

Biodegradable particles as vaccine antigen delivery systems for stimulating cellular immune responses

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There is a need for both new and improved vaccination formulations for a range of diseases for which current vaccines are either inadequate or non-existent. Biodegradable polymer-based vaccines fulfill many of the desired properties in achieving effective long-term protection in a manner that is safe, economical, and potentially more practicable on a global scale. Here we discuss some of the work performed with micro/nanoparticles made from either synthetic (poly[lactic-co-glycolic acid] [PLGA] and polyanhydrides) or natural (chitosan) biodegradable polymers. Our attention is focused on, but not limited to, the generation of antitumor immunity where we stress the importance of particle size and co-delivery of antigen and adjuvant.

Introduction

Vaccines have been responsible for the eradication and almost complete elimination of smallpox and poliomyelitis respectively as well as the largely effective control, in first world countries, of a number of other potentially fatal diseases including tetanus, rabies, and rubella.¹ However, there are a number of diseases, such as cancers and those caused by intracellular pathogens, for which there are no effective prophylactic or therapeutic vaccines. These diseases require vaccine formulations that promote strong cellular, or Th1-biased, immune responses as opposed to humoral, or Th2-biased, immune responses only. In addition, in developing countries there is a need for vaccine formulations with long shelf lives that require minimal, or ideally no, follow-up

boosts. With an increasing understanding of the immune system much research has been focused on improving the current approach toward vaccine development in these areas. Particle-based antigen (Ag) delivery can potentially provide many levels of adjuvancy that include: prolonging Ag presence (depot formation); enhancing dendritic cell (DC)-mediated Ag uptake; direct stimulation of DC and promotion of cross-presentation.² In this commentary we discuss three biodegradable polymers that are strong candidates for future use as vaccine carriers and focus particularly on formulations designed to promote cellular immune responses.

Biodegradable Polymer Particles

Biodegradable polymers have a number of desirable qualities which have led to their use as vectors for drugs and proteins over the past 20 years. Such qualities include biocompatibility, sustained release, and tunable release kinetics.³ Particle-based vaccine delivery vehicles formulated from synthetic or natural polymers offer attractive advantages over current formulations, not least of which is their high degree of adaptability.⁴⁻⁶ Structural modifications to these polymers can affect a range of important parameters such as: release kinetics of loaded molecules, targeting to a specific population of cells, shielding properties, and particle size, all of which, along with the encapsulate characteristics, can significantly influence the nature of the immune response generated. Highly desirable goals for new vaccine formulations are that they are safe, inexpensive, stable, and mimic pathogenic

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infection.⁷ Polymer-based vaccines can mimic infection in a number of ways. First, they can act as a depot thereby persisting long enough to generate adaptive immune responses. Second, they can ensure efficient co-delivery of Ag and adjuvant to DCs resulting in effective priming of naïve T-cell responses.⁸⁻¹¹ Third, the size of micro- and nano-particles is often similar to the size of various bacterial and viral pathogens respectively.¹²

Co-delivery of antigen and adjuvant

Possibly one of the most salient properties possessed by polymeric particles is their capacity to co-deliver Ag and adjuvant to the same DC.¹¹ In order to generate effective immune responses against intracellular pathogens and malignant disease a vaccine must ensure both efficient uptake of Ag by immature DCs, and concomitant stimulation with an adjuvant that drives DC maturation. The importance of co-delivery of Ag and adjuvant to the same DC has been elegantly demonstrated to be not only dependent on simultaneous uptake but also on both components being delivered in an associated form.^{11,13} Until recently, the only adjuvant approved for use by the US Food and Drug Administration (FDA) has been alum which is not effective at generating Th1-biased immune responses.¹⁴ The only exception to this is the recently approved adjuvant, ASO4 (a combination of monophosphoryl lipid A and alum), which is used in the human papillomavirus vaccine, Cervarix.¹⁵ The most potent and promising adjuvants for generating Th1-biased immune responses are agonists to Toll-like receptors (TLRs), often referred to as pathogen-associated molecular patterns (PAMPs), examples of which include lipid A and CpG oligodeoxynucleotides (CpG ODN).^{6,11,15,16} Co-encapsulation of Ag and adjuvant within the one particle also avoids uptake of Ag independently, which under certain conditions may lead to induction of tolerance.¹⁷ Aside from co-encapsulation of PAMPs it has been shown that polymeric particles per se are capable of exerting a mild to strong adjuvant effect.^{9,11,18}

