ASIAN JOURNAL OF PHARMACEUTICAL SCIENCES 9 (2014) 227–235

HOSTED BY

ELSEVIER

Available online at www.sciencedirect.com

ScienceDirect

journal homepage: http://ees.elsevier.com/ajps/default.asp

Review

Nanotechnology and vaccine development

Mi-Gyeong Kim [a], *Joo Yeon Park* [a], *Yuna Shon* [a], *Gunwoo Kim* [b], *Gayong Shim* [a,*], *Yu-Kyoung Oh* [a,*]

[a] *College of Pharmacy and Research Institute of Pharmaceutical Sciences, Seoul National University, Seoul 151-742, Republic of Korea*

[b] *Department of Molecular Medicine and Biopharmaceutical Sciences, Graduate School of Convergence Science, Seoul National University, Daehak-dong, Gwanank-gu, Seoul, Republic of Korea*

ARTICLE INFO

Article history:
Received 28 March 2014
Received in revised form
7 June 2014
Accepted 9 June 2014
Available online 18 June 2014

Keywords:
Nanotechnology
Vaccine delivery
Liposomes
Emulsions
Polymeric nanocarriers
Carbon nanomaterials

ABSTRACT

Despite the progress of conventional vaccines, improvements are clearly required due to concerns about the weak immunogenicity of these vaccines, intrinsic instability in vivo, toxicity, and the need for multiple administrations. To overcome such problems, nanotechnology platforms have recently been incorporated into vaccine development. Nanocarrier-based delivery systems offer an opportunity to enhance the humoral and cellular immune responses. This advantage is attributable to the nanoscale particle size, which facilitates uptake by phagocytic cells, the gut-associated lymphoid tissue, and the mucosa-associated lymphoid tissue, leading to efficient antigen recognition and presentation. Modifying the surfaces of nanocarriers with a variety of targeting moieties permits the delivery of antigens to specific cell surface receptors, thereby stimulating specific and selective immune responses. In this review, we introduce recent advances in nanocarrier-based vaccine delivery systems, with a focus on the types of carriers, including liposomes, emulsions, polymer-based particles, and carbon-based nanomaterials. We describe the remaining challenges and possible breakthroughs, including the development of needle-free nanotechnologies and a fundamental understanding of the in vivo behavior and stability of the nanocarriers in nanotechnology-based delivery systems.

1. Introduction

The seasonal outbreaks of pantropic infection diseases have elevated the development of effective vaccines to the status of a global healthcare concern. Vaccines have been developed using killed organisms [1], live attenuated organisms [2], or inactivated toxins [3]. Recently, subunit vaccines [4], and DNA vaccines that encode antigenic pathogenic proteins [5] have been examined as new vaccine modalities. Although subunit vaccines and DNA vaccines have the advantages of a high safety profile over traditional vaccine, these vaccines suffer from a relatively lower immunogenicity. The immunogenicity may potentially be improved by modulating the vaccine formulation using nanotechnology.

* *Corresponding authors*. Tel: +82 2 880 2493; fax: +82 2 882 2493.
E-mail addresses: shimg@snu.ac.kr (G. Shim), ohyk@snu.ac.kr (Y.-K. Oh).
Peer review under responsibility of Shenyang Pharmaceutical University.
http://dx.doi.org/10.1016/j.ajps.2014.06.002

The nanotechnologies developed for use in the field of vaccines encompass nanocarriers having a variety of compositions, sizes, and surface properties [6]. Numerous vaccine nanocarriers have been designed and investigated for their utility in the delivery of antigens and adjuvants to immune cells in an effort to promote a protective immune response. Unfortunately, although antigens may be taken up by the immune cells, insufficient adjuvant activity may result in limited immunogenicity. In some approaches, nanocarriers have been designed to co-deliver both an antigen and an adjuvant [7]. Nanocarriers can facilitate the targeting and/or sustained release of antigens or adjuvants to antigen-presenting cells [8,9].

Working mechanisms of nanotechnology-based vaccine formulations support the utility of nanocarriers in the vaccine fields. Particles smaller than 10 μm are readily taken up by phagocytic cells, such as macrophages and dendritic cells (DC). This property has been used to improve the cellular uptake of antigens, thereby increasing the efficiency of antigen recognition and presentation [10]. Solid nanocarriers can protect protein-based antigen vaccines from degradation and facilitate entry into the gut-associated lymphoid tissue and mucosa-associated lymphoid tissues, rendering them appropriate for vaccine delivery via oral or mucosal routes [11]. Surface-modified nanocarriers may assist the targeted delivery of antigens. Immune cells express a variety of surface receptors, including the mannose receptor, scavenger receptor, and toll-like receptors (TLR) [12]. Nanocarriers coated with immune cell-targeting molecules, such as carbohydrates [13], antibodies [14], and peptides [15], may target these overexpressed receptors to improve the efficiency of antigen and adjuvant delivery toward the promotion of specific and selective immune responses in prophylactic vaccines.

This review provides an overview of recent advances in nanocarrier vaccine systems, including liposomes, emulsions, polymer-based nanodelivery systems, and carbon-based nanodelivery systems (Fig. 1). The current status of in vivo applications of nanocarriers is summarized in Table 1.

2. Nanodelivery systems for vaccines

2.1. *Liposomes*

Since the first report that liposomes can act as immunological adjuvants [16], liposome formulations (Fig. 1A) have been extensively studied for use in vaccine delivery systems. As of this publication, at least 8 liposomal vaccines are in clinical trials or have been approved for human use [17]. The physicochemical properties of liposomes, including their size, lipid composition, and structure, may be modulated according to the properties of the vaccine antigen to maximize immunogenicity. Liposomes are composed of biocompatible phospholipid bilayers and are capable of loading and delivering both hydrophilic and hydrophobic molecules. These properties enable the co-delivery of antigen and other molecules, such as adjuvants. The surfaces of liposomes may be easily modified using the appropriate functionally active lipid as a component of the lipid bilayer. Surface-modified liposomes have been designed to target immune cells, co-deliver immunostimulatory agents, and enhance both the humoral and cell-mediated immune responses simultaneously to improve the efficacy of liposomal vaccines.

