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Polymeric nanogels as vaccine delivery systems

Sílvia A. Ferreira, MSc[a], Francisco M. Gama, PhD[a,*], Manuel Vilanova, PhD[b,c]

[a]*IBB-Institute for Biotechnology and Bioengineering, Centre for Biological Engineering, University of Minho, Braga, Portugal*
[b]*Laboratório de Imunologia Mário Arala Chaves, IMFF-Departamento de Imuno-Fisiologia e Farmacologia, ICBAS-Instituto de Ciências Biomédicas de Abel Salazar da Universidade do Porto, Porto, Portugal*
[c]*IBMC-Instituto de Biologia Molecular e Celular, Porto, Portugal*

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Abstract

Polymeric nanogels find a relevant field of application in the formulation of a new generation of therapeutic and preventive vaccines, aiming at the fine-tuned modulation of the immune response. Intrinsic properties of polymeric nanogels, such as material chemistry, size and shape, surface charge, and hydrophobicity or hydrophilicity, may be determining factors in shaping the induced immune response. These materials can thus work as synthetic adjuvants, which can also be conjugated with immunostimulants. Polymeric nanogels protect vaccine antigens from degradation in vivo and, surface-conjugated with antibodies or specific ligands, could increase active targeting specificity. This review covers the recent published data concerning the modulation of innate and adaptive immune responses by engineered polymeric nanogels and their potential application as delivery systems in vaccination.

From the Clinical Editor: In this review, the utility of polymeric nanogels is discussed as adjuvants and protective agents for enhanced vaccination with more robust immune response and a more uniform outcome.

Key words: Adjuvants; Delivery systems; Polymeric nanogels; Vaccines

The induction of an antigen-specific immune response is a key principle of vaccination. Usually, immunogenicity depends on the action of antigen-presenting cells (APCs), into which antigens must be carried to be internally processed for surface presentation to T cells. In addition to antigen presentation, APC-dependent activation of the T cells also requires the upregulated expression of surface co-stimulatory molecules or secreted factors such as cytokines (Figure 1). Cytokines released from APCs drive the differentiation of T cells, which acquire effector functions including antigen-specific cytotoxicity or specific help to cellular or humoral immunity. In naturally occurring immunizations, especially in the course of infection, the upregulated expression of T-cell co-stimulatory molecules or cytokines by APCs is triggered by ligands characteristic of invading pathogens, generally designated as pathogen-associated molecular patterns. These may be recognized by specific pattern-recognition receptors on the surface, in the cytosol, or inside intracellular compartments, of which Toll-like receptors (TLRs) are the prototypical example.[1]

When pathogen-associated molecular patterns are absent in subunit vaccine formulations, adjuvants might be necessary to potentiate such mechanisms of T-cell stimulation and therefore immunogenicity. Some adjuvants can act on APCs by engaging specific pattern-recognition receptors, thus mimicking signals usually provided by pathogens.[2] In addition, some delivery systems (e.g., liposomes or virus-like particle preparations) can combine adjuvant activity with the targeted delivery of antigens to APCs.[3] Only a few adjuvants are currently licensed for human vaccines, which include alum (aluminum salts), MF59, Adjuvant System 03 (AS03), Montanide ISA 51, Adjuvant System 04 (AS04), and virosomes.[2,4,5] Approved adjuvants are mostly used in preventive vaccines for diseases caused by viruses or extracellular bacteria, for which specific antibodies provide significant protection.[5] This illustrates one of the limitations of current vaccines: their efficacy mostly relies on the induction of protective antibodies rather than on cell-mediated immunity.[6] This may hamper the immune-based prevention of diseases caused by intracellular pathogens or cancer, where cellular immunity is a key effector mechanism.[7] To overcome the limitation mentioned and to improve vaccine performance or potency as well, novel compounds or formulations are currently being rationally designed. Among them, polymeric nanogels have potential to be safe and effective alternatives to the current means of vaccine delivery, being able to induce not only strong and long-lasting

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*Corresponding author: Institute for Biotechnology and Bioengineering, Centre for Biological Engineering, University of Minho, Campus Gualtar, 4710-057 Braga, Portugal.

E-mail address: fmgama@deb.uminho.pt (F.M. Gama).

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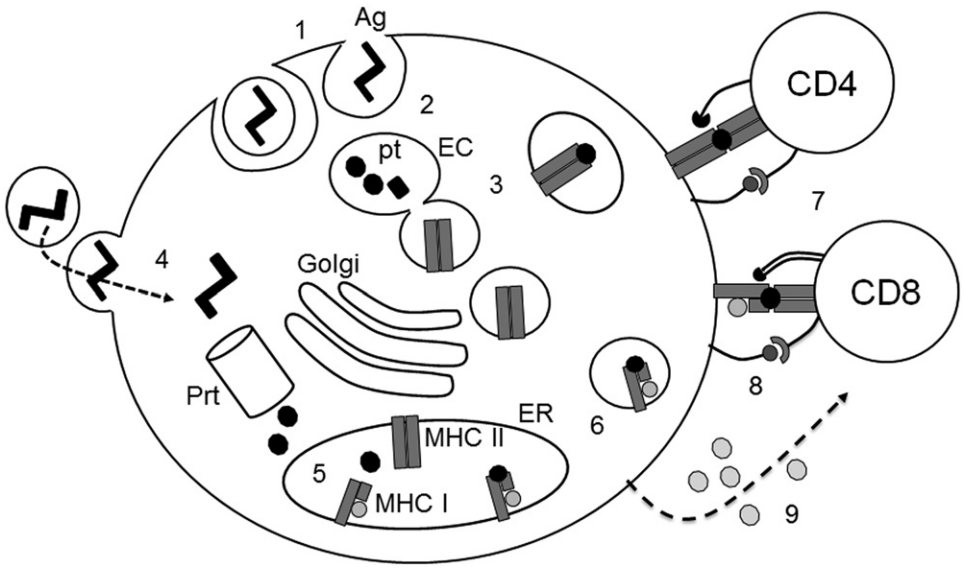

Figure 1. Vaccination antigens (Ag), e.g., purified proteins, may be internalized by antigen-presenting cells (APCs) from the external milieu by phagocytosis or other endocytic processes (1). Within endocytic compartments (EC), antigen is processed at acidic pH into peptide fragments (pt) (2). Peptides are then loaded to major histocompatibility complex (MHC) class II molecules (MHC II) transported in vesicles originating from Golgi apparatus (Golgi) into endocytic compartments (3). Mature endosomes fuse with the plasma membrane where peptides loaded to MHC class II molecules are exposed. Antigens may also be delivered to the cytosol and cleaved within the proteasome (Prt), resulting in peptides (4). These are transported into the endoplasmic reticulum (ER), where they are loaded to MHC class I molecules (MHC I) in a chaperone-assisted process (5). Peptide-loaded MHC class I molecules are transported to the cell membrane (6), where they can be recognized by specific $CD8^+$ T cells, whereas $CD4^+$ T cells recognize peptides presented in the context of MHC class II molecules (7). APCs also provide co-stimulatory stimuli by either surface molecules (8) or soluble factors such as cytokines (9).

antibody responses but also potent cell-mediated immunity based on $CD4^+$ and $CD8^+$ T-cell responses. Polymeric nanogels may combine immunomodulatory properties with targeted antigen delivery features, working as integrated adjuvants.[8,9]

Polymeric nanogels as vaccine delivery or adjuvant systems

Nanometer-sized polymeric hydrogels—nanogels or hydrogel nanoparticles (NPs; size from 1 to 1000 nm)—are swollen networks composed of amphiphilic or hydrophilic polyionic polymers, either natural or synthetic. Nanogels are promising multifunctional polymeric NPs with potential as delivery systems because of their unique properties. These include tunable chemical and physical structures, flexible nanosize, large surface area for multivalent conjugation, high water content, biocompatibility,[10,11] loading capacity, stability, ability to target specific cells and specific cell compartments, immunomodulatory properties, and responsiveness to environmental factors.[12] As nanocarriers must be delivered to specific sites upon injection into body fluids, the possibility of modulating the chemical and physical properties of NPs could be most helpful in overcoming major biological barriers such as the reticuloendothelial system, clearance through kidney glomeruli, and nonspecific accumulation in different organs.