Poly(lactic-co-glycolic acid) (PLGA)

PLGA is a FDA approved biodegradable and biocompatible synthetic polymer which has been extensively studied as a

vaccine delivery system in preclinical settings as well as having been used as sutures and for controlled drug delivery in the biomedical arena.^{4,19-28} PLGA particles as vaccine carriers offer multiple advantages over many other vaccine delivery systems. For example, PLGA is a synthetic polymer which offers high reproducibility during the fabrication process. Ag can be encapsulated in PLGA particles or attached to the particle surface using covalent or ionic bonding.²⁹ Surface modifications of PLGA particles by conjugating targeting antibodies, biotin, polyethylene glycol (PEG), polyethylenimine (PEI),³⁰ and mannose have shown to improve the efficacy of vaccines by increasing bioavailability of particles, improving systemic circulation, or by targeting these particles to DCs.^{29,31-33}

Our laboratory has performed many studies using PLGA formulations fabricated using a double emulsion solvent evaporation technique.^{34,35} As mentioned above, co-delivery of Ag and adjuvant to DCs is important for optimal immune responses, and we emphasized this through murine vaccination studies revealing PLGA co-encapsulating Ag and CpG ODN could invoke stronger Ag-specific IgG (IgG₁ and IgG_{2a}), and IFN- γ responses than soluble Ag and CpG ODN.³⁶ Improved immune responses at later time-points using PLGA particles co-encapsulating Ag and CpG ODN were also observed over a covalently fused product of Ag and CpG ODN, thereby demonstrating the sustained release benefits of the particle based system. There is nevertheless the problematic issue of immunosuppressive elements that may curtail any potential beneficial outcome on host survival derived from a cancer vaccine that co-delivers Ag and adjuvant. We have shown that when Ag, in the form of tumor lysate, and adjuvant, CpG ODN, are co-delivered in PLGA microparticles as a prophylactic vaccine, protection against subsequent tumor (melanoma) challenge was only effective when regulatory T cells (Tregs) were diminished.³⁷ Certain chemotherapeutic agents, aside from imparting direct cytotoxic effects on tumor cells, are capable of diminishing immunosuppressive populations such as Tregs and/or myeloid derived suppressor cells.^{38,39} Our work with two of these agents,

cyclophosphamide and 5-fluorouracil, in conjunction with PLGA (encapsulating Ag and CpG ODN) or a model adenoviral cancer vaccine respectively, independently suggest that such combinations may ultimately have synergistic therapeutic effects for cancer patients while reducing the severity of side effects often associated with high dose chemotherapy.^{37,40}

The size of biodegradable micro/nano-particle-based vaccines is likely to be of significant immune consequence both qualitatively and quantitatively for reasons which include impact on efficiency of uptake by DCs vs. other cell types (e.g., somatic cells or macrophages) along with the influence on release kinetics of the antigenic load.¹² We and others have established the optimal size(s) required for PLGA based vaccines (encapsulating Ag) to generate appropriate or effective immune responses. Early studies in mice showed that Ag encapsulated in PLGA particles of approximately 500 nm (in diameter) were superior at generating Ag-specific cytotoxic T-lymphocyte responses over 2 μ m and 7 μ m particles after multiple intraperitoneal (IP) vaccinations.⁴¹ The subsequent discovery of various adjuvants enhancing the immunogenicity of particle-based vaccines, led our laboratory to investigate the optimal size required for PLGA-based vaccine formulations, co-encapsulating Ag and adjuvant, to generate effective Th1-type immune responses after IP administrations. Our *in vivo* murine vaccination studies revealed that 300 nm PLGA particles generated greater Ag-specific immune responses (IgG_{2a} and CD8⁺ T lymphocyte) compared with particles of sizes 1 μ m, 7 μ m, or 17 μ m. We also found that particles of 300 nm co-encapsulating Ag and CpG ODN were more readily taken up by DCs and more efficient at stimulating DC maturation *in vitro* than the larger sized particles.³⁵ That particle size and DC uptake are inversely proportional parameters has been previously demonstrated using variously sized latex particles and human DCs, suggesting the generality of such a finding.⁴² Based on the results from the few studies performed comparing differently sized PLGA or poly(lactic acid) (PLA) particles encapsulating Ag (with or without adjuvant) there is a recurring theme of smaller