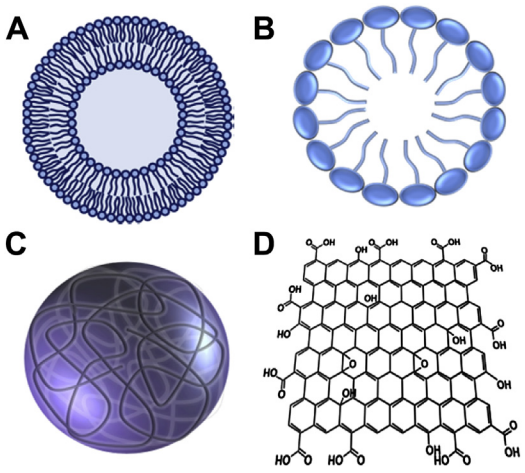

Fig. 1 – Structure of nanocarriers for vaccine antigen delivery. (A) Liposomes, (B) emulsions, (C) polymeric nanoparticles, and (D) graphene oxide nanosheets.

The physicochemical properties of liposomes, such as their size and fluidity, are important for the induction of an immune response. The vesicle sizes of liposomes composed of cationic dimethyldioctadecylammonium (DDA) can affect the cell-mediated immune response, but not the humoral immune response [18]. Liposomes larger than 2 μm in diameter were found to effectively promote interleukin-10 production, whereas liposomes 500 nm in diameter promoted a higher level of interferon-γ production in splenocytes.

Small unilamellar vesicles composed of cationic DDA liposomes were found to produce higher CD8 T cell responses compared to the larger multilamellar vesicles [19]. A recent study reported that rigid DDA lipid-based liposomes produced a Th1-directed immune response against antigens that was 100 times greater than the response produced by fluidic dimethyldioleoylammonium (DODA)-based liposomes [20].

The liposomal delivery of protein antigens via surface adsorption methods may be optimized by tuning the surface antigen and lipid ratio. The protein surface antigen-to-lipid ratio can affect the aggregation behavior of liposomes and can impact general vaccine stability during storage [21]. Surface modifications to antigen-carrying liposomes gearing polyethylene glycol (PEG) groups can reduce liposome aggregation. However, the retention of the liposomes at the injection sites was simultaneously reduced, thereby altering the Th1/Th2 immune response compared to the response produced by unmodified liposomes [22].

The liposomal co-delivery of antigens and an immunostimulatory molecule can enhance the generation of a protective immune response. The entrapment of trehalose 6,6-dibehenate (TDB), an immunostimulatory molecule, within a liposome did not affect the physicochemical properties of neutral distearoyl-sn-glycero-3-phosphocholine (DSPC) or cationic DDA-based liposomes, and significantly increased the production of IFN-γ after immunization [23]. Monophosphoryl lipid A (MPL), a poorly soluble TLR 4 agonist, was added to the bilayers of

Table 1 – In vivo applications of nanocarriers for delivery of vaccines.

Delivery system	Composition	Antigen	Route	References
Liposome	DDA, TDB	Ag85B-ESAT-6	Intramuscular	[18]
	DDA, TDB	OVA	Intramuscular	[19]
	DDA, DODA, TDB	Ag85B-ESAT-6	Intramuscular	[20]
	Pegylated DDA, TDB	Ag85B-ESAT-6	Subcutaneous	[22]
	DDA, DSPC, cholesterol, TDB	Ag85B-ESAT-6	Subcutaneous	[23]
	MPL, DDA, TDB	OVA	Intraperitoneal	[24]
	DDA, TDB	Trivalent influenza vaccine	Subcutaneous	[25]
	DOPC, DOPG, MPB	OVA	Subcutaneous	[26]
	EPC, DOGS-NTA-Ni	His-tagged heat shock protein	Intradermal	[27]
	MDMPC, DMPG, cholesterol, MPL	Polyhistidinylated OVA	Subcutaneous	[28]
	Lecithin, cholesterol	Diphtheria toxoid	Subcutaneous	[29]
Emulsion	MF59	Hemagglutinin	Intramuscular	[34]
	MF59	Recombinant meningococcal B protein	Intramuscular	[36]
	MF59	Recombinant meningococcal B protein	Intramuscular	[35]
	W805EC	OVA	Intranasal	[39]
	W805EC	OVA	Intranasal	[40]
	GLA	Falciparum subunit	Subcutaneous	[41]
	GLA-SE	Plasmodium vivax subunit	Subcutaneous	[42]
	GLA-SE	Recombinant hemaagglutinin	Intramuscular	[44]
Synthetic polymer-based system	PLGA	OVA	Subcutaneous	[8]
	PLGA, polylactic acid	Hepatitis B surface antigen	Pulmonary	[45]
	Lipid-coated PLGA	OVA	Subcutaneous	[46]
	Lipid-coated PLGA	Malaria antigen	Subcutaneous	[47]
	Chitosan-coated polycaprolactone	H1N1 hemagglutinin	Intranasal	[48]
	Polyanhydrides	Yersinia pestis antigen	Intranasal	[49]
	Polylactic acid	Hepatitis B surface antigen	Subcutaneous	[51]
	Deacylated cationic polyethyleneimine	HIV CN54gp140 antigen	Pulmonary	[52]
	PEGylated poly [2-(N,N-dimethylamino) rthylmethacrylate]	HIV gag DNA	Intranasal	[53]
Natural biopolymer-based system	N-trimethyl chitosan	OVA	Intranasal	[54]
	Chitosan nanoparticles	HBsAg	Intraperitoneal	[56]
	Cholesteryl-conjugated pullulan	Clostridium botulinum type-A neurotoxin subunit antigen	Intranasal	[57]
Carbon-based system	SWCNT	Tuberculin purified protein derivative	Subcutaneous	[59]
	Carbon nanotube	Azoxystrobin	Subcutaneous Intraperitoneal	[60]
	Carbon magnetic nanoparticles	Hen egg lysozyme	Intravenous	[61]
	Carbon nanoparticles	Bovine serum albumin	Oral	[63]

cationic liposomes composed of DDA and TBD to increase liposome membrane packing and reduce the surface charges. The MPL/DDA/TDB liposomal formulation carried ovalbumin (OVA) as a model antigen. In mice, the presence of MPL in liposomes did not affect the humoral immune response, but significantly enhanced the antigen-specific CD8+ T cell immune response [24]. Some studies reported the use of an adjuvant formulation comprising liposomes containing an immunostimulatory molecule. The co-administration of a trivalent influenza vaccine and cationic liposomes containing TDB was found to augment both the humoral and the cellular immune responses [25].