Nanogels have been designed using different approaches, which can be classified into physical self-assembly of interactive polymers, polymerization of monomers in a homogeneous phase or in a micro or nanoscale heterogeneous environment, cross-linking of preformed polymers, and template-assisted nanofabrication.[13] Several natural biopolymers have been commonly used to develop nanogels, for example, dextran, dextrin, pullulan, mannan, chitosan, poly-L-lysine, poly(γ-glutamic acid) (γ-PGA), heparin, hyaluronic acid, and alginate. Synthetic biodegradable and biocompatible polymers—for example, poly(methyl methacrylate) (PMMA), poly(D,L-lactic acid) (PLA), poly(glycolic acid) (PGA), poly(D,L-lactic-*co*-glycolic acid) (PLGA), and poly(ε-caprolactone) (PCL)—approved for human administration by the US Food and Drug Administration,[14] have frequently been used in the development of potential vaccine delivery systems.

NPs may be engineered so as to either stimulate or suppress the triggered immune response, thus providing the appropriate activity: upregulation or downregulation of the immune response, respectively, in the prevention or treatment of infections and cancer or of allergies and autoimmune diseases.[15] A compilation of studies in which different NPs have been used as delivery systems for antigens or nucleic acids in different experimental or clinical settings is presented in Table 1. The interaction of particulate delivery systems with APCs may stimulate these cells in a way resembling the stimulation triggered by pathogens, which are commonly recognized, phagocytosed, and processed by professional APCs. In vitro studies have shown that exposing dendritic cells (DCs) to polymeric NPs resulted in their activation and maturation, as evidenced by upregulated surface expression of major histocompatibility complex (MHC) class II or co-stimulatory molecules (CD40, CD80, CD83, and CD86),[29,33,68] secretion of cytokines[65] and chemokines, and expression of chemokine receptors.[72] Activated DCs migrate to regional lymph nodes, where they present antigen to T cells, thereby triggering cellular

immunity, which in turn may provide help to humoral immunity. The intrinsic adjuvant properties of NPs to stimulate APCs may thus be an additional advantage in their potential as antigen delivery systems for vaccination. In vaccination, the relationship between the rate of antigen availability and the induction of the immune response is poorly understood as, apparently, no clear or direct correlation could be found between in vitro antigen release profile and the antigen-specific in vivo immune response. Indeed, both rapid and extended in vitro antigen release profiles have been shown to induce similar immune responses in animal studies upon intranasal administration.[39,73] Continuous antigen delivery is usually considered to be more effective in inducing immunity, as prolonged antigen exposure allows enough time for affinity maturation and antibody isotype switching to occur, and immune memory to be generated.[74] Moreover, in a DNA-based vaccine delivery system, controlled release of DNA in synchrony with the natural development of the immune response seems to be crucial for the efficacy of the vaccine.[75] However, it has been suggested that antigen presentation by APCs to naive and effector T cells may be required over only the first few days for an efficient induction of T-cell expansion and differentiation, and that antigen presentation for weeks or months may instead lead to T-cell death, decreased effector expansion, and reduced cytokine production by recovered effectors.[76]

Properties of the nanodevice vs. immune response

Nanogels themselves may be intrinsically immunologically active, by virtue of their particular character or as a result of protein adsorption, being recognized as a "danger signal." Properties of the nanodelivery systems, such as material chemistry, size and shape, surface charge, and hydrophobicity or hydrophilicity, are determining factors in the induced immunity and will be discussed below.

Material chemistry

The molecular weight and the co-polymer composition can modulate the load release mechanism; higher polymer molecular weight results in slower in vitro release of the biological agent.[77] On the other hand, the functional groups at the nanogel surface can be modified with various targeting moieties for site-specific vaccine delivery. A number of materials chemistries have been engineered to promote release of NPs' payload within the endolysosomal compartments, attending to both pH and the reductive-oxidative gradient experienced during endolysosomal processing.[78] Nanomaterials sensitive to acid hydrolysis (orthoesters; hydrazide or acetal bonds)[69,70,79] or to reduction (glutathione-responsive)[80] have been investigated for endosomal release of biological agents. Whereas the endosomal-phagosomal compartment is the aimed target for MHC class II loading, MHC class I presentation requires that the antigen payload be present in the cytosol.[81] Thus, disruption of the endosomal membrane barrier so that exogenous antigens could gain access to the cytosol is an important target and a challenging problem. Endosomal disruption is also necessary for DNA-based vaccination, in which plasmid DNA must be expressed to produce the antigen.[82] To avoid lysosomal trafficking, "smart" polymers have been designed. Both pH-sensitive and reductive-sensitive nanomaterials release oligonucleotides and peptides into the cytosol as the endosome is acidified, avoiding the lysosomal fusion. As a consequence, antigen processing may occur through the cytosolic (MHC class I) pathway instead of the exogenous (MHC class II) pathway, thus promoting cross-presentation. Indeed, endosomal escape following uptake of PLGA NPs loaded with ovalbumin (OVA) have been linked to an increase in the presence of antigen in the cytosol and promoted cross-presentation, enhancing and sustaining antigen presentation via MHC class I to a much higher degree than soluble antigen, in murine bone marrow–derived DCs.[83] Protamine-coated PLGA NPs stimulated murine bone marrow–derived DCs and enhanced the cross-presentation of encapsulated exogenous antigen (OVA) by facilitating antigen uptake and lysosomal escape.[84] Moreover, HIV envelope glycoprotein (gp)120 loaded in hydrophobically modified γ-PGA (γ-hPGA, γ-PGA-graft-L-phenylalanine co-polymers) NPs induced antigen-specific effector and $CD8^+$ T-cell memory response in intranasally (IN) immunized mice.[46] Acid-degradable particles, whose components exert an osmotic pressure on the endosomal-phagosomal membrane, leading to its rupture, have been used to enhance antigen presentation in vitro and vaccination in vivo.[68,85,86] CD205 (dendritic and epithelial cells, 205-kDa integral membrane glycoprotein, or DEC-205)-targeted acid-degradable acetal–cross-linked OVA-loaded particles enhanced antigen presentation by DCs via both MHC class I and II pathways, leading to an improved cellular immune response.[85] Co-delivery with adjuvants [unmethylated cytosine-phosphate-guanine (CpG) and interleukin-10–specific antisense oligonucleotides] increased secretion of interleukin (IL)-12 and maximized the elicited cellular immune response.[86] Acid-degradable acetal–cross-linked NPs encapsulating both OVA and CpG (TLR9 agonist) induced an OVA-specific $CD8^+$ T-cell response.[68] The same system, but with CpG covalently attached, enhanced the efficacy of antigen presentation via MHC class I, leading to a greater cytotoxic T-cell activity, as compared with particles subcutaneously (SC) co-administered with adjuvant in an unbound form in mice.[70] This system effectively induced protective immunity using the MO5 murine melanoma model until the moment when the cancer cells apparently stopped expressing the antigen, due to in vivo selection pressure.[70]

