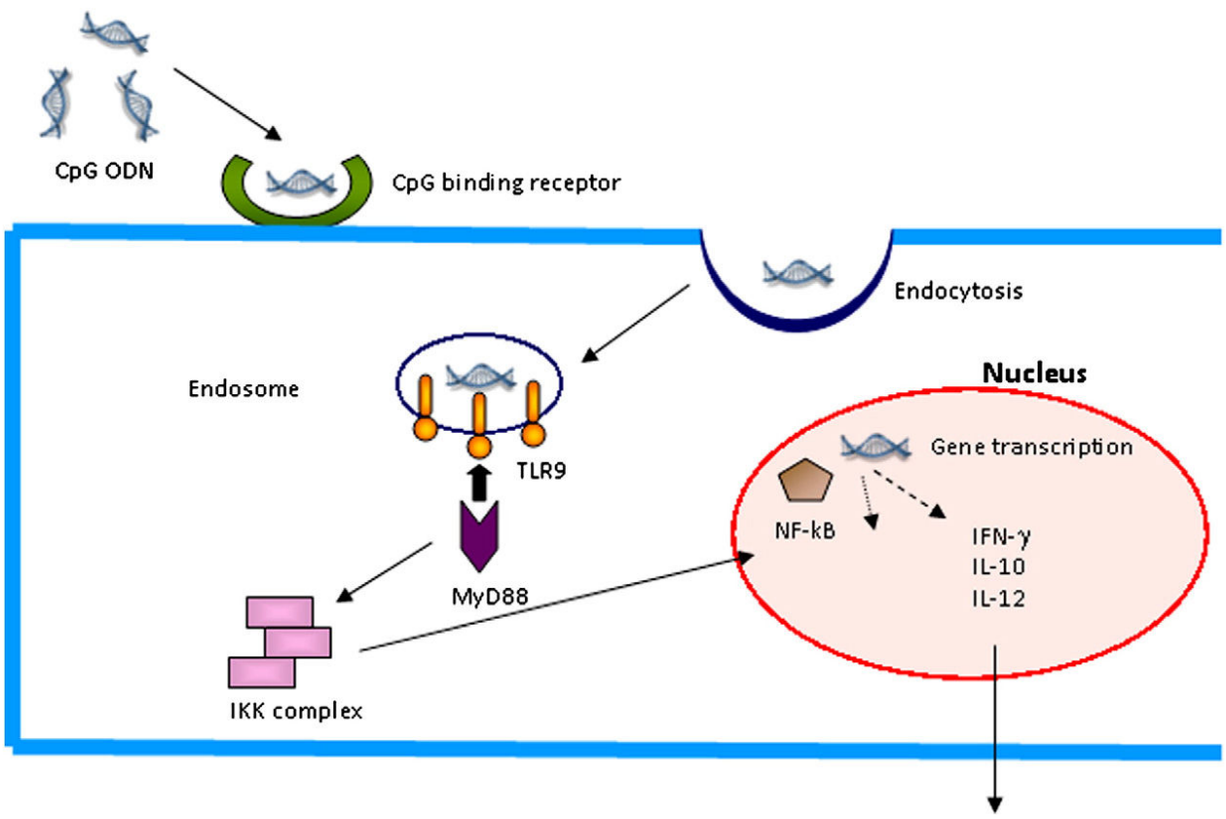

Figure 12. CpG–ODNs are bound by CpG binding protein at the cell surface. The oligonucleotide then undergoes endocytosis where it is bound by TLR9 in the endosome. TLR9 then interacts with MyD88 and the IKK complex and is translocated to the cell nucleus. Once in the cell nucleus, the CpG ODNs activate NF-kB and initiate the induction of cytokines and chemokines.Reproduced with permission from reference [62].Reprinted from Advanced Drug Delivery Reviews, 61 / 3, David E. Fonseca, Joel N. Kline, Use of CpG oligonucleotides in treatment of asthma and allergic disease, 256-272. Copyright 2009, with permission from Elsevier.

