PLGA-based particulate vaccine delivery systems for immunotherapy of cancer

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Chapter 2

PLGA particulate delivery systems for protein- and peptide-based vaccines: linking particle properties to immunogenicity

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Abstract

Vaccination has had a major impact in global health, and continuous efforts in this field have led to the development of newer and safer vaccines based on subunit antigens rather than whole inactivated or live attenuated vaccines. Among the emerging subunit vaccines are recombinant protein- and synthetic peptide-based vaccine formulations. However, proteins and peptides have a low intrinsic immunogenicity. A common strategy to overcome this is to co-deliver (an) antigen(s) with (an) immune modulator(s) by co-encapsulating them in a particulate delivery system, such as poly(lactic-co-glycolic acid) (PLGA) particles. Particulate PLGA formulations offer many advantages for antigen delivery as they are biocompatible and biodegradable; can protect the antigens from degradation and clearance; allow for co-encapsulation of antigens and immune modulators; can be targeted to antigen presenting cells; and their particulate nature can increase uptake and cross-presentation by mimicking the size and shape of an invading pathogen. This review discusses the use of PLGA particulate formulations for subunit vaccine delivery and provides an overview of the formulation parameters influencing their adjuvanticity, such as size, charge, antigen localization, release profile, and the co-delivery of immune modulators and/or specific targeting molecules, and further outlines how these characteristics affect uptake, processing and antigen presentation by dendritic cells and the ensuing immune response. Finally, we address the use of PLGA delivery systems for peptide-based vaccines.

Keywords: Vaccine, delivery systems, antigen, synthetic peptide, adjuvant, dendritic cells, PLGA, nanoparticles, microparticles.

1. Introduction

Vaccination consists of the administration of antigens in order to elicit an adaptive antigen-specific immune response and confer long-term protection against subsequent exposure to the antigen [1]. Traditional vaccine formulations, consisting of either live attenuated or killed pathogens, have been very successful in the last century to prevent widespread infectious diseases [2, 3]. Still, despite their success [4, 5], there are serious safety concerns associated with these vaccines, which include the possibility of reactivation of the attenuated pathogens [6, 7] and the inability of immune-compromised patients to clear the vaccine leading to disease symptoms [8]. These issues have led to the demand for safer alternatives and vaccine development shifted from using whole inactivated pathogens to subunits of the pathogen. These subunits may be antigenic proteins, peptides, capsular polysaccharides or any specific part of the pathogen which has been demonstrated to stimulate a protective immune response. Examples of subunit vaccines include hepatitis B, tetanus, diphtheria, and human papillomavirus (HPV) vaccines. However, the need for eliciting both humoral and cellular immune responses has limited the efficacy of subunit vaccines against certain diseases for which effective vaccines are still unavailable, such as AIDS, malaria, tuberculosis, or cancer. While subunits are safer than whole pathogens, they

generally are less immunogenic, demanding the use of adjuvants [5]. Adjuvants are immunostimulatory molecules and/or delivery systems [9] used in vaccine formulations to enhance the magnitude of antigen-specific immune responses.

Immunostimulatory molecules activate the immune system through their interaction with specific receptors of APCs, which can recognize certain evolutionary conserved molecular motifs associated with groups of pathogens, the pathogen-associated molecular patterns (PAMPs). The identification process is regulated by membrane-bound pattern recognition receptors (PRRs) on the DC surface or internal compartments. PRRs present on DC include nucleotide-binding oligomerization domain (NOD)-like receptors (NLRs), C-type lectin receptors (CLRs) and Toll-like receptors (TLRs). These receptors recognize molecules such as bacterial lipopolysaccharides, viral nucleic acids, bacterial peptides, peptidoglycans or lipoproteins, acting as communicators between innate and adaptive immunity and has been intensively studied over the last few decades [10, 11]. TLRLs have been shown to enhance and modulate the immune response when mixed, conjugated, or co-delivered together with antigen [7, 11]. This knowledge opens the door to the rational design of vaccine formulations that co-deliver TLRLs to increase the immunogenicity of the antigen.

Next to immunostimulatory molecules, subunit vaccines may benefit from encapsulation in particulate delivery systems, which include microparticles (MP) (> 1 μm) and nanoparticles (NP) (< 1000 nm). Particles may promote immunogenicity through the following mechanism:

(1) Stability improvement of the antigen: particulate delivery systems can protect encapsulated or associated antigen from chemical and enzymatic degradation and rapid clearance via the kidneys, resulting in increased residence time [1, 9];

(2) Controlled antigen release: particulate formulations can be tailored to serve as intra- and/or extracellular depot for sustained release of the antigen, increasing antigen exposure to DCs and prolonged antigen presentation [12];

(3) Facilitated DC uptake: particulate delivery systems can mimic the size and shape of an invading pathogen, which facilitates uptake by DCs [11, 13];

(4) Targeted delivery: particles *per se are passively directed to APCs because of their particulate form, but can also be specifically targeted to specific tissues or subsets of immune cells (like DCs) via targeting moieties, such as TLR ligands or DC-specific antibodies* [14-17];

(5) Enhanced cross-presentation: particles may facilitate endosomal escape, which is a known mechanism leading to antigen cross-presentation by DCs and induction of a CTL response [18, 19];

(6) Concomitant delivery of multiple components: particulate formulations can co-deliver a combination of molecules, such as (multiple) antigens and/or immu-

nostimulatory molecules and/or targeting ligands, mimicking pathogens and facilitating uptake by antigen APCs and stimulating immune activation [12, 13];

(7) Regulation of the type of immune response: immunological properties of particles can be tailored by changing their size, surface charge, or hydrophobicity, amongst others [1, 9].

(8) Dose reduction: owing to the potential synergistic effect of all the above-mentioned effects, particles can serve to decrease the dose of antigen required to elicit an immune response [11].

A large number of particulate systems has been reported, such polymeric particles, liposomes, virus like particles and virosomes, immune stimulatory complexes (ISCOMs), emulsions, or inorganic nanobeads, but particularly, poly(D,L-lactide-co-glycolide) (PLGA)-based delivery systems are well studied and are promising candidates for antigen delivery [20]. Since the initial description of PLGA particle as potential adjuvants by O'Hagan et al [21], PLGA particles have been formulated in a wide variety of ways resulting in various size, charge, antigen stability, loading capacity and release profiles. These key formulation aspects can greatly affect the end product characteristics and consequently the potency of the vaccine. These factors will be discussed in detail in this review, as well as the latest advances in peptide-based vaccines using PLGA-based particulate systems.

2. Poly(D,L-lactide-co-glycolide) particulate systems for subunit vaccine delivery

Poly(lactic-co-glycolic acid) (PLGA) and its derivatives are aliphatic polyesters that are available in different ratios of lactic acid and glycolic acid, various molecular weights, and type of end groups (ester-terminated (capped) or carboxylic acid terminated (uncapped)). PLGA polymers have been widely studied over the past few decades for several biomedical applications due to their excellent safety records, varying from sutures to bone reconstruction, as well as in implants for sustained drug delivery, and it has long been approved for parenteral human use by the FDA [22-24]. After their administration, PLGA particles undergo degradation by bulk erosion, during which water diffuses into the polymeric matrix, hydrolyzing the ester bonds throughout the polymer and reducing its molecular weight until degradation products are formed that can be dissolved [9]. This process increases porosity in the matrix, allowing the sustained release of the entrapped material as degradation continues. Finally, PLGA is hydrolyzed into the original monomers, lactic acid and glycolic acid, which are by-products of various metabolic pathways and therefore are not associated with significant toxicity [25]. Degradation rate of PLGA is related to molecular weight, hydrophilicity and crystallinity, but also other factors such as pH of the medium, water uptake rate, process of ester hydrolysis, swelling ratio and degradation by-products [9, 26]. Lower molecular

weight molecules degrade faster, as shorter molecules can be more easily hydrolyzed and dissolved, leaving the polymeric matrix. Higher hydrophilicity can also lead to faster degradation: the hydophilicity is mainly influenced by the monomers' ratio, with glycolic acid being more hydrophilic than lactic acid, so the higher the content of glycolic acid, the more hydrophilic, increasing hydrolysis rate [25]. An exception to this rule is the co-polymer with 50:50 lactide:glycolide ratio, which has the fastest degradation rate, even among polymer compositions with higher glycolic acid content. This is due to the influence of crystallinity: the higher the crystallinity, the slower the degradation, and at a 50:50 ratio the polymer is the least crystalline, resulting in the fastest degradation rate [9, 27]. Uptake of PLGA particles by APCs may further expedite the degradation of PLGA, as the acidic environment of the endosomal compartment (pH ~4.5 – 6.5) [28] accelerates degradation compared to physiological pH (pH 7.4) since low pH catalyzes breakage of the ester linkage of the polymer backbone enhancing polymer erosion [29, 30].Thus, depending on the type of PLGA polymer used, PLGA particles can be made with distinct release kinetics [15, 31-33]. Next to release characteristics various other physical trades of PLGA particle can manipulated including particle size and size distribution, zeta potential, polydispersity index, encapsulation efficiency and drug loading [26]. All these characteristics can be controlled during the synthesis of the particles which can be manufactured according to good manufacturing practice in a scalable, affordable and reproducible way [25]..

While many properties are favorable and controllable, there are also drawbacks in using PLGA particles as a delivery system, especially concerning the stability of encapsulated protein antigens, which will be discussed in more detail later on. Therefore, antigen stability after encapsulation and storage should be evaluated, and each formulation should be specifically customized for each antigen, accordingly to its properties [5].

3. PLGA particle characteristics affecting adjuvanticity

Depending on the preparation method and conditions, PLGA particles can be made with diameters ranging from 80 nm to 250 μm [7]. Moreover, various experimental conditions can be chosen and varied, such as type of solvents and polymer, polymer molecular weight, polymer concentration, type and concentration of surfactants, homogenization mechanism, duration and intensity, or volume ratio of phases. Each of these different factors can affect the particle size, size distribution, zeta potential, encapsulation efficiency, drug loading and release profile [26], which in turn affect the immunogenicity of the formulation. In following section we will systemically review these effects.

3.1. Particle size

Particle size is one of the most critical factors affecting interaction with APCs as well as their biodistribution. Particle size is strongly dependent on the type and concentration of surfactants, polymer concentration, phase volume ratios and homogenization speed [26]. Higher polymer concentration leads to bigger particles, due to higher viscosity of the oil phase, making it harder to break the droplets, as well as and higher (w1/o)/w2 ratios; while higher surfactant concentrations lead to more stable emulsions and can produce smaller particles [26]. The method of homogenization and its speed are also among the most important factors: for instance, microparticles are usually produced using an homogenizer and/or magnetic stirring, whereas nanoparticles are produced by sonication, since the higher the homogenization speed, the smaller the particles.