Immune potentiation can also be achieved by activating the complement system. Triggering of complement activates a series of proteins and enzymes that can promote inflammation, macrophage phagocytosis, anaphylaxis, B-cell activation, and T-cell response, as well as enhance antigen presentation to B cells by follicular DCs.[9] Certain primary hydroxyls[87] or amine groups[88] on the pathogen molecules or on the material surface can bind to the exposed thioester of C3b to activate complement by an alternative pathway.[87,89] Furthermore, activating materials also facilitate the binding of factor B to C3b, forming the C3 convertase, which catalyzes the cleavage of more C3, thus amplifying the response.[89] Interestingly, C1q binds to hydrophobic molecules or aggregates, such as lipopolysaccharide (LPS) and liposomes.[90] Hence, the incorporation of hydrophobic domains could activate complement through the classical pathway. In summary, although much of biomaterials research seeks to avoid interactions with the complement system,

Table 1
Polymeric nanogels as delivery systems for antigens or nucleic acids

Polymeric nanogels[†]	Antigen or nucleic acid	Model[‡]	Response	Route(s)	Reference
CHP	HER2	Human	IgG, $CD4^+$, $CD8^+$ T cells	SC	16-18
CHP	NY-ESO-1	Human	IgG, $CD4^+$, $CD8^+$ T cells	SC	18-20
Chitosan–deoxycholic acid	MAGE-3 peptide	615 mice	IFN-γ, cytotoxic T cells	SC	21
Chitosan	TT	BALB/c	IgG, IgA	IN	22
Chitosan	TT	BALB/c	IFN-γ, IgG	IN	23
Chitosan	HBsAg	BALB/c	IgG	IM/IN	24
Chitosan-alginate (+ *CpG*)	HBsAg	BALB/c	IgG, IgA	IN	25
Chitosan	*Leishmania* superoxide dismutase	BALB/c	IgG	SC	26
Chitosan	Plasmid DNA encoding peanut allergen	AKR/J mice	Decreased IgE	Orally	27
Chitosan	Plasmid DNA encoding HBsAg	BALB/c	IFN-γ, IL-2, IgG, IgA	IN	28
Chitosan	Plasmid DNA encoding *M. tuberculosis* peptides	HLA-A2 transgenic mice	IFN-γ	IM/pulmonary	29
Chitosan	Plasmid DNA encoding VP1 of CVB3	BALB/c	IgG, IgA, cytotoxic T cell	IN	30
Chitosan	Plasmid DNA encoding RSV antigens	BALB/c	IFN-γ, IgG, IgA, cytotoxic T cells	IN	31
Chitosan/TMC	OVA	BALB/c	IgG	intraduodenally	32
TMC	OVA	BALB/c	IgG	ID	33
TMC	OVA	BALB/c	IgG	IM	34,35
TMC	OVA	BALB/c	IgG, IgA	IN	35
TMC (+ *LPS/CTB/ PAM_3CSK_4/MDP/CpG*)	OVA	BALB/c	IgG, IgA	ID/IN	36
TMC–hyaluronic acid	OVA	BALB/c	IgG	IN/ID	37
TMC	HBsAg	BALB/c	IgG/IgA	IN	38
TMC	DT	BALB/c	IgG	ID	33
TMC	Influenza A subunit H3N2	C57BL/6	IgG/IgA	IM/IN	39
TMC-MCC	TT	BALB/c	IgG	IN	40
TMC-alginate	Urease	Kunming mice	IgG/IgA	SC/oral	41
γ-hPGA	Listeriolysin peptide	C57BL/6	Survival	Rear footpad	42
γ-hPGA	Tax peptide	C3H mice	Cytotoxic T cells	SC	43
γ-hPGA	gp100 peptide	C57BL/6	IFN-γ	SC	43
γ-hPGA	EphA2 peptide	C57BL/6	IFN-γ, cytotoxic T cells	IP	44
γ-hPGA	OVA	C57BL/6	Cytotoxic T cells	SC	45
γ-hPGA	OVA	C57BL/6	IgG, cytotoxic T cells	Rear footpad	42
γ-hPGA	HIV-1 gp120	BALB/c	IFN-γ, cytotoxic T cells	IN	46
γ-hPGA	HIV-1 p24	BALB/c	IFN-γ, IgG	SC	47
γ-hPGA	Influenza HA	BALB/c	IFN-γ, IL-4, IL-6, IgG, cytotoxic T cells	SC	48
γ-hPGA	Influenza HA	BALB/c	IFN-γ, IL-4, IL-6, IgG/IgA	SC/IN	49
PEI/γ-PGA	Plasmid DNA encoding *P. yoelii* MSP-1 C-terminal	C57BL/6	IFN-γ, IL12p40, IgG	IV	50,51
PLA (+ *poly-U*)	OVA	C57BL/6	IFN-γ, IgG	SC	52
PLA	HIV-1 p24 and/or gp120	BALB/c	IL-2, IL-6, IL-10, IgG, cytotoxic T cells	SC	53,54
PLA	HBsAg	Wistar rats	IFN-γ, IgG	IM	55
PLGA	Allergen Bet v 1	BALB/c	IFN-γ, IL-10, decreased IgG1	SC	56
PLGA (+ *7-acyl lipid A*)	TRP-2 peptide	C57BL/6	$CD8^+$-derived IFN-γ	SC	57
PLGA, PLGA/TMC	OVA	BALB/c	IgG	IM	35
PLGA (+ *7-acyl lipid A*)	OVA	BALB/c	IFN-γ, $CD4^+$, $CD8^+$ T cells	IP/SC	58
PLGA (+ *MPL*)	OVA	BALB/c	IgG, IgA	Orally	59
PLGA (+ *CpG)*	TT	C57BL/6	IFN-γ, IgG	SC	60
PLGA	*B. pertussis* toxoid and filamentous HA	BALB/c	IFN-γ, IL-5, IgG	IP/orally	61
PLGA	BSA	BALB/c	IgG	SC/orally/IN	62
PLGA	Protein from *N. meningitides* type B	CD1 mice	IgG	IM/IP	63
PLGA (+ *LTK63*)	HIV-1 gp140	BALB/c	IgG	IN + IM	63
PLGA (+ *MPL)*	HBcAg	C57BL/6	IFN-γ	SC	64
PLGA-PEI	Rv1733c DNA /protein	BALB/c	IFN-γ	IM/pulmonary	65
PCL, PLGA, PLGA-PCL	DT	BALB/c	IFN-γ, IL-6, IgG	IN/IM	66

Table 1 (*continued*)

Polymeric nanogels[†]	Antigen or nucleic acid	Model[‡]	Response	Route(s)	Reference
PCL-based (+ *CTB*)	*S. equi* proteins	BALB/c	IL-2, IL-4, IgG	IN	67
Acid-degradable polyacrylamide (+ *CpG*)	OVA	C57BL/6	Cytotoxic T cells	SC	68-70
PMMA–Eudragit L100-55	HIV-1 Tat protein	BALB/c	IFN-γ, IL-4, IgG, cytotoxic T cells	IN	71

BSA, bovine serum albumin; CHP, cholesterol-bearing pullulan; CpG, cytosine-phosphate-guanine; CTB, cholera toxin B subunit; DT, diphtheria toxoid; EphA2, ephrin type-A receptor 2; γ-hPGA, hydrophobically modified poly-(γ-glutamic acid), γ-PGA-graft-L-phenylalanine co-polymers; HA, hemagglutinin; HBcAg, hepatitis B core antigen; HBsAg, hepatitis B surface antigen; HER2, human epidermal growth factor receptor 2; ID, intradermally; IFN-γ, interferon-γ; Ig, immunoglobulin; IL, interleukin; IM, intramuscularly; IN, intranasally; IP, intraperitoneally; IV, intravenously; LPS, lipopolysaccharide; MAGE-3, melanoma-associated antigen 3; MCC, mono-*N*-carboxymethyl chitosan; MDP, muramyl dipeptide; MPL, monophosphoryl lipid A; MSP-1, merozoite surface protein 1; NY-ESO-1, New York-esophagus 1 protein; OVA, ovalbumin; PAM_3CSK_4, Pam_3Cys-Ser-$(Lys)_4$; PCL, poly(ε-caprolactone); PEI, poly(ethylenimine); γ-PGA, poly-(γ-glutamic acid); PLA, poly(D,L-lactic acid); PLGA, poly(D,L-lactic-*co*-glycolic acid); PMMA, poly(methyl methacrylate); poly-U, poly(uridylic acid); SC, subcutaneously; RSV, respiratory syncytial virus; TMC, *N*-trimethyl chitosan; TRP-2, tyrosinase-related protein-2; TT, tetanus toxoid.