Table 1. Summary of murine vaccination experiments comparing immunogenicity of PLGA/PLA particles encapsulating Ag (+/-adjuvant)

Compared sizes	Polymer	Vaccination route	Cargo	Results summary		References
				Output	Optimum size(s)	
500 nm, 2 μ m, and 7 μ m	PLGA 50:50	IP	OVA	Th1 + CTL responses	500 nm	41
300 nm, 1 μ m, 7 μ m, and 17 μ m	PLGA 50:50	IP	OVA and CpG	IgG _{2a} + CTL responses	300 nm	35
200 nm, 500 nm, and 1 μ m	PLGA 50:50	subQ or Oral	BSA	IgG responses	1 μ m	43
200–600 nm vs 2–8 μ m	PLA	IM	HBsAg	Th1 responses	200–600 nm	44
				Th2 responses	2–8 μ m	
50–150 μ m, 10–70 μ m, 2–8 μ m and < 2 μ m	PLA	IM	Tetanus Toxoid	IgG responses	2–8 μ m	45
1 μ m and 5 μ m	PLGA 50:50	Oral	BSA	IgG responses	1 μ m	46

IP, intraperitoneal; subQ, subcutaneous; IM, intramuscular; HBsAg, hepatitis B surface antigen; CTL, cytotoxic T lymphocyte.

particles (300–600 nm) promoting Th1-biased immune responses while larger particles (2–8 μ m) tending to promote Th2-biased responses (see Table 1).

Polyanhydrides

Biodegradable amphiphilic polyanhydrides (PAs) represent a class of synthetic polymers that have attractive properties when considering them for use as vaccine vectors. They can be easily manufactured by double emulsion techniques and the degraded by-products are readily metabolized and non-toxic.⁴⁷ The properties of particles made from PAs, such as release kinetics, Ag retention and adjuvant effect, can be tailored based on polymer composition.⁴⁸ Of particular importance when using PA formulations, is to limit the degree of hydrophobicity which, if too high, can negatively affect antigenicity of the protein cargo through the induction of protein aggregation and/or the creation of an overly acidic microenvironment during polymer degradation.^{47,49}

Amphiphilic PA microparticles based on 1,8-bis(p-carboxyphenoxy)-3,6-dioxaoctane (CPTEG) and 1,6-bis(p-carboxyphenoxy) hexane (CPH) copolymers have been shown capable of stabilizing the structural integrity of encapsulated Ag and providing sustained protein release.^{47,50} The observed sustained Ag release derives from the general property of PAs to degrade through surface erosion thus ensuring zero order release of encapsulated molecules.^{51,52}

This is in contrast to PLGA particles which degrade through bulk erosion, a process that may lead to unwanted aggregation of encapsulated proteins as well as accumulation of acidic byproducts in the depot of PLGA, resulting in undesired inflammatory reactions.⁵³ It has been reported that PA particles can act as strong TLR agonists and that 50:50 CPTEG:CPH particles in particular can promote DC maturation in a manner similar to LPS.^{18,54,55} In a recent study, involving informatics analysis, it was shown that 50:50 CPTEG:CPH nanoparticles possessed characteristics that resembled those of intracellular pathogens such as *E. coli* and *Y. pestis*.⁸ To elaborate, 50:50 CPTEG:CPH nanoparticles could persist in late endosomes in a similar fashion to many microbial pathogens thereby suggesting that the particles were being channeled through the exogenous pathway for antigen processing and presentation. In addition, it was observed that 50:50 CPTEG:CPH possessed both structural and functional similarities with lipopolysaccharide (LPS). These particles promoted upregulated surface expression of MHC class I, MHC class II, CD86, and CD40 on DCs, paralleling results obtained with LPS. On the other hand, 50:50 CPTEG:CPH nanoparticles did not induce an inflammatory cytokine response often associated with LPS and in vivo administration of PA consequently led to minimal damage at the site of injection.⁵⁶ In a separate murine study it was shown that a single intranasal vaccination of 50:50 CPTEG:CPH particles encapsulating a