Polymer-based nanoscale assemblies based on cationic polymers have been intensively searched for biomedical applications [146-148]. Poly(2-aminoethyl methacrylate) (PAEM) cationic homopolymers with defined chain length and narrow molecular weight distribution were synthesized using atom transfer radical polymerization (ATRP), and PAEM/plasmid DNA polyplexes interaction with DCs showed its potential for DNA vaccines [149]. PAEM of different chain lengths (45, 75, and 150 repeating units) showed varying strength in condensing plasmid DNA into narrowly dispersed nanoparticles with very low cytotoxicity. Longer polymer chain length resulted in higher levels of overallcellular uptake and nuclear uptake of plasmid DNA, but shorter polymer chains favored intracellular and intranuclear release of free plasmid from the polyplexes [149]. When a model antigen-encoding ovalbumin plasmid was used, transfected DCs stimulated the activation of naïve CD8 (+) T cells to produce high levels of interferon-γ. The efficiency of transfection, DC maturation, and CD8 (+) T cell activation showed varying degrees of polymer chain-length dependence [149].These structurally defined cationic polymers may have much potential as efficient DNA vaccine carriers and immunoadjuvants.

Other strategies involve the production of hybrid nanoparticles. For example, biocompatible polymeric particles of poly caprolactone were coated with chitosan for effective immunization against influenza using recombinant Influenza A virus (A/California/07/2009) H1N1 hemagglutinin (HA) protein, for the induction of humoral, cellular and mucosal immunity [150]. CS-PCL nanoparticles (cationic nanoparticles) produced humoral (both systemic and mucosal) and cellular immune responses upon nasal administration demonstrating high potential for their use as a carrier adjuvant for nasal administered influenza antigens [150]. Other example is represented by biocompatible core-shell cationic nanoparticles, composed of an inner hard core of poly(methylmethacrylate) (PMMA) and a hydrophilic tentacular shell bearing positively charged groups and poly(ethyleneglycol) chains covalently bound to the core prepared by emulsion polymerization and characterized *in vitro* and *in vivo* for DNA vaccine applications [151]. The nanoparticles reversibly adsorbed large amounts of DNA, mainly through electrostatic interactions, preserved its functional structure, efficiently delivered it intracellularly, and were not toxic *in vitro* or *in mice* [151]. Furthermore, two intramuscular (i.m.) immunizations (4 weeks apart) with a very low dose (1 microg) of the plasmid pCV-tat delivered by these nanoparticles followed by one or two protein boosts induced significant antigen-specific humoral and cellular responses and greatly increased Th1-type T cell responses and CTLs against HIV-1 Tat [151].

Chitosan-based DNA vaccines against Japanese encephalitis virus using transdermal immunization were prepared by using the associative feature of cationic chitosan and anionic DNAs which formed nanoscale complexes [152]. The expression of green fluorescent protein (GFP) reporter gene was observable and traceable in epidermis and spleen over 3 days. The expressions of GFP and the activation of dendritic cells (DCs) were evident and co-localized in hair follicles and epidermis. Mice immunized with the developed chitosan-JEV DNA vaccines elicited desired JEV specific antibodies, whereby the mice maintained high survival rates against 50xLD(50) JEV challenge. The low-pressure-gene-gun mediated chitosan-based JEV

DNA vaccines have proven to be convenient and efficacious, thereby with high capacity in deployment for future prophylaxis against JEV outbreaks [152].

