



New advances in microsphere-based single-dose vaccines

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Abstract

Polymer microspheres have shown great potential as a next generation adjuvant to replace or complement existing aluminum salts for vaccine potentiation. Microsphere-based systems can now be made to deliver subunit protein and peptide antigens in their native form in a continuous or pulsatile fashion for periods of weeks to months with reliable and reproducible kinetics, often obviating the need for booster immunizations in animal models. Microspheres have also shown potential as carriers for oral vaccine delivery due to their protective effects on encapsulated antigens and their ability to be taken up by the Peyer's patches in the intestine. The potency of these optimal depot formulations for antigen may be enhanced by the co-delivery of vaccine adjuvants, including cytokines, that are either entrapped in the polymer matrix or, alternatively, incorporated into the backbone of the polymer itself and released concomitantly with antigen as the polymer degrades. In this article we review the use of polymer microspheres for single-step immunization and discuss future applications for the improvement of vaccines and immunotherapies by utilizing encapsulation technology. © 1997 Elsevier Science B.V.

Keywords: Biodegradable microspheres; Antigen delivery; Vaccine delivery; Antigen stability; Immunization; Controlled release; Microsphere preparation; Mucosal immunization; Vaccine adjuvants

Contents

1. Introduction	98
2. Microsphere mechanisms of adjuvanticity	98
3. Preparation and characterization of antigen-loaded microspheres	99
4. Sterility and manufacturing considerations	101
5. Antigen stability during microsphere preparation and release	102
6. Potency of parenteral microsphere-based vaccines	103
7. Combination preparations: Antigen-microspheres plus adjuvants	107
8. Polymers with built-in adjuvants: Tyrosine-containing polymers	108
9. Mucosal immunization with antigen-microspheres	100
10. Comment on animal models for single-step immunization	111
11. Cytokine delivery for tumor vaccines	111
12. Challenges and future directions	112
12.1. Importance of release pattern for specific antigens (continuous and pulsatile)	112
12.2. Improving the stability of liposomal vaccine preparations	112
12.3. Improving efficiency of oral immunization	113

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12.4. Achieving cellular immunity with microsphere preparations.....	113
13. Conclusions	114
References	114

1. Introduction

Due to advances in biotechnology, many future vaccines will be peptide or protein subunits made by chemical synthesis or recombinant DNA technology. Subunit vaccines are usually very poorly immunogenic compared to whole-cell vaccines, and therefore require several boosters with standard adjuvants (e.g., aluminum salts) in order to fully vaccinate an individual. As a result, these new vaccines will, in many cases, require improved adjuvants and delivery vehicles to raise antibody responses to levels which ensure protection against infectious disease [1].

The need for improved methods of delivering antigens has spurred research aimed at the development of a ‘next generation adjuvant’ to complement or replace the currently used aluminum salts. Aluminum salts were among the first adjuvants discovered back in 1926 [2], and remain the only adjuvants used in currently licensed vaccines in the United States. They are effective with many antigens, but repeat administration is necessary to achieve protection against infection [3]. Their main mode of adjuvanticity is due to their ability to provide a short-term depot effect for absorbed proteins [3], slowly ‘leaking’ antigen to the body’s immune system.

In recent years great effort has been made to improve the efficacy of vaccination by using novel adjuvants or antigen delivery systems. Controlled release of antigens from polymer microparticles has been of particular interest to those interested in the development of vaccines which could be effective in a single dose (‘single-step immunization’) [4,5]. In contrast to aluminum salts, polymeric controlled-release systems may be designed to release entrapped antigens for very long times (weeks to months) following a single immunization, thereby eliminating the need for booster doses in many cases. Several recent studies have demonstrated the possible benefits of single-dose vaccines in developed countries [6–9]. There is even greater need in developing countries where health conditions are poor and most individuals do not return for required booster shots,

resulting in millions of deaths annually from immunizable diseases such as tetanus, pertussis and diphtheria [10,11].