Particle size is also known to influence the loading capacity, depot formation and release kinetics [34-36]. The particle size and size distribution are determinant for antigen release rate, as the total surface area for protein delivery depends on the particle size [26]. The smaller the particle, the faster the antigen release, as smaller particles have a larger surface area, and therefore a greater proportion of antigen located near its surface, which can lead to a higher burst release [37, 38]. On the other hand, microparticles have larger cores from which the encapsulated antigen slowly diffuses out, and require more time to be degraded, usually showing very low antigen release rates and overall antigen release when comparing to nanoparticles, affecting the total amount of antigen actually being delivered to DCs [38].

Smaller particles are generally regarded as more effective delivery vehicles, since their size would allow easier travel through membranes and other biological barriers and efficiently reach target tissues [39-41]. However, there is still no definitive answer to which size PLGA particles are the most effective for vaccine delivery, and results of different studies comparing nanoparticles and microparticles are contradictory [32, 35, 36]. A strong correlation between particle size and the mechanism of antigen uptake, processing and presentation by APCs has been reported in different studies [34-36, 42-44]. APCs are known to take up and process particles with dimensions comparable to viruses and bacteria [45]. The way APCs take up the vaccine can determine how it processes the antigen. Soluble antigens which are preferentially presented by the MHC class II pathway and are only poorly cross-presented. Particles in the range of 20-200 nm are efficiently taken up by DCs via endocytosis or pinocytosis and facilitate the induction of cellular immune responses, whereas microparticles of 0.5-5 μm are taken up via phagocytosis or macropinocytosis mainly generating humoral responses [35, 36, 46]. Particles larger than 10 μm are hardly taken up, leading to defective immune activation [47-49]. It has also been postulated that large microparticles (> 10 μm) preferentially attach to the surface of macrophages thus serving as an extracellular depot system for continuous antigen release [36]. Comparative studies about the effect of PLGA particle size on the observed immune response have been summarized on **Table 1**. These studies suggest that the efficiency of internalization significantly affects the resulting immune response. However, one should bear in mind that particle properties other than size may also affect their fate and biological effects (see following

sections).

The size of MPs should not be too large, as Thomas et al. showed that hepatitis B surface antigen (HBsAg) in PLGA MPs with a size of 5 µm elicited a significantly higher serum antibody response than 12 µm MPs upon pulmonary administration in rats [50]. Confocal imaging also showed that smaller particles were taken up more efficiently by alveolar macrophages, which might explain the increased immunogenicity.

A study investigating the immunogenicity of differently sized PLGA particles (200, 500 and 1 µm) encapsulating bovine serum albumin (BSA) reported that 1000 nm-sized particles were capable of inducing stronger IgG responses *in vivo* than 200 and 500 nm NPs, which induced similar IgG titers to soluble BSA and alum, following immunization via intranasal, oral and s.c. routes in mice [51]. Similar studies were conducted also with PLA MPs encapsulating HBsAg, showing that MPs of 2-8 µm induced stronger anti-HBsAg antibody responses than NPs of 200-600 nm after intramuscular (i.m.) immunization of rats [52]. However, NPs were efficiently taken up by macrophages, whereas PLA MPs were not taken up but found attached to the surface of the macrophages. Immunization with PLA MPs (2–8 µm) promoted IL-4 secretion, upregulated MHC class II molecules and favored a Th2 response, whereas immunization with PLA NPs (200–600 nm) was associated with higher levels of IFN-γ production, upregulation of MHC class I molecules along with antibody isotypes related to a Th1 response [52]. Comparable results were obtained with i.m. vaccination of rats with tetanus toxoid (TT) in PLA particles: PLA MPs sized 2 to 8 µm were more effective in generating antibody titers than particles smaller than 2 µm, potentiating humoral immune response [49]. The choice of particle size may be dependent on the type of immune response desired: NPs favored a Th1 bias, whereas MPs promote Th2 based responses.

After comparing the immunogenicity of TT loaded PLGA NPs (500-600 nm) to MPs (4 µm), both types of particles were mixed together into a formulation that included both NPs and MPs loaded with TT [53]. After i.m. immunization of rats, this mixture elicited higher antibody responses compared to the NPs or MPs alone, which elicited similar responses. A mixture of both size classes could also be considered to stimulate both Th1 and Th2 type responses.

Joshi et al. compared 17 µm, 7 µm, 1 µm, and 300 nm sized PLGA particles co-encapsulating OVA and CpG , by selectively recovering different sized particles with different centrifugation cycles, showing a size-dependent burst release over 48 h followed by a plateau, with total OVA and CpG release ranging from 100% for 300 nm NPs to circa 10% for 17 µm MPs [35]. In a direct comparison, they observed that the efficiency of particle uptake and upregulation of MHC class I and CD86 expression on BMDC was correlated with smaller particle size [35]. The same trend was observed following intraperitoneal (i.p.) vaccination, with the 300 nm NP generating the highest antigen-specific cytotoxic T cell responses, and the highest IgG2a:IgG1 ratio of OVA-specific antibodies, in proportion to DC uptake. These results concur with our own observations, since we have recently compared PLGA NP circa 300 nm with MP > 20 µm, co-encapsulating OVA and poly(I:C), with similar compositions and release

Table 1: Comparative studies about the effect of PLGA particle size on the observed immune response.

Formulation	Particle size	Antigen/TLRL	*In vitro* / *in vivo*	Adminstration route	Response	References
PLGA MPs	5 µm, 12 µm	HBsAg protein	*In vitro* & *in vivo*	Pulmonary	5 µm > 12 µm MPs uptaken by rat alveolar macrophages; Ab responses: 5 µm > 12 µm MPs	[50]
PLGA NPs & MPs	200 nm, 500 nm, 1 µm	BSA protein	*In vitro* & *in vivo*	s.c.	Ab responses: 200 nm ~ 500 nm < 1 µm particles.	[43]
PLA NPs & MPs	200-600 nm, 2-8 µm	HBsAg protein	*In vitro* & *in vivo*	i.m.	NPs >> MPs uptaken by macrophages; MPs ↑ anti-HBsAg Ab responses & ↑ IL-4 secretion related to a Th2 response; NPs ↑ IFN-γ production & ↑ Ab isotype related to a Th1 response.	[52]
PLA MPs	< 2 µm, 2-8 µm, 10-70 µm, 50-150 µm	TT	*In vivo*	i.m.	Ab responses ↑ by 2-8 µm MPs > <2 µm MPs >> 10-70 µm ~ 50-150 µm.	[49]
PLGA NPs & MPs	500-600 nm, 3.5 µm	TT	*In vivo*	i.m.	NPs and MPs mixed together ↑ Ab responses > NPs ~ MPs alone	[53]
PLGA NPs & MPs	17 µm, 7 µm, 1 µm, 300 nm	OVA / Cpg ODN	*In vitro* & *in vivo*	i.p.	particle uptake & upregulation of MHC class I and CD86 expression & ↑ OVA-specific $CD8^+$ T cells & ↑ IgG2a:IgG1 following the same size trend: : 17 µm << 7 µm < 1 µm < 300 nm	[35]
PLGA NPs & MPs	300 nm,> 20 µm	OVA / poly(I:C)	*In vitro* & *in vivo*	s.c.	NPs >> MPs internalized by DCs & ↑ $CD8^+$ T cell activation *in vitro*; vaccination with NPs ↑ OVA-specific $CD8^+$ T cells & Ab production, MPs did not	[48]
PLGA NPs & MPs	600 nm, 1 – 1.5 µm	OVA	*In vitro*	n/a	MPs > NPs induced *in vitro* MHC class I Ag cross-presentation	[54]

Ab: antibody; Ag: antigen; <: less/lower than; >: more/higher than; <<: much less/lower than; >>: much more/higher than; ~: similar; ↑: increased/high: ↓: decreased/low

properties, for their capacity to induce MHC class I cross-presentation *in vitro* and improve immune responses *in vivo* [48]. NPs were efficiently internalized by DCs *in vitro*, whereas MP were not. Subcutaneous vaccination of C57BL/6 mice with NPs resulted in significantly better priming of Ag-specific $CD8^+$ T cells compared to MP. NP also induced a balanced TH1/TH2-type antibody response, whereas MP failed to increase antibody titers [48]. NP also outperformed IFA as an adjuvant, by more efficiently boosting $CD8^+$ T cell activation and (IgG2a) antibody production [48]. Conversely, in a study by Lee et al., MPs in the range of 1.11~1.44 μm more efficiently induced *in vitro* MHC class I cross-presentation of OVA peptides via than 0.56 μm NPs [54]. However, since only 1.11 μm MPs were fully characterized and evaluated *in vivo*, we cannot exclude that differences in (burst) release may be responsible for the differences observed *in vitro*, neither if the results would be translated *in vivo*.

The impact of antigen delivery system size on the resultant immune response also depends on the route of administration employed. Particles in the size range of 20-50 nm are suitable for transport through lymphatic vessels to reach lymph nodes, where they can increase the probability of immune cell interaction [9]. In contrast, large particles (500–2000 nm) depended on cellular transport by skin DCs [40]. These studies suggest that particulate vaccines should be formulated in the nano-size range to achieve efficient uptake, significant MHC class I cross-presentation and effective CTL responses.