Y. pestis fusion protein/Ag (delivered with soluble Ag [40 μ g]) was capable of generating long-term protection against lethal *Y. pestis* challenge as well as inducing high pathogen-specific antibody titers with high avidity.⁵⁷

Many of the traits exhibited by 50:50 CPTEG:CPH particles implicate them as strong candidates for cancer vaccine formulations. We therefore recently explored this possibility in the context of a well established tumor model system in mice.⁵⁸ It was found that vaccination (prime plus boost) with 50:50 CPTEG:CPH particles encapsulating a model tumor Ag, ovalbumin (OVA), provided enhanced protection from subsequent OVA-expressing tumor challenge over other formulations which included: (1) particles encapsulating both OVA and CpG ODN, (2) solution of OVA and CpG ODN, and (3) particles encapsulating OVA plus soluble CpG ODN. Unlike the situation with PLGA particles, co-encapsulation of CpG-ODN reduced rather than enhanced the immunogenicity of the encapsulated Ag (Fig. 1). Since it has been reported that LPS can abrogate the effects of CpG ODN it is conceivable that 50:50 CPTEG:CPH may have a phenotypic dominance over CpG ODN.⁵⁹

Chitosan

Chitosan is a biodegradable, non-toxic natural polysaccharide which has been approved by the FDA for wound healing applications.⁶⁰ Chitosan has been studied

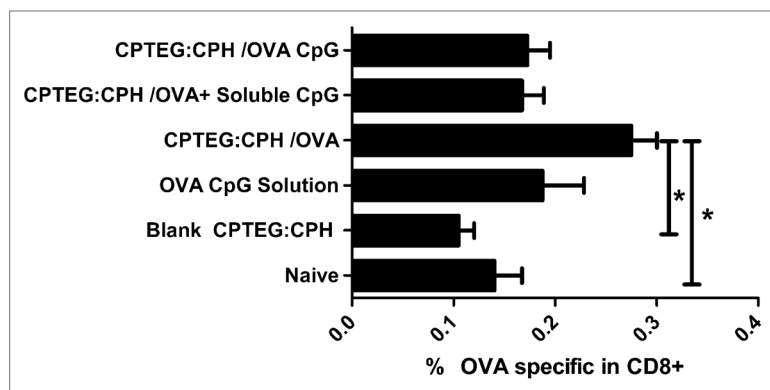


Figure 1. OVA-specific CD8⁺ T-cell frequency in mice vaccinated with polyanhydride microparticles prepared from 50:50 ratio of CPTEG:CPH. Mice were vaccinated twice at 7 d intervals with 100 µg of ovalbumin (OVA) and 50 µg of CpG ODN. Peripheral blood lymphocytes were co-stained using a fluorescently tagged tetramer (binding to OVA/MHC-specific T-cell receptor) and fluorescently labeled anti-CD3 and CD8 antibodies. All groups were statistically compared using ANOVA followed by tukey post-test (*p < 0.05). Adapted from Joshi et al. (2013).⁵⁸

extensively as an orally or nasally delivered vaccine carrier owing to its excellent mucoadhesive properties.^{61,62} It is prepared by deacetylation of N-acetyl-β-D-glucosamine units of chitin. The degree of deacetylation and length of the polymer chain can be varied to change its physiochemical properties.⁶³ Chitosan particles can be prepared by an ionic gelation process, spray drying, complex coacervation, reverse micellar or an emulsion-droplet coalescence method.^{64,65} Chitosan can also be chemically modified at primary amine groups, which facilitates the covalent attachment of Ag (or other molecules) to the chitosan backbone. Surface modified chitosan particles with trans-activated transcription (TAT) peptide, RGD sequence (Arg-Gly-Asp), alginate or PEG sequences have been shown to enhance stability and immunogenicity of vaccines.⁶⁶⁻⁶⁹ Due to its hydrophilicity and positive charge, chitosan possesses a higher protein loading capacity compared with inert polymer delivery systems. Ag or DNA loaded chitosan particles have been shown to induce DC maturation and cytokine production which consequently facilitates T-cell priming, making it a promising vaccine delivery system.^{70,71}