[†] Immunostimulants (if used) are shown in italics within parentheses.

[‡] BALB/c and C57BL/6 are mice strains unless otherwise indicated.

immunobioengineering may exploit surface-mediated complement activation and could affect innate and adaptive immunity in diverse ways.

Size and shape

The size of the polymeric particulate vaccine delivery systems and their interactions with APCs influence the immune response both qualitatively and quantitatively.[13] The nanoscale size is advantageous in vaccine delivery, improving the safety, stability, and targeted delivery of biological agents, enhancing the transport across biological barriers and hence the bioavailability, extending the effect in the target tissue.[12]

Particle size is the critical factor for lymphatic uptake from the interstitial space.[91] Therefore, particles >100 nm frequently remain near the administration site and are internalized by immature peripheral DCs that then migrate to lymph nodes, mature, and present antigen to T cells. Particles <50 nm in diameter are more efficiently carried into lymphatic vessels by the interstitial flow and transported to regional draining lymph nodes, where concentrated populations of resident immature DCs internalize them.[87,92,93] Therefore, the size of particles is a determinant for their applicability toward targeting peripheral vs. lymph node DCs. Interestingly, size may also affect internalization of targeted biomaterials by APCs. DCs have been described to internalize PLGA-based DC-SIGN-targeted NPs and microparticles (MPs; size from 1 to 1000 μm) more effectively than nontargeted controls. However, NPs were more effectively targeted than MPs, as demonstrated by the relatively high nonspecific uptake of MPs by DCs. Contrastingly, scavenging by other phagocytes occurred more efficiently for targeted MPs rather than for NPs.[94]

Transport across mucosal surfaces may also be affected by particle size. Mucosae are both an appealing and challenging route for vaccination. NPs must gain access to the mucosal epithelia for antigen delivery or transfection. Therefore, they must be able to penetrate the mucous layer. The mucus consists of a physically cross-linked, viscoelastic hydrogel, with mesh sizes in the order of 10–100 nm.[95] Barrier penetration has been shown largely restricted for particles greater in diameter than a few hundred nanometers,[95,96] whereas particles of about 50 nm could diffuse in mucus almost as freely as in water.[95] NPs have been described to improve transmucosal transport and transcytosis by microfold (M) cells.[41] Indeed, NPs crossed the mucosal epithelium better than MPs, in that not only M cells overlying the mucosa-associated lymphoid tissues but also the epithelial cells were involved in the transport of NPs.[73] A better uptake by Peyer's patches was observed for negatively charged PLGA particles having a mean diameter of ≤1 μm.[97]

Nanomaterial size may also determine its immunological activity, by influencing uptake by APCs and their maturation.[98] Indeed, it has been shown that cell uptake of NPs was relatively high, when compared to that of MPs.[99,100] The NPs' size-dependent immunomodulation is a key feature in their potential use in vaccination. Immunization with PLA NPs entrapping hepatitis B surface antigen (HBsAg) has been previously linked with higher levels of interferon (IFN)-γ production and with antibody isotypes associated with T helper (T_h)1-type immune response. Conversely, immunization with MPs promoted IL-4 secretion and favored T_h2-type immune response.[55] However, immunization with PLGA MPs loaded with *Bordetella pertussis* antigens elicited a marked T_h1 immune response, whereas similarly loaded NPs favored a T_h2 immune response.[61] In another model, synthetic peptide malaria vaccine SPf66-loaded PLGA NPs proved to be poorly immunogenic, while SPf66-loaded MPs elicited potent, long-lasting systemic antibody levels and mixed T_h1/T_h2 immune response in IN-immunized mice.[101] Therefore, the type of size-dependent polarization of the immune response may also depend or be affected by the particular antigen loaded or other NP characteristics.

In addition to its type, the intensity of the humoral immune response seems to be also affected by particle size, as significant variations on antibody titers were observed after a single immunization, using differently sized PLA particles entrapping HBsAg.[55] NPs have been shown to be efficiently taken up by macrophages but elicited lower antibody titers in comparison to MPs. PLA MPs eliciting the highest and long-lasting antibody titers after single immunization were found attached to the macrophage cell surface, not being internalized.[55] Bovine serum albumin–loaded PGLA particles (1 μm) induced a higher humoral response, immunoglobulin (Ig)G-mediated, than smaller particles administered by oral and intranasal routes.[62] Other studies did not reveal the same size-dependent effect. PLGA NPs

and MPs vaccine systems delivering a recombinant protein antigen from *Neisseria meningitidis* type B [intramuscularly (IM) or intraperitoneally (IP)], and a HIV-1 envelope gp140 (IN followed by an IM boost) elicited comparable immune response in mice.[63]

Although it is not obvious how one specific size range could be optimal for particular vaccine formulations, it is however clear that controlling the size of a vaccine particle could be a means to bias the immune response.[102,103]

The particle geometry has been described as a strategic feature regarding transport through the vasculature, circulation half-life, targeting efficiency, endocytosis, and subsequent intracellular transport.[104,105] Spherical and cylindrical particles have been described to be phagocytosed more effectively than ellipsoid or disk-shaped particles. Elongated particles have been reported to avoid phagocytosis and remained in circulation for longer times, whereas both elongated and flat particles targeted the disease site better than their spherical counterparts.[104]

Surface charge

Surface charge may affect bioadhesivity, entrapment efficiency, percent loading, stability, and in vivo immunogenic performance of a vaccine formulation.[106] As a result of the supercoiled structure and negative charge, the entrapment efficiency and stability of DNA-based vaccine formulations is usually low. Cationic nanomaterials form complexes with plasmid DNA by electrostatic interactions, increasing stability and entrapment efficiency.[30,107] A net positive surface charge can facilitate transfection by favoring the interaction with the negatively charged glycoproteins at the cell membrane. However, electrostatic interactions with solutes or proteins from blood and interstitial fluid can lead to competitive binding, destabilization of the carrier, and subsequent premature release of the nucleic acid payload.[82] These cationic delivery systems have been shown to enhance mucosal and systemic immunogenicity, including the generation of efficient mucosal antibody response and cytotoxic T cells after intranasal administration,[31] hence providing an attractive alternative to parenteral administration. It is therefore critical to control the cationic charge density to minimize the toxicity frequently associated with polycationic materials such as poly(ethylenimine) (PEI), while attaining high immune response.