Pullulan, a fungal exopolysaccharide produced from starch is a water soluble, neutral linear biopolymer consisting of α-1,6-linked maltotriose residues. Recently, a cationic cholesteryl group-bearing pullulan (cCHP) derivative was used to obtain self-assembled nanogels for administering an intranasal vaccine containing a non-toxic subunit fragment of *Clostridium botulinum* type-A neurotoxin BoHc/A (cCHP-BoHc/A) which continuously adhered to the nasal epithelium and was effectively taken up by mucosal dendritic cells after its release from the cCHP nanogel. Vigorous botulinum-neurotoxin-A-neutralizing serum IgG and secretory IgA antibody responses were induced without co-administration of mucosal adjuvant. Importantly, intranasally administered cCHP-BoHc/A did not accumulate in the olfactory bulbs or brain. Moreover, intranasally immunized tetanus toxoid with cCHP nanogel induced strong tetanus-toxoid-specific systemic and mucosal immune responses suggesting that cCHP nanogel can be used as a universal protein-based antigen-delivery vehicle for adjuvant-free intranasal vaccination [153].Recently, the same system was used to deliver a vaccine against pneumonia [154]. This pneumococcal vaccine combined the advantages of pneumococcal surface protein A (PspA) with a nontoxic intranasal vaccine delivery system based on a nanometer-sized hydrogel cCHP. The efficacy of the nanogel-based PspA nasal vaccine (cCHP-PspA) was tested in murine pneumococcal airway infection models. Intranasal vaccination with cCHP-PspA provided protective immunity against lethal challenge with Streptococcus pneumoniae Xen10, reduced colonization and invasion by bacteria in the upper and lower respiratory tracts, and induced systemic and nasal mucosal Th17 responses, high levels of PspA-specific serum immunoglobulin G (IgG), and nasal and bronchial IgA antibody responses. Moreover, there was no sign of PspA delivery by nanogel to either the olfactory bulbs or the central nervous system after intranasal administration showing the effectiveness and safety of the nanogel-based PspA nasal vaccine system as a universal mucosal vaccine against pneumococcal respiratory infection.

Lipid-biopolymer hybrid systems have long been described for incorporation of pDNA [155].Cationic liposomes mixed with pDNA that had been pre-condensed with a polycation (e.g.protamine) yielded the LPD formulation or lipid-polymer-DNA (DOTAP, protamine, pDNA) [156]. As compared to conventional lipoplexes, the double bilayer of LPD confers greater stability and structural homogeneity to the assemblies [156]. Strong antitumor immunity could be generated when a peptide antigen was incorporated into LPD (cationic liposome-polycation-pDNA) nanoparticles [155, 156]. In another study, both the cationic liposome and DNA were shown to be required for the full immunostimulation activity of LPD [157]. The unique ability of LPD to readily move into local lymphoid tissues and to activate antigen-presenting cells might be responsible for its strong immunostimulatory activity [157]. Moreover, cationic liposome stimulates the expression of CD80/CD86 on dendritic cells (DCs), but not the release of TNF-alpha from DCs, suggesting the existence of a NF-kappa B-independent immunostimulation pathway for cationic lipids such as DOTAP [157].The LPD complexes were nanoparticles with less than 100 nm of mean diameter due to the compaction of DNA induced by the polycation [158].

Similarly, cationic maltosylated PEI (mPEI) was electrostatically complexed to a plasmid encoding the human papillomavirus (HPV) type 16L1 protein (pHPV16L1), and further complexed to a maltose binding protein (MBP)-fused human papilloma virus type 16L1 fusion protein (HPV16L1-MBP) [159]. The intracellular co-delivery of protein and plasmid DNA vaccines was significantly higher for mPEI-based triple nanocomplexes than for a simple physical mixture of the proteins and DNA [160]. The high expression levels were comparable to those obtained using dual complexes of mPEI and the plasmid DNA. In vivo, co-immunization of mice with HPV16L1-MBP/mPEI/pHPV16L1 nanocomplexes triggered the highest levels of humoral immune responses among various vaccination groups and there was a significant increase in the number of interferon-γ producing CD8 (+) T cells compared with the use of mixed proteins and plasmid DNA. This approach could enhance the immunogenicity of HPV16L1 vaccines [159].