There has been increasing interest in using chitosan in association with viruses primarily for oral/nasal delivery systems because of the mucoadhesive properties of chitosan.⁷² Simply by mixing virus (usually attenuated or inactivated) with the desired

chitosan solution results in viral units at least partially coated with chitosan. In a clinical trial it was shown that intranasal administration of norwalk virus-like particles and monophosphoryl lipid A mixed with a proprietary chitosan formulation (ChiSys[®], Archimedes Pharma LTD) induced norwalk virus-specific serum IgA antibody responses in 70% of vaccinated human volunteers with a marginal increase in IgG antibodies. The vaccine provided protection against both viral gastroenteritis and norwalk virus associated infection.⁷³ In an immunization study in calves using attenuated human adenovirus encoding a bovine herpes Ag it was shown that addition of glycol chitosan improved virological protection over vaccination with adenovirus alone.⁷⁴ Enigmatically, it was reproducibly observed that the degree of protection did not correlate with the magnitude of the neutralizing antibody responses.^{74,75} One possible explanation is that chitosan may mask the adenoviral surface Ag thus reducing their antigenicity and consequently allowing more time for the adenovirus to persist and allow for increased expression of the encoded herpes Ag. In a separate murine study, N,N,N-trimethyl chitosan was used to coat whole inactivated influenza virus prior to intranasal vaccination.⁷⁶ The presence of chitosan resulted in enhanced viral Ag-specific IgG₁ and IgG_{2a/c} responses as well as increased protection against challenges with live influenza. In a separate murine

study of influenza vaccinations, delivered intramuscularly, it was shown that chitosan dramatically improved protection from influenza challenge when mixed with inactivated influenza strains.⁷⁷ In a tumor protection study performed in our laboratory we observed that, in contrast to expectations, low molecular weight chitosan complexed with adenovirus encoding a model tumor Ag actually reduced Ag-specific CD8⁺ T-cell levels and consequently mice vaccinated subcutaneously with these complexes were more susceptible to subsequent tumor challenge than mice vaccinated with adenovirus alone.⁷⁸ We also discovered that chitosan when complexed with adenovirus interfered with transduction of bone marrow derived dendritic cells (but not other cell types) in vitro. It is possible therefore that chitosan may not be an ideal adjuvant for adenoviral vaccines delivered via non mucosal routes, particularly if strong cytotoxic T-cell responses are desired over humoral responses.

Conclusions

In this article we have stressed that co-delivery of Ag and adjuvant is important in the generation of immune responses in order to ensure that maturation and Ag presentation occurs within the same DC. Co-delivery by micro/nanoparticles, in particular, also promotes cross-presentation of antigen and stimulation of Th1-biased/CTL-mediated immune responses that can be further enhanced by diminishing immunosuppressive cells in conjunction with vaccination delivery (Fig. 2). This commentary describes three polymer types with distinct and adjustable physiochemical properties that may be variably exploited to improve current vaccination strategies or establish new ones. PLGA polymers possibly offer the greater diversity of applications and have shown potential as cancer vaccines when combined with agents that deplete Treg function. Polyanhydrides have the advantage of possessing strong TLR agonist properties obviating the requirement for co-encapsulation of an adjuvant with Ag, however, their versatility requires further investigation. Various modified forms of chitosan have shown great promise as