Interbilayer-crosslinked multilamellar vesicles were recently designed and tested as nanocarriers for protein antigens [26]. The vesicles were formed by crosslinking the head groups of lipid bilayers within anionic mutilamellar vesicles composed of 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC), 1,2-di-(9Z-octadecenoyl)-snglycero-3-phospho-(1'-rac-glycerol) (DOPG) and maleimide-headgroup lipid 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine-N-[4-(p-maleimidophenyl) butyramide (MPB). Interbilayer crosslinking via divalent magnesium cation-based fusion permitted the stable entrapment of protein antigens within the core and lipophilic immunostimulatory molecules within the lipid membranes. OVA was used as a model antigen to demonstrate that the interbilayer-crosslinked vesicles provided the sustained release of antigens and enhanced the antigen-specific CD8+ T cell immune response in comparison with the uncrosslinked liposomes.

Nickel-chelating liposomes were examined for their potential use in His-tag-mediated antigen vaccine loading [27]. Nickel-chelating liposomes 100 nm in size were prepared using egg phosphatidylcholine (EPC) and a nickel-chelating lipid, 1,2-dioleoyl-sn-glycero-3-[N(5-amino-1-carboxypentyl) iminodiacetic acid] succinyl nickel salt (DOGS-NTA-Ni), in a molar ratio of 95:5. The His-tagged heat shock protein from *Candida albicans* was loaded onto the surfaces of the nickel-chelating liposomes via the formation of metallochelating bonds by incubating the antigens with the liposomes.

Nickel-chelating liposomes have also been prepared by incorporating nitrilotriacetic acid lipid derivatives into the liposomes to promote bonding to polyhistidinylated OVA antigens [28]. The liposomes were composed of DOGS-NTA-Ni, dimyristoylphosphatidylcholine (DMPC), dimyristoylphosphatidylglycerol (DMPG), cholesterol, and MPL.

Although these metallochelating liposomes offer meaningful progress toward recombinant protein antigen loading without covalently modifying the antigens, the toxicity of the nickel-chelating lipids presents certain safety concerns for clinical applications.

Liposomes-in-oil adjuvant formulations were found to prolong the immune response after vaccination [29]. Diphtheria toxoids were co-encapsulated with poly (I:C) in a liposome composed of lecithin and cholesterol (9:1 M ratio). The subcutaneous injection of the liposomes suspended in oil (Marcol 52:Montanide 888, 9:1) generated a persistent immune response by reducing antigen transport to the draining lymph nodes.

Cationic liposomes have been examined for their ability to enhance the adjuvanticity of nucleic acid-based TLR agonists. Liposomes composed of the cationic lipid octadecenolyoxy [ethyl-2-heptadecenyl-3 hydroxyethyl] imidazolinium chloride and cholesterol were complexed to the TLR agonist CpG-based plasmid DNA [30]. The intramuscular administration of the combination of lipoplexes and Fluzone, an influenza vaccine, was shown to provide an enhanced antibody response and cell immunity in mice [30] and in elderly rhesus macaques [31].

2.2. Emulsions

Emulsions have been long studied as adjuvant formulations and more recently studied as vaccine delivery systems. Emulsions are dispersions of two or more immiscible liquids composed of oil, emulsifiers, and excipients (Fig. 1B). Two broad classes of emulsions may be formed: water-in-oil emulsions and oil-in-water emulsions. The latter emulsion type is typically used in adjuvant formulations.

The most famous vaccine adjuvant emulsion is MF59. MF59 consists of squalene oil, Span 85, and Tween 80 in a citrate buffer. The MF59-adjuvanted seasonal flu vaccine (Fluad™) was approved in Europe in 1997 [32]. MF59 can effectively increase the immune response in infants and young children [33]. A recent study investigated the role of each component of MF59 toward the adjuvant properties [34]. Span 85 was found to activate the muscle transcriptome. However, Span 85 alone did not provide an efficient immune response comparable to that of MF59.

MF59 was studied for its ability to augment the induction of antibodies against meningococcal protein antigens [35] and the *Neisseria meningitidis* B vaccine [36]. The delivery of recombinant meningococcal B protein antigens in conjunction with the TLR4 agonist, E6020, within MF59 was shown to cause a strong antigen-specific CD4 T-cell response after three staged intramuscular injections into CD-1 mice [35].

AF03, another squalene-based emulsion, has been used as a vaccine adjuvant. AF03, which consists of squalene, sorbitan oleate, and cetheareth-12, is present in the marketed influenza vaccine, Humenza™. Recently, the physicochemical properties of AF03 were characterized to evaluate the long-term stability of the formulation. The surfactants in AF03 were analyzed using mass spectroscopy and high-performance liquid chromatography-mass spectroscopy as quality control tests in emulsion manufacturing processes [37,38].

In addition to the squalene-based MF59 and AF03 emulsions, adjuvant nanoemulsion formulations have been developed without squalene. For example, an aqueous dispersion of $W_{80}5EC$, composed of cetyl pyridinium chloride, Tween 80, ethanol, and soybean oil, in phosphate-buffered saline or 0.9% NaCl was found to promote an immune response to the model antigen OVA via intranasal administration [39]. The nasal adjuvanticity of the $W_{80}5EC$ nanoemulsion was attributed to dendritic cell engulfment of antigen-loaded epithelial cells [40].

Glucopyranosyl lipid A (GLA), a TLR4 agonist, has been tested for its adjuvanticity in the context of oil-in-water emulsion vaccine antigen formulations. For example, GMZ2, a fusion protein component in an anti-falciparum vaccine, was combined with GLA in an oil-in-water emulsion vaccine formulation [41]. The immunostimulatory properties of GLA were then compared with the properties of several other immunostimulatory agents formulated with GMZ2. GLA was found to display the highest antigen-specific IgG2a and total IgG titers. Another study reported that the immunogenicity of a recombinant *Plasmodium vivax* protein vaccine could be enhanced by subcutaneously co-delivering the vaccine with GLA in an oil-in-water emulsion formulation [42]. The intradermal administration of GLA within oil-in-water emulsions to human skin explants was found to enhance the capacity of skin DCs to activate both CD4+ T cells and the emigration of Langerhans cells within skin tissues [43]. Oil-in-water emulsions containing GLA and recombinant hemagglutinin are currently in phase 2 clinical trials for the prevention of seasonal influenza [44]. The adjuvanticity of the hemagglutinin emulsion prepared with GLA was found to increase the titers of hemagglutinin-specific antibodies relative to the formulation prepared without GLA, following intramuscular administration to healthy adults.

The successful commercialization of squalene-based emulsions in marketed vaccines suggests that emulsion adjuvant approaches warrant further exploration. Formulation stability during storage and the development of biocompatible oil components may be crucial concerns for the development of new emulsion formulations for vaccine delivery.