The electrostatic interactions between the mucus—an anionic polyelectrolyte—and the cationic NPs, resulting in mucoadhesion, may provide sufficient residence time for an efficient antigen uptake. Mucoadhesive, hydrophilic NPs have received much attention to deliver protein antigens via the nasal route.[22,73,108,109] Mucoadhesive NPs improve mucosal absorption, because they strongly attach to the mucosa and increase the viscosity of mucin. Thereby, they significantly decrease the nasal mucociliary clearance rate and thus increase the residence time of the formulation in the nasal cavity. For instance, carriers of chitosan and derivatives—polyampholyte mono-*N*-carboxymethyl chitosan (MCC) and positively charged *N*-trimethyl chitosan (TMC) loading tetanus toxoid (TT)—enhanced mucosal immune response in IN-immunized mice.[108] MCC induced relatively lower IgG titers for TT when compared with TMC and chitosan, yet producing the smallest NPs, with narrower size distribution and higher loading capacity.[108] TT-loaded TMC-MCC NPs, obtained without using any organic solvent or cross-linker, induced both mucosal and systemic immune responses in IN-immunized mice.[40]

Hydrophobicity and hydrophilicity

Certain material features can mimic pathogen surfaces leading to the activation of innate immune pathways. Some biomaterials, particularly polymers that contain hydrophobic domains, exhibit natural adjuvant behavior. A positive correlation was observed between hydrophobicity of diphtheria toxoid–loaded PLGA, PCL, and PLGA-PCL NPs, their in vitro uptake, and the serum levels of antigen-specific IgG achieved in IN-immunized mice.[66] The mechanism(s) by which biomaterials hydrophobicity affects the inflammatory and antibody responses, although not fully elucidated, may involve the complement system, TLRs, or both. TLR4 binds to a variety of structurally dissimilar ligands, many of them (including LPS and bacterial fimbriae) having hydrophobic domains. The hydrophobic domains of these ligands might be sensed as a "danger signal" by TLRs to initiate innate immune response.[110] Similarly, hydrophobic portions of polymers in vehicles might interact specifically with TLRs and induce DC maturation and adaptive immunity.[90] For example, LPS-free γ-hPGA NPs stimulated DCs through TLR2 and TLR4, possibly through the hydrophobic regions.[72] This was reached through MyD88-mediated nuclear factor–kappa B activation and p38 mitogen-activated protein kinase pathways, in a manner somewhat similar to LPS signaling through TLR4.[42,72,111,112]

Once exposed to a biological environment, hydrophobic material surfaces are obscured by protein adsorption faster than the hydrophilic ones,[82] affecting the phagocytosis and clearance by macrophages (e.g., through scavenger receptors) and hence potentially affecting distribution and delivery to the intended target sites.[113] Immunoglobulins, complement components, or other opsonins adsorption might be advantageous to induce immunity.[113] A study with DC-SIGN-targeted PLGA NPs, coated with hydrophilic poly(ethylene glycol) (PEG) of various chain lengths to shield nonspecific interactions, demonstrated that PEG chains cannot be extended beyond a certain length without compromising the efficacy of targeted delivery.[114] The addition of PEG and other hydrophilic polymers can also result in lower transfection efficiency.[82]

A hydrophilic surface—for example PEG, poly(ethylene oxide), Pluronic, or poloxamers—is relevant to withstand aggregation and adsorption of particles to components of the mucus and permit their transport as individual particles. Shorter, denser graft layers of PEG tend to sterically stabilize the NPs surface, whereas longer, sparser grafts allow interpenetration of the grafted chains and the mucous network, leading to adhesion to the mucus, associated with entanglement and disentanglement, and unfavorable slower NPs penetration.[115] Therefore, PEG chains long enough (2 kDa)[96] to prevent adsorption, but not long enough (10 kDa)[115] to lead to entanglement, are desirable. PEG coating of PGLA NPs has been shown to enhance diffusion in human cervical mucus in a manner strongly dependent on PEG molecular weight and density[116]; in PLA NPs, PEG coating has favored penetration across rat nasal

mucosa.[78] The role of the hydrophobicity–hydrophilicity character of the transmucosal nanocarriers is controversial in different reports and remains a dilemma.[117]

Multifunctional vaccine delivery systems

A range of technologies and approaches have been used for the development of nanosized vaccine delivery systems, aiming at improving preventive and therapeutic vaccination methods.[77] They are designed to protect antigen from enzymatic degradation,[12,29,118] to extend antigen release,[74] to closely mimic the size, shape, surface molecular organization,[119] composition, and immunological processing of actual pathogens; to actively or passively target APCs for efficient delivery[120]; direct the nature of the resulting immune response, and finally, to induce APCs maturation by interacting with elements of the innate immune system, such as TLRs.[102,119] Polymeric vehicles also offer the significant benefit of reducing the toxicity due to inflammatory cytokines often observed after injection, a common side effect of immunostimulants, by directly targeting APCs.[121]

Vaccines may include synthetic peptides representing an epitope of a pathogen protein; a full-length protein carrying several epitopes that may be recognizable by B and T cells, produced either by pathogens, synthetically or recombinantly; or a gene encoding a particular protein fused into a DNA or RNA plasmid. These vaccines offer considerable advantages over traditional empirical vaccines, based in live-attenuated, inactivated, or killed pathogens, in terms of safety, stability, and production cost. However, in most cases subunit vaccines have limited immunogenicity and require the addition of adjuvants to induce a protective and long-lasting effective immune response.[86,102] Antigens in subunit vaccines are taken up by DCs but usually lack the necessary "danger signals" to induce DC maturation. Several immunostimulants may therefore be co-administered either by co-injection or by physical linkage to the carrier via surface adsorption and co-encapsulation.[36,60,122] An antigen–adjuvant mixture stimulates the activation of immature DCs, but an antigen–adjuvant conjugate increases the chance of simultaneous uptake of both adjuvant and antigen to the same endocytic compartment, resulting in higher numbers of mature antigen-carrying DCs,[34] which are necessary to ensure optimal antigen presentation to $CD4^+$ T cells, cross-presentation and induction of $CD8^+$ T-cell response,[123] and increased humoral immune response.[34,52]

Adjuvants are molecules, compounds, or macromolecular complexes that evoke or enhance the potency and longevity of a specific immune response against co-inoculated antigens.[4,6,77] The adjuvants chosen should meet several criteria, including target site, antigens, type of desired immune response, route of administration, animal species to be vaccinated, duration of immunity, prevention of adverse effects, and stability of the vaccine.[4,124] An optimally formulated adjuvant must be able to promote an antigen-specific immune response and should be safe, intrinsically nonimmunogenic, biocompatible, readily biodegraded and eliminated, inexpensive to produce, stable before administration, and physicochemically well defined to facilitate quality control important to ensure reproducible manufacturing and activity.[4,6]

Multivalent vaccines that encapsulate not only a combination of multiple antigens[31,67] (necessary in many diseases associated with multiantigenic variability and shedding), but that also combine the synergy between different adjuvant mechanisms[25,36] using mixtures of immunostimulants and delivery systems, have been under preclinical study.

Peptide-based vaccines

Peptide epitopes might be recognized by antibody or immune cells. Synthetic peptide-based immunogens are easily produced, are free of bacterial or viral contaminating substances, are devoid of oncogenic potential, present low adverse reactions, have low cross-reactivity and high stability, but also have poor inherent immunogenicity.[77] Peptide-based vaccines can include several peptide epitopes corresponding to subtypes of a pathogen, different stages in the life cycle of a pathogen, or even epitopes from multiple pathogens.[125]

To overcome the limitations of using single cytotoxic T-cell epitopes imposed by MHC polymorphism, mixtures of separate peptides or polytope vaccines have been designed by producing recombinant proteins consisting of a combination of T_h and/or cytotoxic T-cell epitopes. Physical linking of T_h and cytotoxic T-cell peptide epitopes further increased the magnitude of the cytotoxic T-cell response, suggesting that presentation of both T_h and cytotoxic T-cell peptide epitopes on a single APC is more efficient than when the two epitopes are presented on different APCs, which may occur when these epitopes are delivered as a mixture.[126,127]

Peptide-based vaccine efficacy is determined by how the peptides are recognized by the immune system. Specific immune response can be significantly affected by the presence of T_h epitopes, peptide concentration, multivalency, secondary structure,[102] geometry,[128,129] orientation (N terminus or C terminus of B-cell epitopes could determine antibody specificity), chemical linkage between separately synthesized peptide modules,[127] association with adjuvants (self-adjuvanting lipopeptides, such as tripalmitoyl-*S*-glyceryl cysteine coupled to appropriate synthetic epitopes), and size. Long synthetic peptides are not able to bind directly to MHC class I or II molecules and are therefore taken up, processed, and presented by APCs.[102]

The induction of robust $CD8^+$ T-cell response requires a sustained presentation of antigen in a stimulatory context. Carrier-induced epitope suppression and in vivo biodegradation should be avoided. Biodegradation escape can be achieved by using non-natural "protease-resistant" derivatives of cytotoxic T-cell epitopes that still retain the antigenicity and immunogenicity of the parental peptide, or by using a high number of repetitive injections with minimal cytotoxic T-cell peptide epitopes within a week and for several courses.[126] Although vaccines of small peptides can be rapidly biodegraded, larger peptides are relatively protected and may actually benefit from additional extracellular processing.[126]

Some examples of polymeric nanogels tested as potential peptide-based vaccine delivery systems with chitosan, γ-PGA, and PLGA are summarized below.