3.2. Controlled antigen/adjuvant release

In addition to their ability to protect antigens, favor uptake by APCs and enhance the immune response, controlled release systems can extend antigen release for prolonged periods of time [55, 56]. Antigen/adjuvant release from PLGA particles is dependent on a variety of factors, such as size, polymer composition, porosity of the matrix, antigen loading or the way it is associated with PLGA particles, i.e. encapsulated/entrapped or adsorbed onto the surface. In the first case, antigen release depends on the degradation, erosion or dissolution of the polymer; whereas in the second case it is dependent on the interactions between the polymer and the antigen [57]. Entrapment of the antigen within the particle matrix protects antigen from external environment but may lead to incomplete release, which could lead to a weak immune response; in contrast, adsorption may lead to high burst release, prematurely releasing the antigen from the particulate carrier before uptake by DCs, which can lead to deficient immune responses [37]L. Frequently, a combination of adsorbed and encapsulated antigen occurs, resulting in a characteristic triphasic release profile with an initial burst release followed by a lag phase and a final sustained release phase of the encapsulated antigen dictated by polymer erosion [57, 58]. Initial burst release of antigen can be generally explained by two mechanisms: either it occurs mainly due to the release of antigens that are adsorbed or located in the surface layer, or the morphology of NP/MP causes the initial antigen escape through pores and cracks that may form during the NP/MP fabrication process [59-61]. Several factors affect burst release:

higher hydrophilicity, lower molecular weight and lower polymer concentration can lead to higher burst release [26]. The higher glycolide content makes the polymer more hydrophilic, facilitating water uptake from the release medium which results in a higher initial burst release, whereas the higher lactide content makes it more hydrophobic, thus resulting in much slower release [62]. Other parameters may be altered that impact release, such as switching the molecular weight or to ester end-capped version of the respective polymer [33]. By adding salts to the inner water phase (w1), the porosity of the resulting particles can be controlled by increasing the osmotic gradient and the flux of water from w2 into the w1/polymer phase, increasing antigen release rate [48]. Suspensions of sugars [63] or salts in the oil phase are expected to act in a similar way, resulting in a major increase in water-uptake, e.g., by incorporation of suspended NaCl, which has been shown with PLGA films [64]. A larger inner surface, induced by a higher porosity of the particles, can potentially increase the uptake of the release medium into the particles and accelerate the drug pore-diffusion and release [65]. After burst, the release of encapsulated material from such systems is dependent on diffusivity through the polymer barrier (a more hydrophobic polymer will create a higher barrier), porosity, size of antigen molecule and distribution throughout the matrix, leading to prolonged antigen release, thereby enhancing the duration of antigen exposure to APCs and thus the potency of the resultant response [66].

Antigen release kinetics regulates the antigen exposure to the immune system. If most of the cargo is burst released immediately after immunization and before uptake, antigen will be delivered to APCs in soluble form, losing the benefit of particulate delivery [37]. In contrast, if the release profile is too slow or incomplete, there will not be enough antigen available for presentation by APCs. Moreover, Hailemichael et al. showed that Montanide-based persisting vaccine depots can induce specific T cell sequestration, dysfunction and deletion at vaccination sites; whereas short-lived formulations may overcome these limitations and result in greater therapeutic efficacy of peptide-based cancer vaccines [67]. Still, sustained release of antigen/adjuvant seems crucial to properly activate DCs, whereas a low burst eliminates potential antigen loss before uptake, increasing antigen presentation and $CD8^+$ T cell activation [12, 37]. Kanchan et al. reported that slow and continuous release of antigen/adjuvant may prolong MHC antigen presentation, which play a key role in T cell stimulation and activation, and played a critical role in eliciting memory antibody responses [68]. It has been reported that extended antigen release may enhance not only the level, but also the quality of immune responses [36]. Johansen et al. demonstrated that antigenic delivery increasing exponentially over time induced more potent $CD8^+$ T cell responses and antiviral immunity than a single dose or multiple equivalent doses (zero order) [34]. Shen et al. showed that OVA-loaded PLGA MPs enhanced exogenous antigen MHC class I cross-presentation at 1000-fold lower concentration than soluble antigen, and served as an intracellular antigen reservoir, leading to sustained MHC class I presentation of OVA for 72 h [19]. Likewise, Waeckerle-Men et al. showed that MHC classes I and II-restricted presentation of encapsulated proteins and peptides by DCs was markedly prolonged and presented 50-fold more efficiently on class I molecules than soluble antigens [69]. A difference in performance between PLGA NPs connected to the kinetics of antigen delivery was showed by Demento et al., with “slow” releasing NPs eliciting prolonged antibody titers comparing to “fast” releasing ones, and favored long-term effector-

memory cellular responses [12]. Finally, Zhang et al. formulated OVA-loaded PLGA NPs by encapsulating antigen within NPs or by simply mixing soluble antigen with the NPs, observing that the combined formulations induced more powerful antigen-specific immune responses than each single-component formulation. The enhanced immune responses elicited by the combined vaccine formulation may be ascribed to the combination of a depot effect at the injecton site, adequate initial antigen exposure and long-term antigen persistence leading to prolonged antigen presentation [70].

3.3. Surface characteristics

Surface characteristics such as shape, hydrophobicity, and zeta potential are reported to influence phagocytic uptake by APCs. Because cells are negatively charged, cationic particles induce more efficient phagocytic uptake than anionic particles owing to electrostatic attraction to the negatively charged APC membranes [71, 72]. Strategies aimed at improving the efficacy of PLGA particles as antigen delivery vehicles involve coating them with ionic surfactants or polymers such as poly(ethylene glycol) (PEG), sodium dodecyl sulphate (SDS), aminodextran, chitosan, poly(ethylene imine) (PEI), poly(L-lysine), protamine or cetyltrimethylammonium bromide (CTAB) [57, 73, 74]. Coating can be achieved either by incorporating these agents in the particle matrix either together with the polymer or in the external aqueous phase during the emulsification process, or by adsorption to the surface of pre-formed particles by resuspending them in a solution containing the coating and incubating for a determined amount of time. Besides changing surface charge, some of these molecules have bioadhesive properties, such as chitosan [1], which has been employed to develop formulations for mucosal delivery. Polycations can also aid in phagosomal/endosomal escape after being internalized by APCs [1], potentially influencing the antigen presentation pathway and type of immune response.

Wishke et al. studied the impact of the surface properties of MPs (5 – 10 μm) on phagocytosis and the phenotype of DCs, using bovine serum albumin bearing fluorescein isothiocyanate groups (FITC-BSA) as model antigen [74]. Anionic particles were obtained by using polyvinyl alcohol (PVA) as stabilizing agent; whereas for cationic surfaces CTAB and chitosan/PVA or diethylaminoethyl cellulose (DEAE)-dextran/PVA blends were evaluated. Whereas CTAB modified MPs lost their positive charge and aggregated due to CTAB desorption, the modification with chitosan and DEAE-dextran resulted in stable MPs. DEAE-dextran modified MPs increased the cellular uptake of the protein loaded MPs.

Positively charged PLGA microspheres (1 – 5 μm) containing hepatitis B surface antigen (HBsAg) were prepared with cationic agents stearylamine and PEI in the external aqueous phase [71]. Compared to unmodified formulations, positive surface charge enhanced both the systemic and mucosal immune response upon immunization of rats via the intranasal route, showing increased levels of IgG in serum and sIgA in salivary, vaginal and bronchoalveolar lavages. PLGA microspheres coated with chitosan

were developed for nasal immunization using recombinant HBsAg [75]. The modified PLGA microspheres showed the lowest nasal clearance rate and a 30-fold increase of serum IgG levels in comparison with unmodified PLGA microspheres upon nasal administration. OVA-loaded PLGA nanoparticles coated with N-trimethyl chitosan (TMC) were more efficiently taken up by DCs and showed a longer nasal residence time than uncoated particles [76].

Protamine, a cationic polypeptide, has been used as a surface coating material because of its ability of increasing cell penetration [77]. Protamine coating of PLGA microparticles (~7 μm) encapsulating the purified phospholipase A2 (PLA2) from bee venom or ovalbumin (OVA) as model antigens injected s.c. in mice led to increased antibody and T-cell responses as compared to uncoated particles (~3 μm). This was most likely mediated by an increased uptake, as protamine-coated particles (~3 μm) carrying green fluorescent protein plasmid were efficiently internalized in vitro by non-phagocytic cells and impressively increased transfection [77]. In another study from the same group, PLGA microparticles containing PLA2 allergen in combination with adsorbed protamine and CpG (~8 μm) resulted in strong PLA2-specific antibody responses and the induction of the Th1-associated isotype IgG2a [78].

The CTL-restricted OVA peptide SIINFEKL was microencapsulated into bare PLGA MPs (~2.6 μm), chitosan-coated PLGA MPs with CpG either covalently coupled or physically adsorbed onto the MP surface (~3.1 μm), and protamine-coated PLGA MPs with adsorbed CpG (~2.2 μm) [79]. For the covalent coupling, chitosan coated MPs were functionalized with sulfo-MBS (m-maleimidobenzoyl-N- hydroxysuccinimide ester), a water-soluble heterobifunctional cross-linker which forms amide bonds with primary amino groups via the N-hydroxysuccinimide ester while the maleimido residue reacts with sulfhydryl groups to form stable thioether bonds, and then incubated with 5′-thiol-modified CpG. However, only the uncoated PLGA MP with adsorbed CpG mediated a prominent CTL response in mice after s.c. immunization, eliciting the production of 1.2% of IFN-γ secreting and SIINFEKL-specific $CD8^+$ T cells, with failure of the other formulations being ascribed to the low release of antigen and CpG.

During the encapsulation process antigens are exposed to the organic solvents required to dissolve the polymer (e.g. dichloromethane, ethyl acetate), emulsification steps (which may include interfaces, temperature excursions, sonication, and vigorous shaking) and drying (usually lyophilization), all of which may compromise antigen stability [9]. Moreover, after administration the degradation of the polymer matrix creates an acidic and potentially harmful microenvironment which can result in denaturation, chemical degradation or aggregation of the antigen, which may endanger the integrity and immunogenic potential of the vaccine [80, 81]. In particular, protein antigens tend to aggregate or degrade upon entrapment into PLGA or during release from the matrix [81]. Though peptide antigens may be less susceptible to the above-mentioned stress factors than proteins, as they do not possess a defined tertiary structure, that can also lead to deamidation or formation of peptide adducts due to acylation of lysine residues with lactic and glycolic acid units [82]. This may be partially solved by optimized manufacturing methods or addition of stabilizing agents, such as basic inorganic salts (e.g. magnesium hydroxide, calcium carbonate, sodium bicarbonate, zinc carbonate),

other proteins, surfactants or sugars [81, 83-85].

Attachment of antigens via covalent chemistry or adsorption through electrostatic interactions onto the surface particles might be an alternative to encapsulation to prevent antigen instability. Adsorption procedures can be performed under milder condition than encapsulation, the antigen is released faster and is immediately available from the particle surface to be processed following uptake, not being dependent on polymer degradation to release entrapped antigen, and antigen loading is often more efficient [21, 86]. However, adsorption may also result in burst or premature release, therefore release kinetics should be taken into account when considering adsorption or encapsulation of antigens into PLGA particles.