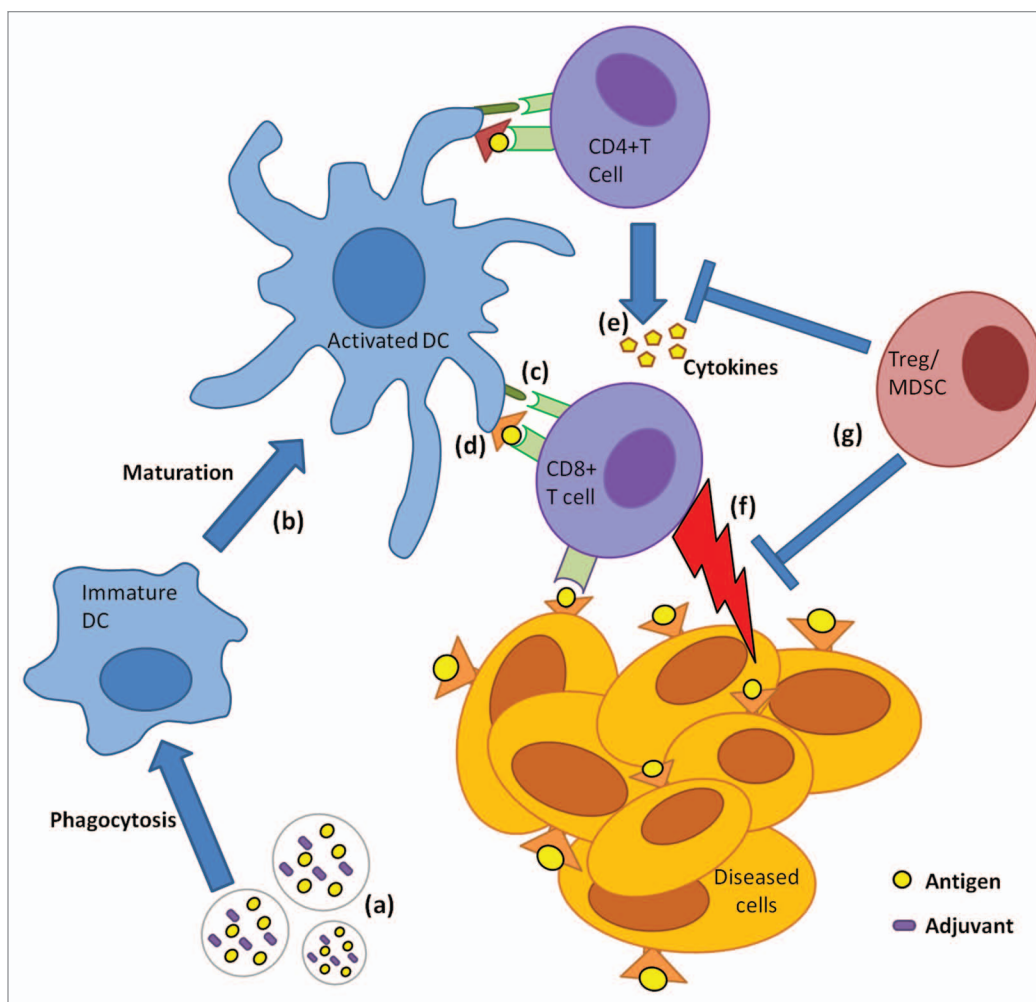


Figure 2. General mechanism of induction of antigen-specific CD8⁺ T-cell immunity by biodegradable particles co-encapsulating Ag and adjuvant. Particles co-encapsulating Ag and an adjuvant (A) are phagocytosed by dendritic cells (DCs). DCs undergo maturation (B), upregulating costimulatory molecules (C) and simultaneously cross-presenting Ag in context of MHC class I (i.e., presentation to CD8⁺ T cells) (D). In the draining lymph node CD8⁺ T cells become activated (i.e., cytotoxic T cells) and concomitantly activated CD4⁺ T cells secrete cytokine help (E) to activated CD8⁺ T cells. Cytotoxic T cells travel via peripheral blood to tumor sites and eradicate Ag expressing cancerous cells or those infected with intracellular pathogens (F). Suppressor cells such as Tregs secrete inhibitory cytokines that prevent T-cell effector activation/function (G) leading to dampening of immune response.

oral/nasal influenza and cancer vaccine carriers, however, the route of administration and formulation characteristics must be carefully considered prior to testing and use. All three polymers have shown enough promise as vaccine vectors to warrant further preclinical and translational studies with a particular focus on diseases requiring Th1-biased immune responses.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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