Chitosan

Chitosan-conjugated deoxycholic acid NPs, self-assembled with melanoma-associated antigen 3 (MAGE-3)–derived $CD4^+$-$CD8^+$ T-cell peptide epitopes in SC-immunized mice, have been

linked to the generation of MAGE-3–targeted cytotoxic T cells, killing MAGE-3–specific tumor cells and causing regression of the growth of mouse forestomach carcinoma cell line.[21]

γ-PGA

Mice immunized with γ-hPGA NPs carrying the listerolysin$_{296–307}$ CD8$^+$ T-cell peptide epitope have proved to be protected from a lethal infection with *Listeria monocytogenes* without the need of additional adjuvant.[42] γ-hPGA NPs entrapping an endoplasmic reticulum transport system containing an endoplasmic reticulum–insertion signal sequence–conjugated antigenic peptide (Tax$_{38–46}$ peptide derived from human T-cell leukemia virus type 1 and gp100$_{25–35}$ human melanoma peptide) markedly amplified and activated cytotoxic T cells and IFN-γ–secreting cells specific for the antigen in SC-immunized mice.[43] Additionally, in a murine model of tumor metastasis, intraperitoneal (IP) vaccination with γ-hPGA NPs loaded with the tumor-associated antigen (TAA)–derived peptide, the ephrin type A receptor 2 (EphA2), have been reported to exhibit an enhanced EphA2-specific CD8$^+$ T-cell activation and have demonstrated an antitumor effect by eliciting immunity equivalent to that of the antigen administered with complete Freund's adjuvant.[44]

PLGA

PLGA NPs encapsulating both the tyrosinase-related protein 2 (TRP-2)$_{180–188}$ (self-TAA peptide) and 7-acyl lipid A (TLR4 agonist) have been shown to induce therapeutic immunity against highly aggressive B16 melanoma in SC-immunized mice, breaking immunotolerance to cancer-associated self-antigens and leading to tumor growth control through the induction of TRP-2–specific cytotoxic T cells. Activated TRP-2–specific CD8$^+$ T cells have been shown to secrete IFN-γ in the lymph nodes and spleens of the vaccinated mice. Within the tumor microenvironment there was reversal of the immune-suppressive milieu through an upregulation of pro-inflammatory cytokines (IL-6, IL-12, IFN-γ, tumor necrosis factor-α) and a downregulation of the proangiogenic vascular endothelial growth factor.[57]

Protein-based vaccines

A suitable vaccine must elicit a T-cell response in a background of many different human leukocyte antigen (HLA) class I and II alleles. Vaccines providing the immune system with complete proteins are ideal in contrast to vaccines containing a single peptide epitope, in that the latter may not contain all important epitopes suitable for loading onto antigen-presenting molecules of individuals with different HLA haplotypes.[126] Intact recombinant proteins are thus more likely to bear peptide sequences and to originate peptides that could bind MHC class I or II molecules of more than one HLA haplotype.

Some examples of polymeric nanogels currently being tested as potential protein-based vaccine delivery systems are summarized below under the name(s) of the polymer(s) chiefly modified: mannan and pullulan; chitosan and derivatives; γ-PGA; PLA and PLGA; PCL; PMMA.

Mannan and pullulan

Cholesterol-bearing mannan or pullulan (CHM or CHP) in complex with human epidermal growth factor receptor 2 (HER2) oncoprotein has been successfully used to induce CD8$^+$ cytotoxic T cells against HER2$^+$ tumors. Mice immunized SC with CHM-HER2 or CHP-HER2 before or early after tumor challenge successfully rejected HER2-transfected tumors.[130,131] In addition, vaccination with CHM-HER2 complexes led to a strongly enhanced production of IgG against HER2.[130] In another study, CHP was used in combination with New York-esophagus-1 (NY-ESO-1) protein (CHP–NY-ESO-1) to pulse DCs, which efficiently activated both CD4$^+$ and CD8$^+$ T cells in vitro.[123] This further indicated the suitability of CHP for use as a vaccine delivery system in cancer therapy. The evaluation of CHP-based protein vaccine in clinical trials yielded encouraging results. In a phase I clinical trial conducted in HER2-expressing cancer patients, the CHP-HER2 complex vaccine, administered SC, proved to be safe and to induce HER2-specific CD8$^+$ and/or CD4$^+$ T-cell immune responses[16]; in a second clinical trial with this vaccine, it was further shown to induce a HER2-specific humoral immune response that was increased by co-administration of granulocyte-macrophage colony-stimulating factor.[17] Despite the CHP-HER2 formulation's effectiveness in raising the production of antibodies specific for the immunogen used, these antibodies were not able to bind to or promote the lysis of HER2-expressing tumor cells. Their usefulness, however, could reside in their usage as surrogate markers for the T cell–mediated immune response.[17] In a phase I clinical trial, CHP–NY-ESO-1 vaccine elicited potent humoral[19] and increased CD4$^+$ and CD8$^+$ T-cell responses in immunized cancer patients.[20] Despite the NY-ESO-1–specific immunity induced in cancer patients by CHP–NY-ESO-1, tumor growth was nevertheless observed upon vaccination.[132] Combined CHP–NY-ESO-1 and CHP-HER2 vaccines administered SC to esophageal cancer patients elicited limited mild adverse events.[18] Targeting multiple tumor antigens proved to be feasible, without antigenic interactions. The combination vaccine elicited a response to NY-ESO-1 comparable to that obtained with the single vaccine, while inducing a lower antibody production specific for HER2.[18] Although the induction of antigen-specific T-cell responses upon vaccination is a promising result, further studies will be necessary to fully understand the true potential or effectiveness of CHP- and CHM-based cancer vaccination. The identification of immunological biomarkers that could allow a more accurate evaluation of the clinical response to cancer immunotherapeutic approaches[133] will certainly be useful in this regard.

Chitosan and derivatives

Chitosan-based vaccines have shown superb effectiveness in preclinical models and promising results in clinical trials. Nevertheless, further optimizations for these systems will be necessary for clinical approval.[109,134] Formulations of superoxide dismutase B1 in chitosan NPs administered SC to mice have been reported to increase the NPs' immunogenicity toward cell-mediated immunity (T_h1 cells, IgG2a) and to be effective against *Leishmania*.[26] Hydrophobic NPs (PLA or PLGA) coated with hydrophilic polymers (PEG or chitosan) and NPs made solely of hydrophilic polymers have proved suitable to deliver proteins across the nasal and intestinal mucosae,[23] as exemplified by chitosan NPs loaded with TT, which elicited high and long-lasting IgG[22,23] and secretory immunoglobulin A (sIgA)

response in IN-immunized mice.[22] The association of recombinant HBsAg with the alginate-coated chitosan NPs in IN-immunized mice proved to be able to elicit a mucosal but not a systemic humoral immune response. However, antigen-specific systemic antibodies of T_h1-associated isotypes were detected when NPs were used together with CpG.[25] In another study the recombinant HBsAg-loaded chitosan NPs induced levels of IgG specific to HBsAg as much as nine times that of the alum-adsorbed vaccine in IM-immunized mice.[24] Colloidal polyelectrolyte complexes, free of chemical cross-linkers and surfactants, were obtained by chitosan and dextran sulfate macromolecular assembly and loaded with HIV-1 p24. In the murine model, upon SC vaccination with these NPs, a strong, specific p24-specific antibody production and cytokine release suggested that both arms of immunity have been stimulated, although the immune response could be T_h2 biased.[135]