A single s.c. injection of HBsAg adsorbed to PLGA-CTAB microspheres (~5-7 μm) gave similar humoral and cellular responses a two injections of HBsAg in alum [87]. Anionic MPs with a mean size of 1 μm were prepared by adding SDS to the external water phase in the preparation process in order to adsorb p55 Gag protein [88]. SDS-coated PLGA particles with adsorbed p55 gag were able to induce high specific CTL responses after i.m. administration in mice. IgG titers were significantly increased compared to soluble p55 Gag protein [89]. The same group also investigated particles consisting of PLGA coated with the anionic surfactant dioctylsulfosuccinate (DSS). 1 μm sized PLGA-DSS particles with Neisseria meningitidis B antigen adsorbed elicited high antigen-specific IgG titers in mice after i.p. administration compared to an antigen-alum formulation [90]. Similar particles were used to adsorb gp120dV2, a recombinant HIV glycoprotein with an isoelectric point of 8.5 [91]. In contrast with encapsulated antigen, surface adsorption of this protein did not affect its binding capability to CD4 [91, 92].

In conclusion, modifying the surface charge may help increase particle uptake efficiency and result in a stronger immune response, especially when considering mucosal delivery. Furthermore, modification of the particle surface using either polycations or polyanions has been used to create cationic or anionic particles to which charged antigens/adjuvants can be adsorbed. Most protein antigens and/or adjuvants are negatively charged, so positive charge would allow such antigens to be adsorbed to the surface instead of being encapsulated into particles, which may be beneficial to improve antigen stability.

3.4. Targeted delivery to DCs

3.4.1. TLRL co-delivery in PLGA systems

One of the greatest benefits of particulate antigen delivery systems is their ability to co-deliver antigens and immunomodulators simultaneously to the same APCs [93]. The concomitant delivery of TLRLs and antigens in PLGA particles has been proven.

successful to enhance antigen-specific CTL responses [79, 94]. The appropriate selection of the TLRL for co-delivery will determine the bias towards Th1 or Th2 responses [93]. Furthermore, as most pathogens simultaneously present multiple TLR agonists to APCs, the combination of multiple TLRLs may result in a synergistic effect and a promising strategy to induce strong protective immune responses [7]. Over the last decades, some of these ligands have been used in several vaccine formulations to target and activate TLRs. A summary of PLGA vaccine formulations containing TLRLs can be found in **Table 2**.

CpG is a ligand to TLR9 which is known to induce a MHC I class driven antigen presentation, resulting in a cellular immune response. Separate groups of C57BL/6 mice were immunized s.c twice with TT and CpG in PLGA NPs (test group), TT-loaded NPs, TT-loaded NPs mixed with soluble CpG, TT and CpG both in solution (reference group), TT alone in solution, and alum adsorbed TT [95]. While CpG increased the immunogenicity of soluble TT antigens when co-delivered s.c. as a soluble adjuvant in mice, its effect was significantly higher when administered in a particulate PLGA formulation. The TT/CpG NPs group showed strong antigen-specific T cell proliferation ex vivo significantly higher than that observed for T cells isolated from the reference group, and was associated with higher levels of interferon γ secretion. IgG titers that were also 16 times higher than the reference group, and differences of 8-fold for IgG1 and IgG3, and 5-fold for IgG2b titers were observed. Overall, the results show that co-delivery of CpG and TT resulted in induction of both Th1 and Th2 immune responses with a bias towards Th1 type. Characterization studies showed that co-encapsulation of CpG and TT in PLGA particles resulted in CpG localization on the outer side of the particles [95]. The importance of co-encapsulation was studied by co-encapsulating ovalbumin (OVA) and CpG-chitosan complexes in PLGA MPs, showing that the co-encapsulation of CpG with OVA in PLGA MPs significantly improved the antibody response and isotype shifting in comparison with mice immunized i.d. with OVA-loaded MPs [96]. Heit et al. compared the *in vivo* immunogenicity of MPs (<10 μm) co-entrapping OVA and CpG with that of CpG-OVA conjugates, or a mixture CpG and OVA [97, 98]. *In vivo*, MP-based vaccination triggered clonal expansion of Ag-specific MHC class I-restricted $CD8^+$ T cells comparable to CpG-Ag conjugates. Conjugation of antigens to adjuvants is however a difficult process that has to be performed and optimized for each individual antigen, whereas particulate formulations offer a more generic approach. Using infection- and tumor-model system, PLGA MP-based single step s.c. vaccination at the tail base of mice conferred protective and even therapeutic immunity against OVA-expressing B16 melanoma tumor cells [98].

TLR3 ligand poly(I:C) is also known to enhance cross-priming of $CD8^+$ cytotoxic T lymphocytes [99]. Poly(I:C) adsorbed to (DEAE)–dextran-coated PLGA MPs containing FITC-BSA were effectively phagocytized by DCs ex vivo and induced a maturation similar to that achieved with a cytokine cocktail or higher concentrations of soluble poly(I:C) [100]. Schlosser et al. demonstrated that MHC I cross-priming in mice after s.c. vaccination with PLGA MPs was enhanced when OVA was co-encapsulated together with either a CpG or poly(I:C) as compared to co-inoculation of OVA-loaded MPs with soluble or separately encapsulated adjuvants [94]. Using four different read-out systems: (i) SIINFEKL/H-2Kb tetramer positive CTLs, (ii) IFN-γ production,

Table 2: Examples of reports of PLGA formulations using Toll-like receptor ligands and their immunological effects.

Receptor	Ligand	Formulation	Antigen	*In vitro / in vivo*	Adminstration route	Response	References
TLR 1/2	Pam3CSK4	PLGA NPs (~350 nm)	OVA24 peptide	*In vitro & in vivo*	s.c.	TLR 2 stimulation ↑ MHC class I presentation of OVA24-NPs by DCs *in vitro* & ↑ prolonged Ag presentation and $CD8^+$ T cell activation *in vivo* after adoptive transfer of NP-loaded DCs	[111]
	Pam3CSK4	PLGA NPs (~500) and MPs (~2 µm; <7 µm)	$CS_{252-260}$ coupled to Pam3CSK4 (Pam-$CS_{252-260}$)	*In vivo*	i.p	Pam-$CS_{252-260}$ particles ↑ cytolytic activity > $CS_{252-260}$-MPs or sPam-$CS_{252-260}$; 500 nm NPs > 2 µm ~ <7 µm MPs inducing CTL responses	[110]
TLR 3	Poly(I:C)	(DEAE)–dextran-PLGA MPs (~3 µm)	FITC-BSA	*In vitro*	n/a	poly(I:C) coated-MPs ↑ expression of CD80, CD86, and CD83 at the DC surface ~ cytokine cocktail or ↑ concentrations of sPoly(I:C).	[100]
TLR 4	MPLA	PLGA MPs (1 – 10 µm)	OVA_{323-39} peptide; MUC1 mucin peptide	*In vivo*	s.c.	Ag/MPLA-MPs ↑ T cell proliferative response & production of IFN-g by T cells, eliciting a specific Th1 immune response > Ag-MPs or Ag mixed with alum	[102, 103]
	MPLA	PLGA NPs (350 – 450 nm)	OVA protein	*In vitro & in vivo*	i.p. or s.c.	OVA/MPLA-NPs ↑ $CD8^+$ T cell proliferative responses & IFN-g *in vitro* & >13-folds increase in clonal expanded $CD4^+$ T cells *in vivo* > OVA-NPs	[104]
	MPLA	PLGA NPs (~300 nm)	HBcAg protein	*In vivo*	s.c.	HBcAg/MPLA-NPs ↑ Th1 cellular response with predominant IFN-g profile > sHBcAg, sHBcAg/sMPLA, or HBcAg-NPs	[105]
	MPLA	PLGA NPs (~500 nm)	$HBcAg_{129–140}$	*In vivo*	s.c.	$HBcAg_{129–140}$/MPLA-NPs ↑ Th1-type response > control formulation of $HBcAg_{129–140}$ in CFA	[132]

TLR 4	MPLA	PLGA NPs (350 – 450 nm)	OVA; MUC1 lipopeptide (BLP25)	*In vitro & in vivo*	n/a	OVA/MPLA-NPs ↑ *in vitro* and *in vivo* antigen-specific primary Th1 immune responses > OVA-NPs or sOVA/sMPLA after adoptive transfer of antigen-pulsed DCs; MUC1/MPLA-NPs delivery to DCs ↑ MUC1 reactive T cells *in vitro* > MUC1-NPs, MPLA-NPs, sMUC1, or sMUC1 with MPLA-NPs	[13]
	7-acyl lipid A	PLGA NPs (350 – 410 nm)	$TRP2_{180-188}$ peptide	*In vivo*	s.c.	$TRP2_{180-188}$/7-acyl lipid A-NPs ↑ $CD8^+$ T cell-mediated anti-tumor immunity & therapeutic anti-tumor effect & levels of IFN-g and pro-inflammatory Th1-related cytokines > $TRP2_{180-188}$-NPs	[107]
	MPLA	PLGA NPs (~80 nm)	$TRP2_{180-188}$ peptide	*In vitro & in vivo*	i.d.	NP ↑ uptake *in vitro* & *in vivo*; $TRP2_{180-188}$/MPLA-NPs ↓ growth of s.c. inoculated B16 melanoma cells in a prophylactic setting > $TRP2_{180-188}$-NPs, $sTRP2_{180-188}$/sMPLA	[108]
	MPLA or RC529	PLGA MPs (3 – 5 μm)	gp120 protein; Men B	*In vivo*	i.p.	Ag adsorbed on TLRL-MPs ↑ IgG serum titers > Ag adsorbed-MPs with sTLRL.	[109]
TLR 9	CpG ODN	PLGA NPs (~300 nm)	Tetanus toxoid (TT)	*In vitro & in vivo*	s.c.	TT/CpG-NPs ↑ antigen-specific T cell proliferation ex vivo & IFN-γ secretion & 16-fold IgG titers > sTT/sCPG; co-encapsulation ↑ Th1 and Th2 immune responses towards Th1 type bias.	[95]
	CpG ODN	PLGA MPs (<10 μm)	OVA protein; CpG-OVA conjugate	*In vitro & in vivo*	s.c.	OVA/CpG-MPs were uptaken by DCs *in vitro*; OVA/CpG-MPs ↑ Ag-specific $CD4^+$ and $CD8^+$ T cells ~ CPG-OVA conjugates *in vivo*. In a tumor challenge, MPs caused complete tumor regression in four out of five mice.	[98]
	CpG ODN	PLGA MPs (<25 μm)	PLA2 protein	*In vivo*	s.c.	PLA2/CPG-MPs ↑ PLA2-specific Ab responses & ↑ Th1-associated isotype IgG2a. The effect of CpG ↑ when protamine was co-encapsulated for complexation of CpG.	[78]