2.3. Synthetic polymer-based nanodelivery systems

Polymeric nanocarriers (Fig. 1C) have been examined as vaccine delivery systems to take advantage of their ability to offer the controlled release of antigens or adjuvants. Poly(lactic-co-glycolic acid) (PLGA) is the most widely used biodegradable synthetic polymer nanocarrier with a relatively long history of biomedical use. The PLGA particle size has been varied and surface modifications have been introduced into vaccine formulations for use in oral, mucosal, and systemic delivery.

The sizes, surface modification, and release profiles of PLGA particles were shown to affect the immunogenicity of entrapped antigens. The average size of an aerosolized PLGA or poly(L-lactic acid)-based nanoparticle pulmonary hepatitis B virus vaccine formulation was suggested to influence the immunogenicity of the antigen [45]. Nanoparticles larger than 500 nm induced the generation of antigen-specific secretary IgA more effectively than smaller nanoparticles (<500 nm) in rats.

The surfaces of the PLGA polymeric nanoparticles were modified with lipids to enable the co-delivery of antigens and adjuvants [46]. In a recent study the OVA antigen was covalently anchored to a pegylated phospholipid bilayer coating formed on the PLGA nanoparticle surfaces, and a lipid adjuvant, such as MPL or α-galactosylceramide, was incorporated into the lipid bilayer. Subcutaneous co-delivery of OVA with MPL in the surface lipid coating of PLGA nanoparticles was shown to enhance the antigen-specific IgG titer values more than 10 folds. Other groups have conjugated a candidate *P. vivax* malaria antigen, VMP001, to the surfaces of lipid-coated PLGA particles, and MPL has been incorporated into the lipid membranes [47]. In the study PLGA particle surfaces were coated with lipids composed of DOPC, DOPG, and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide].

The prolonged release of antigens delivered by PLGA nanoparticles was shown to enhance the immune response to the model OVA antigen [8]. PLGA nanoparticles provided an OVA release profile that extended over 10 weeks and was much slower than the release profile obtained from a liposomal formulation. In line with the sustained release profile, an OVA–PLGA particulate vaccine formulation induced a higher antibody titer compared to the liposome formulation 8 weeks after subcutaneous administration in mice.

The synthetic PLGA vaccine SEL-068 (Selecta Bioscience, Inc., USA) is now in phase I clinical trials for the prevention of nicotine addiction and relapse (http://www.selectabio.com). SEL-068 was designed to facilitate smoking cessation and is the first nano-vaccine to enter clinical trials. The SEL-068 formulation incorporates a universal peptide antigen for eliciting T helper cell response and an immunostimulating TLR agonist into the PLGA polymer matrix. Nicotine, a B cell antigen, is covalently linked to the nanoparticle surfaces. No dose-limiting systemic toxicities were observed in a repeat-dose GLP safety and efficacy study in cynomolgus monkeys.

Other biodegradable polymers, including poly (ε-caprolactone) and polyanhydrides, have been tested for their utility in vaccine delivery applications. The H1N1 hemagglutinin antigen was incorporated into chitosan-coated polycaprolactone nanoparticles. Intranasal delivery of the chitosan–polycaprolactone nanocarriers containing H1N1 hemagglutinin was shown to produce serum IgG levels and secretory IgA levels in nasal and lung lavage that were higher than those produced following intranasal delivery of the soluble antigen to Balb/c mice [48].

Polyanhydride nanocarriers have been tested as vaccine delivery systems to take advantage of their biodegradable and safety profiles. Polyanhydride nanoparticles containing *Yersinia pestis* antigens were examined for intranasal vaccination [49]. The recombinant protein F1–V was co-loaded with the adjuvant MPL to induce the generation of F1–V-specific antibodies detected 23 weeks post-vaccination. Polyanhydride microparticles were shown to be taken up by DC cells and to induce antigen-specific proliferation of both CD4+ and CD8+ T cells [50].

Numerous polymeric nanoparticles have been designed to physically entrap antigens within polymer matrices. Electrostatic polyplexes have also been tested for their utility in delivering subunit antigens [51] or plasmid DNA vaccines [52,53]. Hepatitis B surface antigen was loaded onto cationic L-poly lactic acid microspheres via the formation of electrostatic complexes [51]. A single subcutaneous immunization with HBsAg complexed onto the surfaces of cationic microspheres was found to induce comparable levels of serum IgG responses and higher cellular immune responses as compared to two injections of HBsAg with adjuvant alum in a mouse model.

Cationic polymers have been used to form complexes with negatively charged plasmid DNA vaccines. Deacylated cationic polyethyleneimine was used to form complexes with the human immunodeficiency virus (HIV) CN54gp140 antigen [52]. Pulmonary administration of the polyplexes elicited an immune response and protected the immunized mice against viral challenge. PEGylated poly [2-(N,N-dimethylamino)ethyl methacrylate] was used to deliver HIV gag DNA vaccine [53]. Intranasal administration of the polyplexes was observed to enhance the gag-specific serum IgG levels and IFN-γ secreting splenocytes in mice as compared to the naked DNA vaccine-treated group.

Synthetic polymer-based vaccine delivery systems can provide a sustained release profile for vaccine antigens over prolonged periods of time. The release profile may be tuned by controlling the molecular weights of the synthetic polymers and the encapsulation conditions. Relatively few biodegradable and biocompatible synthetic polymeric delivery systems have been tested to date, and the safety profiles of the biodegradation products have not been extensively studied. Progress in the field of biodegradable polymer development is anticipated to accelerate polymer-based vaccine delivery studies.

2.4. *Natural biopolymer-based nanodelivery systems*

Natural biopolymers, including chitosan and pullulan, have been studied as vaccine or adjuvant delivery systems. Chitosan is biocompatible, nontoxic, biodegradable, and provides a cationic charge that may facilitate endocytosis. In the context of vaccine delivery, chitosan displays valuable properties, including mucoadhesiveness and an adjuvanticity that acts by promoting the maturation of DCs.

The mucoadhesiveness of chitosan has been exploited in mucosal vaccine delivery formulations. A recent study showed that an N-trimethyl chitosan nanocarrier carrying a model antigen OVA provided prolonged residence in nasal mucosa compared to PLGA nanoparticles bearing the same antigen [54]. Whereas intramuscularly administered N-trimethyl chitosan-based vaccine formulations provided immune responses that were comparable to those generated by the PLGA-based vaccine, intranasally administered N-trimethyl chitosan-based vaccine formulations induced higher antigen-specific secretory IgA levels compared to PLGA carriers. These results suggest the importance of mucoadhesiveness in vaccine carriers for enhancing the induction of mucosal immunity.