TMC NPs carrying monovalent influenza A subunit H3N2 have been described to significantly enhance systemic IgG and local sIgA immune responses in mice (administered IM or IN), compared to soluble influenza vaccine.[39] Urease, a target antigen used in vaccination against *Helicobacter pylori* infection, loaded into TMC NPs, have elicited specific IgG and sIgA responses when orally administered, but only IgG in SC-immunized mice.[41] TMC NPs have induced humoral and mucosal immune responses against recombinant HBsAg in IN-immunized mice.[38] Another study reported that TMC-based formulations containing either OVA or diphtheria toxoid were able to elicit high titers of IgG specific for both antigens, in intradermally (ID) immunized mice.[33] TMC-OVA conjugate (OVA covalently linked to TMC) caused higher OVA-specific IgG levels than plain OVA or a physical mixture of TMC and OVA in IM-immunized mice, and slightly elevated levels when compared to those achieved with TMC-OVA NPs obtained by ionic complexation.[34] Intraduodenal vaccination of mice with OVA-loaded chitosan and TMC NPs led to significantly higher antibody response than immunization with OVA alone. TMC NPs could induce OVA-specific antibodies after only a priming dose. TMC NPs but not chitosan or PLGA NPs had an intrinsic adjuvant effect on DCs.[32] Among similar-size OVA-loaded PLGA, TMC, and TMC-coated PLGA (PLGA-TMC) NPs, only mucoadhesive TMC was able to increase the nasal residence time of OVA compared to OVA alone. All nanosystems administered IM induced higher IgG titers than OVA alone—PLGA and TMC being superior to PLGA-TMC. Slow antigen-releasing PLGA and PLGA-TMC NPs did not induce detectable antibody titers, whereas positively charged, fast antigen-releasing TMC NPs led to high sIgA and serum antibody titers in IN-immunized mice. Therefore, particle charge and antigen release pattern of OVA-loaded NPs must be adapted to the intended route of administration.[35] Additionally, covalently stabilized TMC–hyaluronic acid NPs loaded with OVA have shown adequate loading efficiency, somewhat greater particle integrity, and enhanced adjuvanticity as evidenced by higher IgG titers, as compared with nonstabilized particles in ID- and IN-immunized mice.[37]

γ-PGA

The potential of using self-assembled γ-hPGA NPs in triggering murine immunity has been demonstrated for several protein antigens, such as HIV-1 p24,[47] HIV-1 gp120,[46,99] influenza hemagglutinin,[48,49] and OVA.[42,45,111] In macaques (IN and SC), HIV-1 gp120–carrying γ-hPGA NPs have shown great potential for the induction of specific cellular and humoral immunity. However, the macaques intravenously (IV) challenged with simian and human immunodeficiency chimeric virus (SHIV)-KU-2 have presented an increased viral load when immunized with those NPs. Thus, the induced immune response was not effective for protection but actually enhanced the infection in rhesus macaques.[136] Furthermore, γ-hPGA NPs proved to be promising adjuvants and allergen-delivery systems for allergen-specific immunotherapy; human monocyte-derived DCs from allergic subjects stimulated in vitro with a mixture of γ-hPGA NPs and extract of grass pollen allergen *Phleum pratense* increased allergen-specific IL-10 production and proliferation of autologous $CD4^+$ memory T cells.[72] More recently, OVA–benzalkonium chloride–γ-PGA complex administered SC to mice has been described to induce IgG1−T_h2-type−IgG2a, and IgG3−T_h1-type antibody isotypes, indicating the ability of this complex to induce humoral and cellular responses. This complex was able to inhibit the growth of OVA-expressing tumor cell line E.G7 and caused complete tumor rejection.[137]

PLA and PLGA

PLA NPs coated with HIV-1 p24 have been described to induce enhanced cellular and humoral immune responses in mice, rabbits, and macaques immunized by the SC route.[53] Co-adsorption of HIV-1 p24 and gp120 to these NPs preserved their antigenicity and immunogenicity.[54] In another study in which mice were also immunized SC, OVA and poly(uridylic acid) (poly-U, a TLR7/8 agonist), co-encapsulated in PLA NPs, increased the specific humoral immune response and the levels of IFN-γ–secreting T cells.[52]

PLGA NPs have been reported as effective vehicles for sustained and targeted antigen delivery to APCs by efficiently trafficking through local lymphoid tissues.[138,139] Co-delivery of hepatitis B core antigen (HBcAg) and monophosphoryl lipid A in PLGA NPs promoted HBcAg-specific T_h1 cellular immune response with IFN-γ production in a murine model immunized SC.[64] Encapsulated West Nile virus envelope protein antigen conferred host protection in a murine model of viral encephalitis.[140] In another study, PLGA particulate delivery of OVA and 7-acyl lipid A to DCs led to an increased antigen-specific $CD8^+$ and $CD4^+$ T cell–mediated response.[58] The expanded T cells were capable of cytokine secretion and displayed an activation and memory surface phenotype.[58] Oral administration to mice of OVA and monophosphoryl lipid A co-delivered in PLGA NPs proved to induce both systemic and mucosal immune responses.[59] LPS-modified PLGA NPs, in SC-immunized mice were able to effectively enter APCs, eliciting both humoral and cellular immunity against encapsulated OVA, without toxicity, therefore proving to be an effective vaccine vector through both TLR and inflammasome activation.[140] DEC-205–targeted OVA-loaded PLGA NPs have been demonstrated to induce DCs to produce IL-10, with levels correlating with the amount of DEC-205–specific monoclonal antibodies conjugated on the particle surface, both in vitro and in IP-immunized mice.[141] This delivery system induced DCs and T

cells to produce both pro-inflammatory (IL-12, IL-5, IFN-γ) and anti-inflammatory (IL-10) cytokines. The DEC-205–associated pathway elicited the DC production of IL-10 and T-cell production of IL-10 and IL-5 without impeding IL-12–mediated DC priming of a T_h1-type response characterized by IFN-γ production, as a result of the PLGA component. Multivalent cross-linking of the DEC-205 receptors was required for the response and was associated with the upregulation of the scavenger receptor CD36 on the DCs.[141]

PCL

PCL NPs modified by different adjuvants (mucoadhesive polymers alginate or glycol chitosan; and absorption enhancers spermine or oleic acid), with *Streptococcus equi equi* (*S. equi*) surface proteins adsorbed or encapsulated, have been shown to induce significantly higher specific systemic and mucosal immune responses to *S. equi* antigens in IN-immunized mice.[67] The inclusion of cholera toxin B subunit in the formulations further activated the pathways leading to T_h1 and T_h2 cells differentiation.[67]

PMMA

Vaccine formulations composed of HIV-1 Tat protein and anionic surfactant–free polymeric core-shell NPs and MPs with an inner core constituted by PMMA and a hydrophilic outer shell composed of a hydrosoluble co-polymer (Eudragit L100-55) have been demonstrated to induce robust and long-lasting cellular and humoral immune responses in mice after systemic and/or mucosal immunization.[71]

DNA-based vaccines

In DNA-based vaccines, the peptide/protein targets of immune response are encoded in DNA and produced within the body's own cells, which can mimic actual infection more closely than injection of traditional nonreplicating vaccines.[142] The DNA vector is made of a bacterial-derived plasmid equipped with eukaryotic or viral promoter–enhancer transcription elements and a gene encoding the antigen of interest followed by a transcript termination–polyadenylation sequence.[143]

DNA-based vaccines accumulate desirable qualities, such as immunogenicity (expression of multiple antigens or epitopes in a single vector inducing antigen-specific humoral and cellular immune responses), safety (low cytotoxicity and reduced immunogenic reactions), versatility (i.e., vaccine targets can be simply, rapidly, and economically changed by selecting the appropriate sequence of the plasmid DNA), easy to scale up and manufacture (low cost and reproducible large-scale production and isolation), stability (long shelf-life), and mobility (ease of storage and transport, not likely to require a cold chain).[82,143,144] The principal drawbacks lie in the challenging intracellular delivery of DNA in the appropriate cell type (APCs or a bystander cell) and the low levels of transfection that may consequently limit the immune response.[82] The approach of co-inoculating plasmids encoding different cytokines, co-stimulatory factors, or other fusion constructs to enhance or modify the immune response generated by the vaccine plasmid has been used successfully.[144]

Different polymers have been extensively studied as nonviral DNA carriers for vaccine delivery,[120] and some examples are summarized below.