TLR 9	CpG ODN	bare, chitosan-coated, and protamine-coated PLGA MPs (<10 µm)	SIINFEKL peptide	*In vivo*	s.c.	Only uncoated SIINFEKL-MPs with adsorbed CpG ↑ IFN-g secreting and SIINFEKL-specific $CD8^+$ T cells.	[79]
	CpG ODN	PLGA MPs (~1 – 1.5 µm) coated with CTAB or DSS	p55 gag or gp120 env proteins	*In vitro & in vivo*	i.m.	CpG adsorbed to PLGA-CTAB MPs co-administered with gp120 env or p55 gag proteins adsorbed to PLGA-DSS MPs ↑ Ag-specific serum IgG titers, as well as CTL responses against p55 gag > sCpG/sAg,	[88-91]
	CpG ODN-chitosan complexes	PLGA 502 and 752 MPs (~1 – 2 µm)	OVA protein	*In vivo*	i.d.	OVA/CpG-MPs ↑ Ab response and isotype shifting to Th1 > OVA- MPs.	[96]
	CpG ODN or Poly(I:C)	PLGA MPs (<5 µm)	OVA protein	*In vivo*	s.c.	CpG/OVA- or poly(I:C)/OVA-MPs ↑ (i) SIINFEKL/H-2Kb tetramer positive CTLs, (ii) IFN-g production, (iii) *in vivo* cytotoxicity & (iv) protection from vaccinia virus > to OVA-MPs with sTLRL or OVA-MPs with TLRL-MPs.	[94]
	CpG ODN & Poly(I:C)	PLGA MPs (~0.5 - 5 µm)	OVA protein	*In vivo*	s.c.	OVA/CpG-MPs with MP-poly(I:C) ≥ IFA in eradication of preexisting tumors and suppression of lung metastases	[101]
	CpG ODN or/and Poly(I:C)	PLGA NPs (~1 µm)	OVA protein	*In vitro*		poly(I:C)/OVA- or CpG/OVA-NPs ↑ prolonged MHC class I- & II-restricted presentation & ↑ OVA-specific $CD8^+$ and $CD4^+$ T cells; combination of both TLRLs synergistically ↑ MHC class I-restricted, but not class II, Ag presentation.	[99]

Ab: antibody; Ag: antigen; <: less/lower than; >: more/higher than; <<: much less/lower than; >>: much more/higher than; ≥: equal or higher than; ~: similar; ↑: increased/high: ↓: decreased/low; CFA: complete Freund's adjuvant; sX: soluble X.

(iii) *in vivo* cytotoxicity against SIINFEKL-charged target cells, and (iv) the protection from infection with vaccinia virus encoding OVA, they found that co-encapsulated TLR ligand and antigen consistently yielded stronger CTL responses. For instance, a single immunization with MPs containing co-encapsulated OVA and CpG yielded 9% SIINFEKL/H-2Kb tetramer positive CTLs, whereas approximately half of the response was detected when a mixture of the two MP preparations was administered. This effect was observed for both adjuvants examined, though the responses to poly(I:C) were generally lower. Mueller et al. compared the immune response to IFA and a mixture of PLGA MPs combining both OVA and CpG (MP-OVA/CpG) or a mixture of MP-OVA/CpG and MP-poly(I:C), observing that the PLGA MP mixture was as efficient as or superior to IFA in eradication of pre-existing tumors and suppression of lung metastases [101]. PLGA MPs opsonized with mouse IgG, either loaded with OVA and either poly(I:C) or CpG, were incubated with DCs and significantly increased and prolonged both MHC class I- and class II-restricted OVA presentation, with each TLRL showing similar potency. A combination of the two TLR agonists synergistically increased the MHC class I-restricted, but not the class II-restricted, presentation of exogenous antigen [99].

Monophosphoryl lipid A (MPLA) is a detoxified form of lipid A derived from LPS which is a potent TLR4 agonist [7]. Immunization of mice s.c. with PLGA MPs incorporating MPLA with an OVA peptide, consisting of residues 323–339 containing Th and B epitopes of OVA, resulted in an increase in the production of IFN-γ, eliciting a specific Th1 immune response [102] Similar results were obtained with cancer-associated 24mer human MUC1 mucin peptide [103]. Delivery of MUC1 mucin peptide by Poly(d,l-lactic-co-glycolic acid. Hamdy et al. studied the co-delivery of MPLA and OVA in PLGA NPs (OVA/MPLA NPs). The primary $CD4^+$ T cell responses to OVA/MPLA NPs were investigated using OVA-specific T cells from DO11.10 transgenic mice. Following adoptive transfer of these cells, mice were immunized s.c. by NP formulations. For assessing the $CD8^+$ T cell responses, bone marrow derived DCs were pulsed with different OVA formulations and co-cultured with $CD8^+$ T cells from OT-1 mice. Co-delivery of MPLA and OVA in PLGA NPs induced a higher $CD8^+$ T cell proliferative responses and IFN-γ *in vitro* and >13-folds increase in clonal expanded $CD4^+$ T cells *in vivo* following either i.p. or s.c. route of immunization, compared to OVA-PLGA particles without MPLA [104]. The expanded T cells were capable of cytokine secretion and expressed an activation and memory surface phenotype. Similar studies also showed a significantly stronger cell-mediated response in mice after s.c. vaccination with hepatitis B core antigen (HBcAg) protein or peptide ($HBcAg_{129–140}$) and MPLA in PLGA nanoparticles than when using the free antigen, the free antigen with MPLA, or particles loaded with antigen alone [105, 106]. Elamanchili et al. demonstrated that delivery of OVA and MPLA in PLGA NPs to DCs induced potent *in vitro* and *in vivo* antigen-specific primary Th1 immune responses in mice compared to OVA encapsulated alone or soluble OVA and MPLA, after adoptive transfer of antigen-pulsed DCs [13]. Similarly, co-delivery of MUC1 lipopeptide (BLP25, a cancer vaccine candidate) and MPLA loaded in PLGA NPs to human DCs significantly enhanced proliferation of antigen-specific T cells *in vitro* comparing to NP-MUC1, NP-MPLA, soluble MUC1, or soluble MUC1 mixed with NP-MPLA [13]. This combination of MUC1 and MPLA in PLGA NPs was shown to break tolerance and elicit strong T-cell responses against self-antigens *in vivo* after

adoptive transfer of DCs loaded with the vaccine formulations. Hamdy et al. showed that s.c. vaccination of mice bearing melanoma B16 tumors with PLGA NPs co-encapsulating the poorly immunogenic melanoma antigen, tyrosinase-related protein 2 (TRP2), along with TLR4 ligand (7-acyl lipid A, an analog for MPLA) was able to induce potent $CD8^+$ T cell-mediated anti-tumor immunity and therapeutic anti-tumor effect in comparison to antigen encapsulated alone [107]. Zhang et al. observed that i.d. vaccination of mice with PLGA NPs carrying melanoma antigenic peptide $TRP2_{180-188}$ and MPLA significantly delayed growth of subcutaneously inoculated B16 melanoma cells in a prophylactic setting compared to NPs with $TRP2_{180-188}$ alone, or soluble $TRP2_{180-188}$ mixed with MPLA [108]. In another approach, enhanced immunogenicity in mice was obtained with i.p. vaccination with HIV-1 gp120 protein or antigen from Neisseria meningitidis serotype B (Men B) adsorbed to the anionic surface of PLGA MPs treated with ionic surfactant DSS that contained either encapsulated MPLA or the synthetic LPS analog RC529 [109]. Delivery of MPLA or RC529 encapsulated in PLGA MPs with antigen adsorbed onto the MPs resulted in even further enhancement of IgG serum titers over those obtained with soluble TLRLs. This effect was observed for both antigens regardless of whether or not the TLRL and the antigen were used with the same or with separate particles.

In addition, PLGA NPs (~450 - 600 nm) and MPs (~1 – 3 μm or ~6 – 32 μm) have been used to deliver a lipid modified peptide (lipopeptide) from berghei circumsporozoite protein ($CS_{252-260}$, SYIPSAEKI), coupled to TLR2 agonist Pam3Cys with a Ser-Lys-Lys-Lys-Lys spacer (here designated Pam-$CS_{252-260}$). Particles of mean size < 500 nm were better inducers of CTL than larger microparticles (> 2 μm). Pam-$CS_{252-260}$ loaded PLGA particles administrated i.p. to mice elicited higher levels of cytolytic activity than $CS_{252-260}$-MPs or soluble Pam-$CS_{252-260}$ [110]. Recent work by our group using a 24 residue synthetic long peptide (SLP) from OVA SLP-OVA24 as vaccine antigen and TLR2 ligand Pam3CSK4 co-encapsulated in PLGA NPs showed that TLR2 stimulation enhanced MHC class I presentation by DCs and significantly enhanced prolonged antigen presentation and $CD8^+$ T cell activation *in vivo* after adoptive transfer of antigen-loaded DCs [111].

The best way to deliver adjuvants in PLGA particles, either to entrap or to adsorb them onto their surface, is yet to be resolved. The better choice likely depends on the cellular location of their target receptors: if they act on the cell surface, it might be desirable to have the adjuvant readily available on uptake; but if they need to be internalized to interact with endosomal receptors, encapsulation within the particle might be preferable [112].

3.4.2. Targeted delivery to other DC receptors

Aside from TLR ligands, there are many more targeting ligands that have been used with PLGA particles to increase the immunogenicity of subunit vaccines (see **Table 3**). This can be achieved by modifying the particle surface with ligands that can target specific surface receptors of APCs, by either physical association or conjugation reactions

Table 3: Examples of studies of PLGA particles targeted to DCs.