Chitosan nanoparticles were studied as mucosal vaccine delivery systems [55]. Chitosan nanoparticles that encapsulated the hemagglutinin-split influenza virus were prepared by ionically crosslinking the chitosan polymers in the presence of sodium tripolyphosphate. Two intranasal vaccination doses of the hemagglutinin-split influenza virus entrapped in chitosan nanoparticles induced higher systemic and mucosal

antibody responses compared to the responses generated by a vaccine prepared from the hemagglutinin-split influenza virus alone in mice.

The intracellular fate of the HBsAg encapsulated in chitosan nanoparticles has been examined [56]. The cellular fates of the HBsAg and chitosan nanoparticles were followed by fluorescently labeling the HBsAg and chitosan with Cy3 and Cy5, respectively. The HBsAg-containing chitosan nanoparticles were found to be endocytosed by bone marrow-derived DC, and the HBsAg was found to dissociate from the nanoparticles 6 h after cellular uptake. The dissociation of HBsAg from the chitosan nanoparticles was thought to result from the chitosan matrix degradation in the lysosome.

Cholesteryl-conjugated pullulan polymer-based nanoscale hydrogels were studied as potential vaccine vehicles [57]. A non-toxic subunit fragment of the *Clostridium botulinum* type-A neurotoxin BoHc/A was loaded into the nanogel and administered intranasally. The viscosity of the nanogel promoted the retention of BoHc/A in the nasal mucosal layer. After release from the nanogel, BoHc/A was taken up by the mucosal DCs. The intranasal nanogel formulations provided high titers of the antigen-neutralizing serum IgG and antigen-specific sIgA in the absence of additional mucosal adjuvants.

Synthetic and natural polymer hybrid vaccine delivery systems have been investigated. For example, chitosan-coated polycaprolactone nanoparticles were developed for the intranasal delivery of the recombinant influenza A virus (A/California/07/2009) H1N1 hemagglutinin protein [48]. About 66.5% of entrapped antigens were released from the nanoparticles over 63 days. A single intranasal immunization with the antigen-loaded chitosan-coated polycaprolactone nanoparticles resulted in a total IgG response that exceeded the response achieved after a booster intramuscular administration of the free subunit antigen solution in Balb/c mice.

2.5. *Carbon-based nanodelivery systems*

Carbon-based nanomaterials and carbon nanotubes in particular were recently investigated as antigen delivery systems [58]. Carbon-based nanodelivery systems are insoluble, non-degradable, and mimic bacteria in size and shape. Carbon nanotubes are not intrinsically immunogenic, display a low toxicity, are capable of carrying multiple antigens, and are taken up rapidly by antigen-presenting cells. Such features support the feasibility of using carbon nanotubes as antigen carriers.

Zeinali and colleagues examined the utility of single-walled carbon nanotubes (SWCNTs) in vaccine delivery systems [59]. A purified tuberculin protein derivative was loaded onto a carboxyl group-functionalized SWCNT through covalent binding. Following subcutaneous administration, the antigen–SWCNT conjugate was found to stimulate the production by Th1 cells of cytokines, such as IFN-γ and IL-12, at levels comparable to those generated through administration of the conventional tuberculosis BCG vaccine.

A recent study reported the importance of carbon nanotube type on the induction of immunogenicity [60]. In the study, a commercially available fungicide azoxystrobin was loaded onto four types of carbon nanotube (short and single-walled, short and multi-walled, long and single-walled, and long and multi-walled). The resulting immune responses were then compared. The short and multi-walled carbon nanotubes 0.5 μm in length and 50 nm in diameter were found to provide the highest anti-azoxystrobin IgG antibody titers in rabbits.

Carbon magnetic nanoparticles were constructed to permit the tracing of carbon-based vehicles [61]. Magnetic resonance imaging data showed that magnetic nanoparticles 20–80 nm in diameter were rapidly distributed to the spleen, followed by the kidneys and the inguinal lymph nodes. The preferential targeting of carbon magnetic nanoparticles to DCs was used to enrich DCs ex vivo using an external magnet. The biotinylated model antigen hen egg lysozyme protein and the biotinylated TLR ligand CpG were attached to avidin-coated carbon magnetic nanoparticles to improve the induction of T cell activation and antigen-specific IFN-γ responses compared to the free protein antigen.

The nanostructures of carbon-based materials were reported to affect the antigen presentation process [62]. Graphene oxide nanosheets (Fig. 1D) differed from C60 fullerenes in its ability to promote antigen presentation to the T cells. C60 fullerenes stimulated MHC class I antigen presentation of OVA, whereas graphene oxide suppressed antigen presentation by DCs. Moreover, unlike the C60 fullerenes, graphene oxide reduced the immunoproteasomes in DCs.

Although most carbon-based vaccine delivery systems have been tested through systemic administration, a recent study examined the feasibility of using carbon nanoparticles for oral vaccine delivery [63]. Carbon nanoparticles 470 nm in diameter bearing 40–60 nm pores were synthesized using silica as a template. Instead of loading the antigens via surface adsorption, the model antigen, bovine serum albumin, was encapsulated within the meso- and macropores of the nanoparticles. The rigid structure of the nanoparticles resisted destruction in the oral digestive tract and appeared to protect the antigens from the harsh environments of the alimentary canal after oral delivery. In this study, the IgG and cytokine immune responses elicited by the oral bovine serum albumin entrapped in the porous nanoparticles were comparable to those elicited by parenteral administration of bovine serum albumin with Freund's adjuvant.

Carbon-based nanomaterial vaccine delivery systems are relatively new and less well studied than other systems. Several investigations have indicated the feasibility of carbon-based systems for systemic or oral antigen delivery. Future developments should demonstrate whether the lack of biodegradability will promote or reduce the safety of a carbon-based nano delivery system.