Chitosan

Chitosan NPs containing a cocktail of DNA encoding nine immunogenic antigens of respiratory syncytial virus have been demonstrated to elevate the production of IFN-γ in the lungs, and to induce high levels of IgG and sIgA and cytotoxic T cells with antiviral action in a mice model.[31] Plasmid DNA expressing different *Mycobacterium tuberculosis* epitopes loaded on chitosan NPs, when administered by the pulmonary route in mice, proved to increase IFN-γ secretion from T cells.[29] Chitosan NPs loaded with DNA encoding VP1, a major structural protein of coxsackievirus B3, induced high levels of IgG and sIgA and a strong cytotoxic T-cell response that effectively eliminated coxsackievirus B3 viruses in IN-immunized mice.[30] The chitosan complexes with plasmid DNA encoding HBcAg in IM-immunized mice displayed stronger immunogenicity than naked DNA vaccines, with a higher level of specific antibody, elevated IFN-γ secretion, and increased specific cell lysis.[118] Plasmid DNA encoding HBsAg loaded on chitosan NPs induced humoral, both systemic and mucosal, and cellular immune responses in IN-immunized mice.[28] Low-molecular-weight chitosan, although having lower binding affinity to plasmid DNA encoding human cholesteryl ester transfer protein C-terminal fragment, mediated higher transfection efficiency, elicited significant systemic immune response, modulated plasma lipoprotein profile and attenuated the progression of atherosclerosis in IN-immunized rabbits.[145] Oral delivery of chitosan-DNA vaccine encoding mite dust allergen from *Dermatophagoides pteronyssinus* generated high gene expression levels in mice, and preferentially activated a specific T_h1 immune response, thus preventing subsequent sensitization toward T_h2 cell–regulated specific IgE response.[146]

γ-PGA

DNA vaccination with PEI/γ-PGA NPs loaded with a plasmid encoding *Plasmodium yoelii* merozoite surface protein 1 C terminus, administered IV in mice, has been shown to generate an antigen-specific IgG response dominated by IgG1 and IgG2b and to induce weak T_h1 (IFN-γ and IL-12 p40) and strong T_h2 (IL-4) cytokines responses.[50] In another study, the same complex when administered IV and IP, provided complete protection against lethal challenge with a significant increase in levels of immunoglobulins and T_h1 and T_h2 cytokines, but in the group vaccinated SC, only half of mice were protected and marginal levels of specific antibody were measured.[51]

PLA and PLGA

A single dose of plasmid DNA encoding β-galactosidase encapsulated in PLA-PEG NPs induced a significant systemic antibody response to the encoded protein in IN-immunized mice.[78] Multifunctional core-shell polymeric NPs, comprising a hydrophobic PLGA core loaded with fluorescent quantum dots and a reporter gene electrostatically adsorbed onto the positively charged glycol chitosan shell, could be delivered transdermally in a mouse model via gene gun bombardment. The loaded DNA was intracellularly released via a pH-mediated mechanism, directly into epidermal Langerhans cells, which then migrated and expressed the encoded gene products in the skin-draining lymph nodes.[147]

The PLGA-PEI NPs combined with DNA encoding *M. tuberculosis* latency antigen Rv1733c, when applied to the lungs, increased T-cell proliferation and IFN-γ production more potently than the same formulations given IM to mice. The strongest immunogenicity was obtained by pulmonary priming with NPs-adsorbed Rv1733c DNA followed by boosting with Rv1733c protein.[65]

RNA-based vaccines

Significant challenges continue with respect to delivery of RNA-based NPs.[148] The RNA-based vaccines, in contrast to those of DNA, offer a simpler delivery directed to the cytoplasm, thus bypassing dependence on cellular transcription machinery and transport of nucleic acids to and from the nucleus, excluding any potential for integration into host chromosomes. Nevertheless, RNA is relatively labile and expensive to manufacture at a commercial scale.

Efficient transfection of DCs with messenger RNA (mRNA) expressing TAA, followed by vaccination with the RNA-pulsed DCs, has shown promising results in murine models and recently in humans. In this context, prior identification and characterization of individual gene sequences encoding the TAA seems to be nonessential, as preparations of total mRNA isolated directly from tumors may also be used.[149]

The mRNA-based vaccines in vivo may have to deal with potency issues related to limited transfected mRNA copies into each cell and insufficient levels of expressed protein antigen to stimulate the desirable immune response. A smart strategy to increase the intracellular levels of mRNA comprises the incorporation of replication elements derived from RNA viruses (alphaviruses, flaviviruses, and picornaviruses), which together program the cytoplasmic self-amplification of RNA within transfected cells. To avoid the production of any detrimental infectious virus, essential virus genes such as those encoding the structural "coat" proteins are excluded, originating modified RNA vaccine vectors, termed "replicons."[149]

Biodegradable core-shell NPs—comprising a pH-responsive poly(β-amino ester) core, selected to promote endosome disruption, enveloped by a phospholipid bilayer shell to reduce the polycation core toxicity—were designed for in vivo mRNA delivery with possible usage in noninvasive delivery of mRNA-based vaccines. These NPs loaded with luciferase-encoding mRNA led to statistically higher expression of the reporter protein luciferase than in the naked-mRNA treatment group, when administered IN to mice.[150]

Concluding remarks and future perspectives

Polymeric nanogels effectively perform as targeted carriers protecting vaccine antigens from degradation in vivo. Following internalization of the biomaterial vehicles by APCs, the loaded antigens are released intracellularly and enter MHC class II– and class I–dependent antigen presentation pathways, and, therefore, can induce both $CD4^+$ and $CD8^+$ T cell–mediated immunity. Moreover, the surface of the biomaterial vehicle can be conjugated with antibodies or other specific ligands to improve tissue, cellular, or subcellular targeting specificity, steer specific immune response by improving the efficacy achieved at a much lower antigen dose, and/or reduce inflammatory side effects associated with some "danger signals." Biomaterials themselves can function as synthetic adjuvants, which can also be conjugated with immunostimulants that activate APCs and induce subsequent T-cell immunity. Advantages in the usage of polymeric nanogels as antigen delivery systems comprise their simplicity of formulation, loading capacity, stability of the resulting dispersifon, nontoxicity, lower cost, and easiness of manufacture and scale-up.

Although peptide- and RNA-based vaccines are currently less developed than DNA- or protein-based vaccines, major advances in these newer vaccines can be expected in the near future. A comprehensive evaluation of all of the latest vaccination concepts, together with a better understanding of disease pathology, advances in biomaterials science and technology, and regulated systematic experiments, will provide more delivery systems (proven safe, effective, and targeted) that actually advance preventive and therapeutic vaccines to the next level, achieving a major goal in global public health.

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