Receptor	Formulation	Antigen / adjuvant	*In vitro / in vivo*	Administration route	Response compared to untargeted particles	References
Integrin, lectin & mannose	PLGA MPs (~2.5 µm) c.c. to RGD peptide; WGA; mannose-PEG3-NH2	-	*In vitro*	n/a	↑ uptake of targeted MPs	[117]
Integrin	PLGA NPs (~200 nm) c.c. to RGD peptide	OVA	*In vitro & in vivo*	oral	↑uptake by M cells & ↑ IgG responses *in vivo*	[116]
	PLGA MPs (~1 µm) with alginate or c.c. RGD-alginate	SPf66; S3	*In vivo*	i.d.	↑ Ab and cellular responses and more balanced Th1/Th2 responses; ↑ IFN-γ secretion and splenocyte proliferation	[118]
Mannose	Mannan c.c. to PLGA NPs (~400 nm)	OVA	*In vitro & in vivo*	s.c.	↑ antigen-specific $CD4^+$ and $CD8^+$ T cell responses *in vitro* and & vivo	[123]
	Mannan-coated on or c.c. to PLGA NPs (~400-500 nm)	-	*In vitro*	n/a	↑ DC uptake & cell surface markers (CD40, CD86) & secretion of inflammatory cytokines (IL-12, IL-6 and TNF-α)	[122, 120]
DC-SIGN	PLGA MPs (2 µm) and NPs (200 nm) c.c. to humanized hD1 anti-DC-SIGN antibody	BSA; TT	*In vitro*	n/a	MPs were taken up nonspecifically; NPs effectively targeted DCs: ↑ uptake & Ag-specific T cell responses at 10–100 fold lower concentrations	[15]
DEC-205	PLGA NPs (~200 nm) c.c. to bfFp containing anti-DEC-205 antibody fragment	OVA	*In vitro & in vivo*	s.c.	2-fold ↑ receptor-mediated uptake of bfFp functionalized NPs *in vitro*; ↑ OVA-specific IgG responses *in vivo*	[127]
DEC-205	PLGA NPs (~200-250) c.c. to anti-DEC-205 mAb	OVA / KRN	*In vitro & in vivo*	footpads	↑ antigen-specific humoral & CTL responses & promoted potent antitumor responses	[129]

DEC-205; CD40; CD11	PLGA NPs (200 nm) c.c. either with anti-DEC-205, -aCD40 or -CD11 mAbs	OVA / poly(I:C) & R848	*In vitro & in vivo*	s.c.	↑ uptake of targeted NPs & IL-12 production & expression of IFN-g *in vitro*; ↑ OVA-specific $CD8^+$ T cell responses *in vivo*	[14]
CD40	PLGA NPs (200 nm) c.c. with anti-aCD40 mAb	OVA; HPV-E7 / poly(I:C) & Pam3CSK4	*In vitro & in vivo*		↑ selective delivery to DCs & ↑ $CD8^+$ T cell priming *in vitro*; ↑ tumor control & prolonged survival of tumor-bearing mice *in vivo*	[17]

Ab: antibody; Ag: antigen; <: less/lower than; >: more/higher than; <<: much less/lower than; >>: much more/higher than; ≥: equal or higher than; ~: similar; ↑: increased/high: ↓: decreased/low; CFA: complete Freund's adjuvant; sX: soluble X; c.c.: chemically conjugated; bfFp: bifunctional fusion protein of strepatividin

[1, 5]. Physical association is driven by electrostatic and hydrophobic interactions, whereas preformed PLGA nanoparticles with carboxyl end groups can be chemically conjugated with molecules with terminal amine groups via amide coupling reactions using carbodiimide reagents [113]. To do that, the surface of PLGA is first derivatized by PEG-NH2 with functional end groups that can react with different ligands [113]. For instance, surface modification with biotin-PEG-NH2: as avidin and its homologues show very high affinity to biotin, biotinylated PEG-PLGA particles allow noncovalent binding with avidin-ligand conjugates or vice versa, allowing targeting ligands such as antibodies to be attached to PLGA particles using these methods [113]. Interaction between PLGA particles functionalized with specific ligands and/or antibodies against DC receptors may improve targeting to DCs, increase particle uptake by DCs through receptor-mediated endocytosis and modulate DC maturation, and thereby enhance the effectiveness of the vaccine formulation [10].

M-cell targeting can be considered if the vaccine is administered at a mucosal tissue [114, 115]. Integrins are heterodimeric transmembrane subunits that have specific affinities toward peptides with an arginine-glycine-aspartate (RGD) sequence [113]. Grafting of the integrin binding targeting peptide RGD can be used to promote the uptake of NPs via interaction with β1 integrins on M cells. Garinot et al. improved the efficiency of orally delivered vaccines in mice, using PEGylated OVA-loaded PLGA NPs displaying RGD molecules at their surface, which were able to elicit IgG responses *in vivo* [116]. Brandhonneur et al. studied the influence of target ligand-grafted PLGA MPs (~2.5 µm) on the rate of uptake by alveolar macrophages using three different ligands: WGA (lectin weat germ agglutinin, which interacts with lectin receptors), a RGD (arginine-glycine-aspartate) containing peptide (interacting on integrins), and mannose-PEG_3-NH_2 (interacting with mannose receptor) covalently coupled the particle surface using the carbodiimide method, showing that a much higher uptake was observed for targeted MPs because of the specific mechanism of phagocytosis. [117]. Mata et al. developed PLGA MPs (1 µm) containing a small percentage of alginate (PLGA-alg MP) or RGD-modified alginate (PLGA-alg-RGD MP). Two malaria synthetic peptides, SPf66 and S3, were microencapsulated into PLGA, PLGA-alg and PLGA-alg-RGD MP. Intradermal (i.d.) vaccination of mice demonstrated that incorporation of alginate elicited higher humoral and cellular immune responses leading to more balanced Th1/Th2 responses, and that the addition of RGD increased cell targeting, enhancing cellular responses such as IFN-γ secretion and slenocyte proliferation [118].

C-type lectin receptors (CLRs) are endocytic receptors that recognize exogenous and endogenous carbohydrates which are present on the surface of DCs and macrophages [113]. Antigens associated with specific sugar residues can target to these receptors on DCs, including the mannose receptor, DEC-205 (also known as CD205), and DC-specific intracellular adhesion molecule-3 (ICAM3)-grabbing non-integrin (DC-SIGN) [119]. Two main strategies can be used to target CLRs, either by grafting particles with specific sugar residues which are natural ligands to these endocytic receptors (e.g. sugars with terminal mannose, fucose or N-acetylglucosamine) or by coupling mAbs against them [120, 121]. Many CLRs expressed by DCs are directly implicated in immunoregulatory processes, such as antigen uptake, intracellular trafficking and antigen presentation [119].

PLGA particles decorated with mannan, a natural polymannose isolated from the cell wall of Saccharomyces cerevisiae, have been designed for targeted DC delivery via mannose receptors. Incorporation of mannan in NPs (~400 – 500 nm) made from capped and uncapped PLGA was achieved either through addition in first or second aqueous phases during NP preparation or by attachment onto the surface of freeze dried NPs by physical adsorption or chemical conjugation to COOH terminated polymer. PLGA NPs containing chemically conjugated or physically adsorbed mannan significantly enhanced DC uptake [122] and induced DC maturation, as evidenced by the up-regulation of cell surface markers (CD40, CD86) and secretion of inflammatory cytokines (IL-12, IL-6 and TNF-α) [120]. PLGA NPs (~400 nm) with chemically conjugated mannan showed increased $CD4^+$ and $CD8^+$ T cell immune response against encapsulated OVA in comparison to their untargeted counterparts [123]. In addition to being a targeting ligand, mannan is also reported to have immunostimulatory properties, possibly due to TLR agonism [123]. The type of sugar can determine which receptor is targeted and the immunological outcome. The nature of mannan has been shown to guide the type of immune response, with antigens conjugated to oxidized mannan inducing mostly cellular responses, whereas antigens coupled to reduced mannan induced humoral responses [124]. Oxidized mannan facilitates endosomal escape of the mannosylated antigen into the cytoplasm, resulting in a $CD8^+$ T cell response, whereas formulations with reduced mannan remain in the endosome, being degraded by the lysosomal enzymes, and resulting in a $CD4^+$ T cell response [125]. A drawback of targeting mannose receptors is the fact that they are also expressed by other cell types, such as monocytes, macrophages, and other subsets of other cells, which might result in unspecific uptake [47].

DEC-205 is a CLR that has been used to target DCs *in vivo*, that is mainly expressed on mature DCs, and also by B cells, T cells, monocytes, macrophages and natural killer (NK) cells at lower levels [121]. DEC-205 is a membrane protein which binds to carbohydrates and mediates endocytosis and antigen presentation and [47]. However, delivery of OVA conjugated to mAb targeting DEC-205 without additional maturation stimuli results in the induction of regulatory T cells and immune tolerance, whereas concomitant delivery of agonistic α-CD40 mAb with targeted OVA results in strong induction of OVA-specific $CD4^+$ and $CD8^+$ T-cell responses [126]. Raghuwanshi et al. prepared OVA-loaded PLGA NPs (~200 nm) for targeted delivery to dendritic cells [127]. First, NPs were prepared by coupling of biotin-PEG2000-amine to the carboxylic group of PLGA. Then, NPs were mixed with a recombinant bifunctional fusion protein (bfFp) of strepatividin and a single chain antibody fragment that recognizes mouse DEC-205 receptors. *In vitro* studies showed a two-fold increase in uptake of targeted NPs, and s.c. immunization of mice using the targeted NPs in combination with a DC maturation agent (anti-CD40 mAb) showed enhanced IgG titers against OVA. However, co-administration of anti-CD40 mAb was shown to be required as DEC-205 targeting in its absence leads to antigen-specific tolerance, as shown in previous studies [126, 127].

Invariant Natural Killer T (iNKT) can enhance cross-priming in a CD1d-dependent manner as they recognize (glyco)lipid antigens [128]. The most extensively studied

CD1d antigen is high-affinity α-galactosylceramide (α-GalCer), an immunostimulatory ligand that can elicit the secretion of a wide array of cytokines by iNKT cells upon recognition, enhancing DC activation and acting as a strong adjuvant for Th1 and CTL responses [27]. Targeted delivery of immunostimulant α-GalCer analogue KRN7000 and OVA by anti-DEC-205 conjugated NPs (~200 - 250 nm) triggered optimal antigen-specific humoral and CTL responses and promoted potent antitumor responses mediated by iNKT cells [129].