Platelet-derived growth factor-bb (PDGF-BB) and fibroblast growth factor-2(FGF-2) are known to induce chemotaxis, proliferation, differentiation, and matrix synthesis so that growth factor therapy is an emerging treatment modality that enhances tissue vascularization, promotes healing and regeneration and can treat a variety of inflammatory diseases or promote healing of chronic wounds [160]. Chitosan was combined with PDGF-BB and FGF-2 genes to induce the therapeutic expression of these genes in mice. The percentage of the residues that are glucosamine is called the degree of deacetylation (DDA). PDGF-BB and FGF-2 genes were amplified from human tissues by RT-PCR. To increase the secretion of FGF-2, a recombinant 4sFGF-2 was constructed bearing eight amino-acid residues of the signal peptide of FGF-4. PCR products were inserted into the expression vector pVax1 to produce recombinant plasmids pVax1-4sFGF2 and pVax1-PDGF-BB, which were then injected into BALB/C mice in the format of polyelectrolyte nanocomplexes with specific chitosans of controlled DDA and molecular weight, including 92-10, 80-10, and 80-80(DDA-number average molecular weight or M(n) in kDa) [160]. ELISA assays on mice sera showed that recombinant FGF-2 and PDGF-BB proteins were efficiently expressed and specific antibodies to these proteins could be identified in sera of injected mice, but with levels that were clearly dependent on the specific chitosan used [160]. High DDA low molecular weight chitosans were efficient protein expressors with minimal or no generation of neutralizing antibodies, while lowering DDA resulted in greater antibody levels and correspondingly lower levels of detected recombinant protein. Histological analyses corroborated these results by revealing greater inflammatory infiltrates in lower DDA chitosans, which produced higher antibody titers [160]. The subcutaneous delivery of the plasmids was more efficient than the one by intramuscular injection [160]. Certain chitosans can be efficient, non-toxic and therapeutic protein delivery systems or vectors for DNA vaccines [160]. The recent advances that have led to a more rational design of chitosan polyplexes has recently been reviewed [161]. Recent progress in preparation and characterisation of chitosan based polyplexes has enabled coupling analysis of chitosans structural parameters that has led to increased transfection efficiency by tailoring of chitosan's structure in accordance with a more rational design of chitosan polyplexes [161].

Genetic vaccination against leishmaniasis has been intensively pursued by Rafati´s group [162-166]. Leishmaniasis is a major health problem in many tropical and sub-tropical countries

and development of a safe and easily-available vaccine has high priority. Several antigens potentially capable of inducing protective immunity have been studied like cathepsin L-like cysteine proteinases (CPs) and type Iand II CPs used in a heterologous prime-boost vaccination regime for experimental visceral leishmaniasis in dogs. Due to the promising results of the mentioned vaccination regime, cationic solid lipid nanoparticles (cSLNs) for in vitro delivery of these antigens were used as a cocktail DNA vaccine to deliver immunogenic CP genes efficiently. Briefly, the cationic lipid DOTAP (0.4% w/v) was dissolved in hot aqueous phase which was then added to the melted cetyl palmitate and cholesterol (5.1%w/v) phase containing tween 80 as a nonionic surfactant at 3.2:1molar ratio. Emulsification was carried out by stirring the mixture at 2000 rpm for 10 min at 90°C. Samples were then homogenized using a high shear homogenizer at 18,000 g for 15 min and the cSLN dispersion was obtained by direct cooling of hot O/W microemulsion on an ice-bath while stirring at 1000 rpm. The cSLNs were washed by centrifugation (6000 g, 10 min, three times) using 100 kDa ultra centrifugal filters to purify the suspension from the excess of surfactant. Efficiency/cytotoxicity ratio of cSLN-pDNAs formulations was comparable to linear PEI-25KD-pDNAs polyplexes while exhibiting significantly lower cytotoxicity [162].In addition, C-terminal domain deletion in CPs enhanced the protective activity of cpa/cpb loaded solid lipid nanoparticles against *Leishmania major* in BALB/c mice [163]. cSLN's were able to boost immuneresponse magnitude of cpa/cpb(-CTE) cocktail vaccination against leishmaniasis so that the average parasite inhibition percent could be increased significantly [163].The cSLNs were used to formulate three pDNAs encoding *L. major* cysteine proteinase type I (cpa), II (cpb) and III (cpc)\[164]. BALB/c mice were immunized twice with a 3-week interval, with SLN-pcDNA-cpa/b/c, pcDNA-cpa/b/c, SLN, SLN-pcDNA and PBS.Footpad assessments, parasite burden, cytokine and antibody responses were evaluated. Mice vaccinated with SLN-pcDNA-cpa/b/c significantly ($p<0.05$) showed higher protection levels with specific Th1 immune response development compared to other groups [164]. This was the first report demonstrating cSLNs as a nanoscale vehicle boosting immune response quality and quantity [164].