3. Current & future developments

Nanotechnology-based vaccine delivery systems have been developed to enhance the immunogenicity of a vaccine antigen by modulating antigen delivery to the immune cells. Nanocarrier formulations of vaccines offer the advantages of co-delivery of antigen and immunomodulator [64]. Numerous studies have reported enhanced immunogenicity of nanocarrier-based vaccines upon co-delivery with an immunomodulator. Single immunization of OVA and MPL co-

formulated with the PLGA nanocarrier induced much higher systemic and mucosal immune responses after oral delivery than OVA alone [65]. Micelle formulations that include a PEG-modified cationic polypeptide can co-deliver OVA and TLR3 agonists while increasing vaccine-induced antibody production by more than a factor of 70 [66]. The subcutaneous vaccination of pH-responsive micelle nanoparticles containing amphiphilic diblock copolymers conjugated to OVA and CpG oligonucleotides displayed remarkably higher CD8+ T cell responses compared with the free form or a physical mixture [67].

In addition to the co-delivery of antigens and immunomodulators, modifying the surfaces of nanocarriers can contribute to the delivery of antigens specifically to relevant immune cells. Imiquimod (TLR7 agonist)-entrapped PLGA nanocarriers were coated with a chitosan derivative (*N,N,N*-trimethylated chitosan) to improve the protective response generated by mucosal immunization [68]. Another chitosan derivative, glycol chitosan, was decorated onto the surfaces of PLGA nanoparticles for use in a nasal vaccination [69]. Glycol chitosan-coated PLGA nanoparticles showed a lower clearance rate and a higher local uptake in the nasal cavity compared with chitosan-coated PLGA nanocarriers.

Multifunctional nanovaccines can significantly increase the immune response generated by the target-specific, effective, and stable delivery of an antigen. However, the use of many-component nanovaccines with complex structures can increase production costs and complicate the manufacturing process. Many nanovaccines have been manufactured in small batches for research use [70]. Batch-to-batch variations with respect to particle size, shape, and quality are critical problems in the area of nanoparticle synthesis, and these problems must be addressed during scale-up for clinical trial testing [71,72]. Surface-modified nanoparticulate vaccines require complicated synthesis procedures that can require complex purification processes and high expenses. Scale-up processes tend to be time-consuming for pharmaceutical applications. Self-assembled nanovaccine technologies would be beneficial for reducing obstacles to the development of industrial-scale manufacturing protocols.

4. Conclusions

Nanotechnology platforms present promising strategies for improving the immunogenicity of a vaccine antigen. Nanocarriers that are useful for the formulation and delivery of antigens and adjuvants offer many advantages over alternative adjuvant approaches, including improved stability, sustained release kinetics, lower immunotoxicity, and targeting to specific and selective immune cells. In addition, nanoparticles efficiently deliver antigens due to their nanoscale size, solid form, ease of surface modification, and ability to co-deliver antigens along with adjuvants. Although the applications of nanotechnologies for nanocarrier-based vaccine delivery are currently in a nascent investigational stage, and only a few products are being tested in clinical trials, these vaccine delivery systems may potentially be used more broadly for the prevention and treatment of infectious disease.

Acknowledgment

This work was supported by grants from the Korean Health Technology R&D project, Ministry of Health and Welfare (Grant No. A092010), and from Ministry of Science, ICT and Future Planning (No. 2014023227), and from Business for Cooperative R&D between Industry, Academy, and Research Institute funded Korea Small and Medium Business Administration (No. C0010962).

REFERENCES

[1] Naiman BM, Alt D, Bolin CA, et al. Protective killed leptospira borgpetersenii vaccine induces potent Th1 immunity comprising responses by CD4 and γδ T lymphocytes. Infect Immun 2001;69:7550–7558.
[2] Epstein JE, Tewari K, Lyke KE, et al. Live attenuated malaria vaccine designed to protect through hepatic CD8+ T cell immunity. Science 2011;334:475–480.
[3] Saha A, Chowdhury MI, Nazim M, et al. Vaccine specific immune response to an inactivated oral cholera vaccine and EPI vaccines in a high and low arsenic area in Bangladeshi children. Vaccine 2013;31:647–652.
[4] Norton EB, Lawson LB, Mahdi Z, et al. The A subunit of Escherichia coli heat-labile enterotoxin functions as a mucosal adjuvant and promotes IgG2a, IgA, and Th17 responses to vaccine antigens. Infect Immun 2012;80:2426–2435.
[5] Luke JM, Simon GG, Söderholm J, et al. Coexpressed RIG-I agonist enhances humoral immune response to influenza virus DNA vaccine. J Virol 2011;85:1370–1383.
[6] Peek LJ, Middaugh CR, Berkland C. Nanotechnology in vaccine delivery. Adv Drug Deliv Rev 2008;60:915–928.
[7] Dunkle A, Blanchette C, Boone T, et al. Co-delivery of adjuvant and subunit antigens via a nanoparticle platform induces tissue-associated and systemic adaptive immune responses (P4409). J Immunol 2013;190:205–214.
[8] Demento SL, Cui W, Criscione JM, et al. Role of sustained antigen release from nanoparticle vaccines in shaping the T cell memory phenotype. Biomaterials 2012;33:4957–4964.
[9] Gregory AE, Titball R, Williamson D. Vaccine delivery using nanoparticles. Front Cell Infect Microbiol 2013;3:1–13.
[10] Oyewumi MO, Kumar A, Cui Z. Nano-microparticles as immune adjuvants: correlating particle sizes and the resultant immune responses. Expert Rev Vaccines 2010;9:1095–1107.
[11] Borges O, Borchard G, Verhoef JC, et al. Preparation of coated nanoparticles for a new mucosal vaccine delivery system. Int J Pharm 2005;299:155–166.
[12] Apostolopoulos V, Thalhammer T, Tzakos AG, et al. Targeting antigens to dendritic cell receptors for vaccine development. J Drug Deliv 2013. ePub.
[13] Lepenies B, Lee J, Sonkaria S. Targeting C-type lectin receptors with multivalent carbohydrate ligands. Adv Drug Deliv Rev 2013;65:1271–1281.
[14] Raghuwanshi D, Mishra V, Suresh MR, et al. A simple approach for enhanced immune response using engineered dendritic cell targeted nanoparticles. Vaccine 2012;30:7292–7299.
[15] Misumi S, Masuyama M, Takamune N, et al. Targeted delivery of immunogen to primate M cells with tetragalloyl lysine dendrimer. J Immunol 2009;182:6061–6070.
[16] Allison AG, Gregoriadis G. Liposomes as immunological adjuvants. Nature 1974;252:252.