DC-SIGN is the most DC-specific CLR and is mainly expressed on immature DCs [121]. A recent study by Cruz et al. using BSA- or TT-loaded MPs (2 μm) and NPs (200 nm) consisting of a PLGA core coated with a PEG-lipid layer conjugated to the humanized anti-DC-SIGN targeting antibody hD1 demonstrated that NPs were targeted more efficiently than MPs to DCs, leading to improved antigen presentation by human DCs and activation of antigen-specific T-cell responses at 10–100 fold lower concentrations of antigen compared to the non-targeted NPs [15]. In another study, similar PLGA NPs (~200 - 250 nm) co-encapsulating OVA and TLRLs poly(I:C) and R848, were coupled to different mAbs against distinct DC surface molecules: DEC-205; TNF family receptor with known DC activating properties αCD40; and CD11c, an integrin receptor [14]. Targeted NPs were more efficiently internalized, and increased IL-12 production and expression of IFN-γ *in vitro* than their non-targeted counterparts, with CD40-targeted NPs performing slightly better than DEC-205 or CD11c targeted NPs. Upon s.c. vaccination, all targeted NP consistently showed higher efficacy than non-targeted NP to stimulate $CD8^+$ T cell responses, though no significant differences were observed between NPs targeted to different receptors [14]. In a subsequent study, s.c. injection of αCD40-targeted PLGA NPs (~200 - 250 nm) loaded with OVA or HPV-E7 protein and TLRLs poly(I:C) and Pam3CSK4 led to very efficient and selective delivery to DCs and efficient $CD8^+$ T cell priming. CD40-targeted NPs encapsulating HPV-E7 protein significantly enhanced antigen-specific $CD8^+$ T cells compared to non-targeted NPs for a mixture of soluble HPV-E7-protein and adjuvants. Therapeutic application of CD40-targeted NPs improved priming of $CD8^+$ T cells against the two independent tumor associated antigens and enhanced tumor control and prolonged survival of tumor-bearing mice, showing that targeting to specific DC receptors is an effective way to increase the efficacy of particulate vaccines [17].

4. Recent advances in PLGA particles for peptide-based vaccine delivery

Peptide-based vaccine formulations offer several advantages, as peptides can be easily synthesized and characterized in a highly reproducible manner in large scale and are generally more stable than whole proteins [7]. Furthermore, they may be especially valuable as anticancer vaccines, where the use of the whole protein is inadequate due to its similarity to endogenous human protein or carcinogenic properties; and the lack of redundant components significantly reduces the risk of allergic or autoimmune

responses [72]. However, peptides alone are poorly immunogenic and need to be combined with adjuvants such as immune modulators and/or delivery systems in order to properly activate the innate and adaptive arms of the immune system [7]. Currently available experimental adjuvants such as Montanide are associated with side effects, while commercially available adjuvants are ineffective at inducing cellular immunity [72]. Encapsulation of synthetic peptides in PLGA particles has been shown to promote cross-presentation, resulting in both humoral and cell-mediated immunity [10]. Different synthetic antigenic peptides have been successfully entrapped into PLGA particles resulting in increased immunogenicity of the peptide vaccines. A summary of peptide-based formulations is presented in **Table 4**.

Partidos et al. injected mice i.p. with the CTL epitope (LDRLVRLIG) representing the 51-59 residues from measles virus nucleoprotein (MVNP51-59) encapsulated in PLGA MPs, which elicited a higher specific cytotoxicity compared with results obtained with IFA [130]. However, simply mixing empty PLGA MPs with the peptide resulted in the induction of CTL responses comparable to those induced by the encapsulated peptide formulation, likely due to surface adsorption of the peptide to the particles [131]. PLGA NPs (~500 nm) containing HBcAg129–140 peptide and MPLA induced a strong Th1-type response with a predominant IFN-γ profile after a single s.c. immunization, which was enhanced after a booster immunization, while mice immunized with a CFA-peptide formulation showed a Th2 bias. Furthermore, mice primed with the CFA-peptide formulation still developed a strong Th1-type response when boosted with the NP formulation [132].

Peptides derived from malarial antigens have also been studied. In a study by Men et al., two synthetic peptides, P30B2 (composed of one universal T helper epitope from tetanus toxin P30 and a B cell epitope B2 derived from the repeat sequence of Plasmodium berghei) and (NANP)6P2P30 (composed of P30 and another T helper epitope from tetanus toxin P2 and a B cell epitope B2 derived from the repeat sequence of Plasmodium falciparum), were incorporated into MPs (1-20 μm) of different compositions eliciting strong and sustained proliferative and antibody responses comparable to those obtained with IFA-peptide preparations after s.c. immunization at the tail base [133]. Malarial peptide SPf66, the first chemically synthesized vaccine to elicit a partial protective immune response against malaria, when encapsulated in PLGA MPs (~1 μm) induced a superior immune response in comparison to alum-adjuvanted SPf66, and a similar response compared with the peptide formulated with Freund's complete adjuvant (FCA) [134]; leading to high antibody levels and protection against P. falciparum challenge when administered to Aoutus monkeys [135]. Another study by Mata et al. compared the immune response against the S3 malarial synthetic peptide injected s.c. using PLGA MPs (~3 μm), Montanide, and alum, with Montanide and MPs behaving comparably and resulting in mixed Th1/Th2 immune responses [136]. Prolonged antibody responses were also obtained by Mata et al. with two malarial peptides derived from the constant region of 3D7 and FC27 Plasmodium falciparum MSP2 dimorphic proteins encapsulated in PLGA MP formulations after s.c. or i.d. injections [137]. More recently, Mata et al. developed PLGA MPs (1 μm) containing a small percentage of alginate (PLGA-alg MP) or RGD-modified alginate (PLGA-alg-RGD MP) [118]. Malaria peptides SPf66

Table 4: Examples of PLGA formulations for peptide-based vaccine delivery.

Formulation	Antigen	Adjuvant	*In vitro* / *in vivo*	Administration route	Response	References
PLGA MPs (~1.6 µm)	$MVNP_{51-59}$	n/a	*In vivo*	i.p.	$MVNP_{51-59}$-MPs ↑ specific CTL responses against $MVNP_{51-59}$ & MV for 120 days > $sMVNP_{51-59}$ and in saline or in IFA; mixing empty PLGA MPs with $sMVNP_{51-59}$ ↑ CTL responses ~ $MVNP_{51-59}$-MPs	[130, 131]
PLGA NPs (~500 nm)	$HBcAg_{129–140}$	MPLA	*In vivo*	s.c.	$HBcAg_{129–140}$-NPs ↑ Th1-type response > $HBcAg_{129–140}$ in CFA ↑ Th2-type; mice primed with CFA- $HBcAg_{129–140}$ ↑ Th1-type response when boosted with $HBcAg_{129–140}$-NPs.	[132]
PLA and PLGA MPs (1-20 µm)	P30B2; (NANP)6P2P30	n/a	*In vivo*	s.c.	MPs ↑ Ab responses & specific isotype subclass Ab production ~ IFA-peptide preparations	[133]
PLGA MPs (~1 µm)	SPf66	n/a	*In vivo*	s.c.	SPf66-MPs ↑ immune response ~ SPf66-CFA > alum-SPf66, leading to ↑ Ab levels and protection against P. falciparum challenge in Aoutus monkeys	[134, 135]
PLGA MPs (~3 µm)	S3	n/a	*In vivo*	i.d.	S3-MPs ~ S3 in Montanide resulting in mixed Th1/Th2 immune responses; but < IFN-γ secretion than Montanide. MPs ↑ Ab levels & ↑ isotype IgG2a & IFN-γ levels.	[136]
PLGA MPs (~1 µm)	MSP2 3D7; MSP2 FC27	n/a	*In vivo*	s.c. or i.d.	MSP2 3D7- & FC27-MPs ↑ potent and long-lasting Ab responses; Montanide was effective against the 3D7 peptide but not FC27.	[137]
PLGA MPs (1 – 10 µm); PLGA NPs (500-900 nm);	OVA_{323-39} peptide; MUC1 mucin peptide	MPLA	*In vitro* & *in vivo*	s.c.	Ag/MPLA-MPs ↑ production of IFN-g, eliciting a specific Th1 immune response > Ag-MPs or Ag mixed with alum	[102, 103]
PLGA NPs (350 – 450 nm)	MUC1 lipopeptide (BLP25)	MPLA	*In vitro* & *in vivo*	n/a	MUC1/MPLA-NPs ↑ proliferation of MUC1 specific T-cells *in vitro* > MUC1-NPs, MPLA-NPs, sMUC1, or sMUC1 with MPLA-NPs; MUC1/MPLA-NPs broke tolerance to self-antigen MUC1 *in vivo* after adoptive transfer of NP-loaded DCs	[13]

PLGA NPs (~330 nm)	OVA24	n/a	*In vitro*	n/a	Low-burst release-NPs ↑ MHC class I Ag presentation *in vitro* > high-burst release-NPs > sOVA24	[37]
PLGA NPs (~300 nm)	OVA24	Pam3CSK4	*In vitro* & *in vivo*	s.c.	NPs ↑ MHC class I Ag presentation *in vitro*; ↑ prolonged Ag presentation & $CD8^+$ T cell activation *in vivo* after adoptive transfer of NP-loaded DCs	[111]
PLGA NPs (~350 nm)	OVA24	Poly(I:C) & pam3csk4	*In vitro* & *in vivo*	s.c.	↑ Ag uptake by DCs and activation of T cells *in vitro*; ↑ OVA-specific $CD8^+$ T cell proliferation *in vivo* ~ MF59 and Montanide	Varypataki et al., in prep.
pLHMGA NPs (~400 – 500 nm)	HPV $E7_{43-69}$	Poly(I:C)	*In vivo*	s.c.	HPV E743–69/Poly(I:C)-NPs or HPV E743–69-NPs with sPoly(I:C) ↑ HPV-specific $CD8^+$ T cells & prolonged the survival of mice in a therapeutic tumor setting ~ to IFA.	[143]
PLGA NPs (180-280 nm)	MART-1_{27-35}; $gp100_{209-217}$; $mSTEAP_{326-335}$	n/a	*In vitro*	n/a	Ag-NPs ↑ Ag presentation by DC pulsed with NPs & ↑ CTL activation > sAg or empty NPs	[144]
PLGA NPs (~80 nm)	$TRP2_{180-188}$	MPLA	*In vitro* & *in vivo*	i.d.	NP ↑ uptake *in vitro* & *in vivo*; TRP2180-188/MPLA-NPs ↑ CTL responses > TRP2180-188-CFA; TRP2180-188/MPLA-NPs ↓ growth of s.c. inoculated B16 melanoma cells in a prophylactic setting compared to TRP2180-188-NPs or sTRP2180-188 / sMPLA	[108]
PLGA NPs (350 – 410 nm)	$TRP2_{180-188}$ peptide	7-acyl lipid A	*In vitro* & *in vivo*	s.c.	TRP2180-188/7-acyl lipid A-NPs ↑ $CD8^+$ T cell-mediated anti-tumor immunity & therapeutic anti-tumor effect & levels of IFN-g and pro-inflammatory Th1-related cytokines > TRP2180-188-NPs	[107]
PLGA-PEG-PCL NPs in a 70:15:15 w/w ratio (~140-190 nm) grafted with mannose	Melan-A:26, gp100:209 or gp100:44	Poly(I:C), CpG, and mannose	*In vitro* & *in vivo*	s.c.	Ag/TLRLs-NPs ↑ IgG2c/IgG1 ratios & ↑ IFN-γ and IL-2; mannose-functionalization of NPs ↑ Th1 immune response; MHC class I- or class II-restricted melanoma Ag/TLRLs-mannose-NPs ↓ the growth rate of murine B16F10 melanoma tumors in therapeutic and prophylatic settings	[145]

Ab: antibody; Ag: antigen; <: less/lower than; >: more/higher than; <<: much less/lower than; >>: much more/higher than; ≥: equal or higher than; ~: similar/comparable; ↑: increased/high: ↓: decreased/low; CFA: complete Freund's adjuvant; sX: soluble X.

and S3 were microencapsulated into PLGA, PLGA-alg and PLGA-alg-RGD MP. Intradermal vaccination of mice demonstrated that incorporation of alginate elicited higher humoral and cellular immune responses leading to more balanced Th1/Th2 responses, and that the addition of RGD increased cell targeting, enhancing cellular responses such as IFN-γ secretion and splenocyte proliferation.