Very fundamental questions regarding size and charge of nanoscale cationic assemblies and their role in adjuvanticity have seldom been asked and properly answered [167-169]. The role of surface charge density in cationic liposome-promoted dendritic cell maturation and vaccine-induced immune responses was recently evaluated for a series of DOTAP/DOPC cationic liposomes with different surface densities by incorporating varying amounts of DOPC (a neutrallipid) into DOTAP (a cationic lipid) [167]. The liposomes with a relatively high charge density, such as DOTAP/DOPC 5:0 and 4:1 liposomes, potently enhanced dendriticcell maturation, ROS generation, antigen uptake, as well as the production of OVA-specific IgG2a and IFN-γ [167]. In contrast, low-charge liposomes, such asDOTAP/DOPC 1:4 liposome, failed to promote immune responses even at highconcentrations, confirming that the immunoregulatory effect of cationic liposomes is mostly attributable to their surface charge density. Moreover, the DOTAP/DOPC 1:4 liposome suppressed anti-OVA antibody responses *in vivo*. Overall, maintaining an appropriate surface charge is crucial for optimizing the adjuvant effect of cationic liposomes and enhancing the efficacy of liposome-based vaccines.

Recently, the high-pressure extrusion method was used to obtain cationic liposomes entrapping pDNA over a range of sizes [168]. This is a well-known sizing method for liposomes, but had not been applied for liposomes that are already loaded with pDNA. Liposomes composed of egg PC, DOTAP, and DOPE with entrapped pDNA were prepared by the dehydration-rehydration method and subjected to various extrusion cycles, comparing different membrane pore sizes and extrusion frequencies [168]. At optimized extrusion conditions, liposome diameter and polydispersity were reduced from 560 nm and 0.56 to 150 nm and 0.14, respectively, and 35% of the pDNA was retained [168]. Importantly, gel electrophoresis and transfection experiments with pDNA extracted from these extruded liposomes demonstrated the preservation of the structural and functional integrity of the pDNA. The reduction in size resulted in enhanced transfection of HeLa cells, as detected by functional expression of the fluorescent protein, eGFP. In addition, these liposomes were able to stimulate Toll-like receptor 9, indicating efficient endosomal uptake and release of the included pDNA. In conclusion, high-pressure extrusion is a suitable technique to size cationic liposomes with entrapped pDNA and allows preparation of well-defined nanosized pDNA-liposomes, with preserved pDNA integrity [168]. Their improved transfection efficiency and ability to activate an important pattern-recognition receptor are favorable properties for DNA vaccine delivery vehicles [168].

The formulation of DNA into both liposomal and polymeric cationic nanoparticles was found to block completely vaccination-induced antigen expression in mice and ex vivo human skin [169].This detrimental effect of cationic nanoparticle formulation was explained by immobilization of the nanoparticles in the extracellular matrix, caused by electrostatic interactions of the cationic nanoparticles with negatively charged extracellular matrix components [169]. Shielding the surface charge of the nanoparticles by PEGylation improved *in vivo* antigen expression more than 55 fold [169]. Furthermore, this shielding of cationic surface charge resulted in antigen-specific T cell responses that were similar as those induced by naked DNA for the two lipo-and polyplex DNA carrier systems tested [169]. Charge shielding should be generally applied for the development of intradermal vaccine formulations [169].

Various aspects that could be decisive in the formulation of efficient and stable carrier system(s) for the development of malaria vaccine have recently been reviewed [170]. The capacity of multi subunit DNA vaccine encoding different stage *Plasmodium* antigens to induce CD8+cytotoxic T lymphocytes and interferon-γ responses in mice, monkeys and humans has been observed [170]. Moreover, genetic vaccination may be capable of eliciting both cell mediated and humoral immune responses. The cytotoxic T cell responses are categorically needed against intracellular hepatic stage and humoral response with antibodies targeted against antigens from all stages of malaria parasite life cycle. Therefore, the key to success for any DNA based vaccine is to design a vector able to serve as a safe and efficient delivery system. Also against malaria the development of non-viral DNA-mediated gene transfer techniques based on artificial vectors such as liposomes, virosomes, microspheres and nanoparticles is strongly warranted [170].

4. Conclusions

Cationic nanostructures from lipids, biocompatible polymers, hybrid lipid-polymer or organic-inorganic systems efficiently combine with important biomolecules such as proteins, DNA, epitopes and enhancers of the immune response, most of them negatively charged. Their growing importance for vaccine design becomes evident from the examination of the works briefly described in this overview.

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