[17] Watson DS, Endsley AN, Huang L. Design considerations for liposomal vaccines: influence of formulation parameters on antibody and cell-mediated immune responses to liposome associated antigens. Vaccine 2012;30:2256–2272.
[18] Henriksen-Lacey M, Devitt A, Perrie Y. The vesicle size of DDA: TDB liposomal adjuvants plays a role in the cell-mediated immune response but has no significant effect on antibody production. J Control Release 2011;154:131–137.
[19] Milicic A, Kaur R, Reyes-Sandoval A, et al. Small cationic DDA: TDB liposomes as protein vaccine adjuvants obviate the need for TLR agonists in inducing cellular and humoral responses. PLoS One 2012;7:e34255.
[20] Christensen D, Henriksen-Lacey M, Kamath AT, et al. A cationic vaccine adjuvant based on a saturated quaternary ammonium lipid have different in vivo distribution kinetics and display a distinct CD4 T cell-inducing capacity compared to its unsaturated analog. J Control Release 2012;160:468–476.
[21] Hamborg M, Jorgensen L, Bojsen AR, et al. Protein antigen adsorption to the DDA/TDB liposomal adjuvant: effect on protein structure, stability, and liposome physicochemical characteristics. Pharm Res 2013;30:140–155.
[22] Kaur R, Bramwell VW, Kirby DJ, et al. Pegylation of DDA: TDB liposomal adjuvants reduces the vaccine depot effect and alters the Th1/Th2 immune responses. J Control Release 2012;158:72–77.
[23] McNeil SE, Rosenkrands I, Agger EM, et al. Subunit vaccines: distearoylphosphatidylcholine-based liposomes entrapping antigen offer a neutral alternative to dimethyldioctadecylammonium-based cationic liposomes as an adjuvant delivery system. J Pharm Sci 2011;100:1856–1865.
[24] Nordly P, Agger EM, Andersen P, et al. Incorporation of the TLR4 agonist monophosphoryl lipid A into the bilayer of DDA/TDB liposomes: physico-chemical characterization and induction of CD8+ T-cell responses in vivo. Pharm Res 2011;28:553–562.
[25] Rosenkrands I, Vingsbo-Lundberg C, Bundgaard TJ, et al. Enhanced humoral and cell-mediated immune responses after immunization with trivalent influenza vaccine adjuvanted with cationic liposomes. Vaccine 2011;29:6283–6291.
[26] Moon JJ, Suh H, Bershteyn A, et al. Interbilayer-crosslinked multilamellar vesicles as synthetic vaccines for potent humoral and cellular immune responses. Nat Mater 2011;10:243–251.
[27] Mašek J, Bartheldyova E, Turanek-Knotigova P, et al. Metallochelating liposomes with associated lipophilised norAbuMDP as biocompatible platform for construction of vaccines with recombinant His-tagged antigens: preparation, structural study and immune response towards rHsp90. J Control Release 2011;151:193–201.
[28] Watson DS, Platt VM, Cao L, et al. Antibody response to polyhistidine-tagged peptide and protein antigens attached to liposomes via lipid-linked nitrilotriacetic acid in mice. Clin Vaccine Immunol 2011;18:289–297.
[29] de Veer M, Neeland M, Burke M, et al. Cell recruitment and antigen trafficking in afferent lymph after injection of antigen and poly(I: C) containing liposomes, in aqueous or oil-based formulations. Vaccine 2013;31:1012–1018.
[30] Lay M, Callejo B, Chang S, et al. Cationic lipid/DNA complexes (JVRS-100) combined with influenza vaccine (Fluzone) increases antibody response, cellular immunity, and antigenically drifted protection. Vaccine 2009;27:3811–3820.
[31] Carroll TD, Matzinger SR, Barry PA, et al. Efficacy of influenza vaccination of elderly rhesus macaques is dramatically improved by addition of a cationic lipid/DNA adjuvant. J Infect Dis 2014;209:24–33.
[32] O'Hagan DT. MF59 is a safe and potent vaccine adjuvant that enhances protection against influenza virus infection. Expert Rev Vaccines 2007;6:699–710.
[33] Vesikari T, Knuf M, Wutzler P, et al. Oil-in-water emulsion adjuvant with influenza vaccine in young children. N Engl J Med 2011;365:1406–1416.
[34] Calabro S, Tritto E, Pezzotti A, et al. The adjuvant effect of MF59 is due to the oil-in-water emulsion formulation, none of the individual components induce a comparable adjuvant effect. Vaccine 2013;31:3363–3369.
[35] Singh M, Kazzaz J, Ugozzoli M, et al. MF59 oil-in-water emulsion in combination with a synthetic TLR4 agonist (E6020) is a potent adjuvant for a combination Meningococcus vaccine. Hum Vaccin Immunother 2012;8:486–490.
[36] Brito LA, Chan M, Baudner B, et al. An alternative renewable source of squalene for use in emulsion adjuvants. Vaccine 2011;29:6262–6268.
[37] Klucker MF, Dalencon F, Probeck P, et al. AF03, an alternative squalene emulsion-based vaccine adjuvant prepared by a phase inversion temperature method. J Pharm Sci 2012;101:4490–4500.
[38] Cotte JF, Sonnery S, Martial F, et al. Characterization of surfactants in an oil-in-water emulsion-based vaccine adjuvant using MS and HPLC-MS: structural analysis and quantification. Int J Pharm 2012;436:233–239.
[39] Makidon PE, Nigavekar SS, Bielinska AU, et al. Characterization of stability and nasal delivery systems for immunization with nanoemulsion-based vaccines. J Aerosol Med Pulm Drug Deliv 2010;23:77–89.
[40] Myc A, Kukowska-Latallo JF, Smith DM, et al. Nanoemulsion nasal adjuvant W_{80}5EC induces dendritic cell engulfment of antigen-primed epithelial cells. Vaccine 2013;31:1072–1079.
[41] Lousada-Dietrich S, Jogdand PS, Jepsen S, et al. A synthetic TLR4 agonist formulated in an emulsion enhances humoral and Type 1 cellular immune responses against GMZ2-a GLURP-MSP3 fusion protein malaria vaccine candidate. Vaccine 2011;29:3284–3292.
[42] Lumsden JM, Nurmukhambetova S, Klein JH, et al. Evaluation of immune responses to a *Plasmodium vivax* CSP-based recombinant protein vaccine candidate in combination with second-generation adjuvants in mice. Vaccine 2012;30:3311–3319.
[43] Schneider LP, Schoonderwoerd AJ, Moutaftsi M, et al. Intradermally administered TLR4 agonist GLA-SE enhances the capacity of human skin DCs to activate T cells and promotes emigration of Langerhans cells. Vaccine 2012;30:4216–4224.
[44] Treanor JJ, Essink B, Hull S, et al. Evaluation of safety and immunogenicity of recombinant influenza hemagglutinin (H5/Indonesia/05/2005) formulated with and without a stable oil-in-water emulsion containing glucopyranosyl-lipid A (SE+GLA) adjuvant. Vaccine 2013;31:5760–5765.
[45] Thomas C, Rawat A, Hope-Weeks L, et al. Aerosolized PLA and PLGA nanoparticles enhance humoral, mucosal and cytokine responses to hepatitis B vaccine. Mol Pharm 2011;8:405–415.
[46] Bershteyn A, Hanson MC, Crespo MP, et al. Robust IgG responses to nanograms of antigen using a biomimetic lipid-coated particle vaccine. J Control Release 2012;157:354–365.
[47] Moon JJ, Suh H, Polhemus ME, et al. Antigen-displaying lipid-enveloped PLGA nanoparticles as delivery agents for a *Plasmodium vivax* malaria vaccine. PLoS One 2012;7:e31472.
[48] Gupta PN, Vyas SP. Investigation of lectinized liposomes as M-cell targeted carrier-adjuvant for mucosal immunization. Colloids Surf B Biointerfaces 2011;82:118–125.
[49] Ulery BD, Kumar D, Ramer-Tait AE, et al. Design of a protective single-dose intranasal nanoparticle-based vaccine