An extensively studied application for peptide-based vaccines is for immunotherapy of cancer by encapsulation of tumor-derived peptides in PLGA particles. Vaccination with synthetic long peptides (SLPs), containing the CTL and/or Th epitopes of a TAA, has shown superior efficacy to protein antigen [138] or minimal MHC class I restricted epitopes in mouse models [18, 139]. In contrast to short peptides, SLPs cannot bind directly to MHC molecules, but have to be taken up and processed by DCs like regular pathogens, inducing the activation of both $CD4^+$ and $CD8^+$ T cells and eliciting a stronger cellular immune response [18, 140, 141].

Immunization of mice s.c. with PLGA MPs (1-10 μm) incorporating MPLA with an OVA peptide, consisting of residues 323–339 containing Th and B epitopes of OVA, resulted in an increase in the production of IFN-g, eliciting a specific Th1 immune response [102]. Ovalbumin peptide encapsulated in poly(d,l lactic-co-glycolic acid. Similar results were obtained with PLGA NPs (500-900 nm) loaded with cancer-associated 24mer human MUC1 mucin peptide [103]. Delivery of MUC1 mucin peptide by Poly(d,l-lactic-co-glycolic acid. Elamanchili et al. showed that co-delivery of MUC1 lipopeptide (BLP25, a cancer vaccine candidate) and MPLA loaded in PLGA NPs (340-450 nm) to human DCs significantly enhanced proliferation of antigen-specific T cells *in vitro* comparing to NP-MUC1, NP-MPLA, soluble MUC1, or soluble MUC1 mixed with NP-MPLA [13]. This combination of MUC1 and MPLA in PLGA NPs was shown to break tolerance and elicit strong T-cell responses against self-antigens *in vivo* after adoptive transfer of DCs loaded with the vaccine formulations [13, 142].

Our group has demonstrated that encapsulation of OVA24, a model 24-residue synthetic long peptide (SLP) antigen covering a CTL epitope of ovalbumin (SIINFEKL) in PLGA NPs of circa 330 nm, engineered to diplay a low burst release, showed enhanced MHC class I restricted T cell activation *in vitro* when compared to high-burst releasing NPs and soluble OVA24 [37]. Co-encapsulation of OVA24 with TLR2 ligand Pam3CSK4 in PLGA NPs enhanced MHC class I presentation by DCs *in vitro* and significantly enhanced prolonged antigen presentation and sustained $CD8^+$ T cell proliferation *in vivo* after adoptive transfer of PLGA NP-loaded DCs [111]. Moreover, recent studies comparing the co-delivery of two SLPs containing the CTL and Th epitopes of OVA (OVA24 and OVA17, respectively), and the TLRLs poly(I:C) and Pam3CSK4 in different delivery systems, showed that subcutaneous vaccination in mice with PLGA NPs resulted in enhanced OVA-specific $CD8^+$ T cell proliferation *in vivo* at least comparable to the formulations that are currently used in clinic, MF59 and Montanide (Varypataki et al., manuscript in preparation). NPs (~400 – 500 nm) based on hydrophilic polyester poly(D,L lactic-co-hydroxymethyl glycolic acid) (pLHMGA) were loaded with a 27-mer SLP containing the CTL epitope RAHYNIVTF of HPV E7 oncoprotein (E743–69) together with poly(I:C) and compared to E743–69 and poly(I:C) formulated in IFA [143]. Encapsulation of antigen substantially enhanced the population of HPV-specific $CD8^+$

T cells when combined with poly(I:C) either co-encapsulated or in its soluble form, and vaccination with NPs substantially prolonged the survival of mice (three weeks) in a therapeutic tumor setting, with a therapeutic efficacy in tumor eradication equivalent to that of the IFA formulation. Moreover, as opposed to IFA, subcutaneous administration of pLHMGA NPs was not associated with local adverse effects.

Some melanoma specific antigens that have been studied for cancer vaccines include MART-1, gp100, TRP1, and TRP2. Encapsulation of MHC class Ia, Ib, and class II-restricted peptide epitopes MART-1_{27-35}, $gp100_{209-217}$ and $mSTEAP_{326-335}$ in PLGA NPs (180-280 nm), resulted in a significantly enhanced antigen presentation of the encapsulated peptides upon internalization of the NPs by DCs *in vitro*, and confirmed that the improved peptide presentation is actually associated with more efficient generation of peptide-specific CTL and T helper cell responses [144].

Zhang et al. loaded PLGA NPs (~80 nm) with TAA peptides from melanoma ($hgp100_{25–33}$ or $TRP2_{180–188}$) observing that the NPs were efficiently uptaken by murine DCs *in vitro* and *in vivo* and induced stronger cellular immune responses than the peptides mixed with CFA [108]. Moreover, i.d. vaccination with PLGA-NP carrying both $TRP2_{180-188}$ and MPLA significantly delayed growth of s.c. inoculated B16-F10 melanoma tumor cells in a prophylactic setting compared to NPs with TRP2 alone, TRP2 peptide mixed with MPLA. Additionally, i.p. administration of IFN-γ from day 1 after the tumor inoculation was highly effective for controlling tumor growth in challenged mice.

Similarly, subcutaneous administration of co-encapsulated $TRP2_{180–188}$ and 7-acyl lipid A into PLGA NPs (350-410 nm) to B16-F10 tumor-bearing mice resulted in anti-tumor activity related with increased levels of IFN-γ and pro-inflammatory Th1-related cytokines compared to control groups [107].

Silva et al. studied the co-entrapment of melanoma-associated antigens and the TLRLs poly(I:C) and CpG, in mannose-functionalized aliphatic polyester-based NPs (constituted by a blend of PLGA, poly(D,L-lactic-co-glycolide-b-ethylene glycol) (PEG-b-PLGA) and poly(ε- caprolactone-b-ethylene glycol) (PEG-b-PCL) in a 70:15:15 w/w ratio) to be targeted to mannose receptors on antigen-presenting cells and induce anti-tumor immune responses [145]. The s.c. co-delivery of the model antigen OVA and the TLR ligands was crucial to induce high IgG2c/IgG1 ratios and high levels of IFN-γ and IL-2, while mannose-functionalization of PLGA-PEG-PCL NPs potentiated the Th1 immune response. The nanoparticulate vaccines decreased the growth rate of murine B16F10 melanoma tumors in therapeutic and prophylatic settings. The combination of mannose-functionalized NPs containing MHC class I- and class II-restricted melanoma antigens (Melan-A:26 and gp100:209) co-entrapped in the same mannose-NPs demonstrated to have a detrimental effect on the anti-tumor immune response, whereas the combination of mannose-NPs containing either a MHC class I- or class II-restricted antigens along with both TLRLs demonstrated the most potent anti-tumor immune response, suggesting the importance of the activation of both $CD4^+$ and $CD8^+$ T-cells.

Although there are still no PLGA-based particulate vaccines on the market [1], one has

reached clinical trial. A phase I clinical trial evaluated the safety and immunogenicity of a synthetic HIV peptide (HIV-1 MN V3) administered i.m. with alum or administered orally encapsulated into PLGA microspheres, unfortunately with poor results [5, 146]. Still, antigenic peptides incorporated into PLGA particulate carriers have demonstrated a considerable potential as prophylactic and therapeutic vaccines, and it is expected that more PLGA-based vaccines enter clinical trials in the near future.

5. Conclusions

Vaccination with subunit antigens is not always successful due to their limited bioavailability and poor immunogenicity. Moreover, soluble antigens are often inefficiently cross-presented. Delivery systems can be used in order to overcome these problems, by protecting antigens from degradation and increase their biodistribution and ability to reach and be uptaken by APCs.

Depending on their physicochemical characteristics, delivery systems can modulate the immune response, mainly due to direct influence in the following mechanisms: facilitated uptake by APCs, regulation of the internalization pathways and ability to endosomal escape, and interaction with specific receptors that mediate the immune response towards humoral or cellular bias. The main immunogenic properties of viruses that elicit potent immune responses may serve as a base for rational vaccine design [147].

Most studies are clear: size plays a crucial role in vaccine efficacy. Smaller particles tend to be more immunogenic due to their easier uptake by DCs and more efficient transport in the lymphatic system, where they can reach immature DC subsets; still, microparticles can form stable antigen depots and are more suitable for intranasal or inhalable pulmonary vaccination [1]. Recent studies have suggested that smaller particles mostly induce cellular immunity while larger particles tend to induce humoral responses [1, 36]. Other important factors include release kinetics; surface characteristics; concomitant delivery of antigen and immunostimulants, allowing DCs to associate danger signals with the antigen, while co-encapsulation of multiple TLRLs may result in a synergistic effect; coating or coupling of DC-specific targeting moieties, increasing DC uptake and enhancing antigen presentation to T cells. Future developments in vaccine delivery will likely involve the combination of immunostimulants with delivery vehicles modified with DC-specific targeting ligands/antibodies.

In summary, vaccines that mimic the size, charge, release kinetics and PAMPs of pathogens may be the future of peptide-based immunotherapy of cancer and/or other diseases that cannot be treated by conventional vaccines.

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