platform for respiratory infectious diseases. PLoS One 2011;6:e17642.
[50] Torres MP, Wilson-Welder JH, Lopac SK, et al. Polyanhydride microparticles enhance dendritic cell antigen presentation and activation. Acta Biomater 2011;7:2857–2864.
[51] Saini V, Jain V, Sudheesh MS, et al. Humoral and cell-mediated immune-responses after administration of a single-shot recombinant hepatitis B surface antigen vaccine formulated with cationic poly(l-lactide) microspheres. J Drug Target 2010;18:212–222.
[52] JF1 Mann, McKay PF, Arokiasamy S, et al. Pulmonary delivery of DNA vaccine constructs using deacylated PEI elicits immune responses and protects against viral challenge infection. J Control Release 2013;170:452–459.
[53] Y1 Qiao, Huang Y, Qiu C, et al. The use of PEGylated poly [2-(N,N-dimethylamino) ethyl methacrylate] as a mucosal DNA delivery vector and the activation of innate immunity and improvement of HIV-1-specific immune responses. Biomaterials 2010;31:115–123.
[54] Slutter B, Bal S, Keijzer C, et al. Nasal vaccination with N-trimethyl chitosan and PLGA based nanoparticles: nanoparticle characteristics determine quality and strength of the antibody response in mice against the encapsulated antigen. Vaccine 2010;28:6282–6291.
[55] Sawaengsak C, Mori Y, Yamanishi K, et al. Chitosan nanoparticle encapsulated hemagglutinin-split influenza virus mucosal vaccine. AAPS PharmSciTech 2014;15:317–325.
[56] Lugade AA, Bharali DJ, Pradhan V, et al. Single low-dose un-adjuvanted HBsAg nanoparticle vaccine elicits robust, durable immunity. Nanomedicine 2013;9:923–934.
[57] Nochi T, Yuki Y, Takahashi H, et al. Nanogel antigenic protein-delivery system for adjuvant-free intranasal vaccines. Nat Mater 2010;9:572–578.
[58] Scheinberg DA, McDevitt MR, Dao T, et al. Carbon nanotubes as vaccine scaffolds. Adv Drug Deliv Rev 2013;65:2016–2022.
[59] Zeinali M, Jammalan M, Ardestani SK, et al. Immunological and cytotoxicological characterization of tuberculin purified protein derivative (PPD) conjugated to single-walled carbon nanotubes. Immunol Lett 2009;126:48–53.
[60] Parra J, Abad-Somovilla A, Mercader JV, et al. Carbon nanotube-protein carriers enhance size-dependent self-adjuvant antibody response to haptens. J Control Release 2013;170:242–251.
[61] Schreiber HA, Prechl J, Jiang H, et al. Using carbon magnetic nanoparticles to target, track, and manipulate dendritic cells. J Immunol Methods 2010;356:47–59.
[62] Tkach AV, Yanamala N, Stanley S, et al. Graphene oxide, but not fullerenes, targets immunoproteasomes and suppresses antigen presentation by dendritic cells. Small 2013;9:1686–1690.
[63] Wang T, Zou M, Jiang H, et al. Synthesis of a novel kind of carbon nanoparticle with large mesopores and macropores and its application as an oral vaccine adjuvant. Eur J Pharm Sci 2011;44:653–659.
[64] Hafner AM, Corthésy B, Merkle HP. Particulate formulations for the delivery of poly(I: C) as vaccine adjuvant. Adv Drug Deliv Rev 2013;65:1386–1399.
[65] Sarti F, Perera G, Hintzen F, et al. In vivo evidence of oral vaccination with PLGA nanoparticles containing the immunostimulant monophosphoryl lipid A. Biomaterials 2011;32:4052–4057.
[66] Luo Z, Li P, Deng J, et al. Cationic polypeptide micelle-based antigen delivery system: a simple and robust adjuvant to improve vaccine efficacy. J Control Release 2013;170:259–267.
[67] Wilson JT, Keller S, Manganiello MJ, et al. pH-responsive nanoparticle vaccines for dual-delivery of antigens and immunostimulatory oligonucleotides. ACS Nano 2013;7:3912–3925.
[68] Primard C, Poecheim J, Heuking S, et al. Multifunctional PLGA-based nanoparticles encapsulating simultaneously hydrophilic antigen and hydrophobic immunomodulator for mucosal immunization. Mol Pharm 2013;10:2996–3004.
[69] Pawar D, Mangal S, Goswami R, et al. Development and characterization of surface modified PLGA nanoparticles for nasal vaccine delivery: effect of mucoadhesive coating on antigen uptake and immune adjuvant activity. Eur J Pharm Biopharm 2013;85:550–559.
[70] Marre S, Jensen KF. Synthesis of micro and nanostructures in microfluidic systems. Chem Soc Rev 2010;39:1183–1202.
[71] Sebastian V, Arruebo M, Santamaria J. Reaction engineering strategies for the production of inorganic nanomaterials. Small 2013. ePub.
[72] Gomez L, Sebastian V, Irusta S, et al. Scaled-up production of plasmonic nanoparticles using microfluidics: from metal precursors to functionalized and sterilized nanoparticles. Lab Chip 2014;14:325–332.