2. Microsphere mechanisms of adjuvanticity

Traditionally, the term adjuvant has been used to describe any molecule that improves the immune response to co-administered antigen. Many classical adjuvants, such as bacterial cell walls and their adjuvant-active extracts [12], work by stimulating a non-specific inflammatory response (consisting of the various cells of the immune system) local to the site of antigen when given as a co-injection (for detailed reviews of adjuvants and their mechanisms of action see [3,12–16]). However, well-designed antigen delivery systems significantly enhance immunity without invoking a vigorous inflammatory response. It was initially proposed that controlled release delivery systems enhance immunity by providing a long-term suppository for the antigen [17], a phenomenon known as the depot theory of adjuvant action [13]. In fact, controlled release systems can provide a release of antigens for weeks to months, a time far exceeding the depot effect of aluminum salts or water/oil emulsions such as Freund’s adjuvants. In addition, microspheres can be made to deliver antigen in a continuous or pulsatile fashion over several months (for a review see [5]). Continuous release mimics the delivery of many small boosters given very close together and pulsatile release may mimic the administration of traditional bolus primary and booster immunizations. Both cases obviate the need for multiple doses of vaccine for protection against infection.

It is now known that microspheres enhance the immune response to antigen by several mechanisms in addition to the depot effect. For example, microspheres are capable of providing enhanced antigen processing through their ability to target phagocytosis by professional antigen presenting cells (APC’s). Microspheres less than 10 microns in diameter are readily phagocytosed by macrophages (for a review

see [18]), the primary APC's in the body, leading to direct intracellular delivery of antigen for processing by the major histocompatibility complex (MHC) class II pathway (exogenous antigen). Recently, it has also been shown that the encapsulation of antigen within particulates [19–21], or on their surface [22–24], can lead to antigen presentation by the MHC class I pathway (endogenous antigen) as well. Presentation of antigen by MHC class II molecules generally leads to enhanced antibody production (i.e., the induction of a humoral immune response), whereas antigen presentation by MHC class I molecules primes for a cytotoxic T lymphocyte (CTL)-mediated immune response. A humoral immune response is generally effective for protection from blood-borne pathogens and toxins, while a cellular immune response is thought necessary for the eradication of infected or altered cells of the body, as is the case with cancerous or virus-infected cells.

Microspheres are also capable of protecting antigens from rapid destruction *in vivo*, allowing for presentation of antigen in its native conformation to the various cells of the immune system. Native antigen is of particular importance to antibody affinity maturation, the process by which the immune system selects the pool of B-cells which produce antibody with the highest affinity for the antigen being delivered. If the delivered antigen is not in its native state, one may expect an affinity maturation that selects for antibodies with lower affinity for the native antigen (and, therefore, lower toxin or pathogen neutralizing capacity).

The protection afforded antigen by encapsulation in polymer microspheres further allows antigen delivery via the oral route. The protective polymer coating of microspheres is thought to at least partially shield antigen from destruction by the low pH of the stomach, and the high levels of proteases and bile salts in the intestine. Furthermore, a percentage of microspheres smaller than 10 μm in diameter are apparently taken up from the intestine into the immune-inductive environment of the Peyer's patches where they can induce both mucosal and systemic immune responses [25,26].

Finally, microspheres can deliver adjuvants or be made of polymers that break down into adjuvant-active molecules (for a review see [16]), thereby providing for a long-term delivery of antigen associ-

ated with a vaccine adjuvant for further potentiation of the immune system.

3. Preparation and characterization of antigen-loaded microspheres

Prior to preparation of microspheres, a suitable polymer matrix must be selected. Although there are a number of biological and synthetic polymers available for use in microencapsulation [27,28], a vaccine formulation intended for humans requires the use of a biodegradable and safe polymer. As mentioned above, the only US FDA approved vaccine adjuvant is alum. However, the biodegradable polymer, poly(lactic-*co*-glycolic acid) (PLGA), has been used for many years in resorbable sutures and bone plates [29–31] and in several commercial formulations, such as Lupron Depot[®], consisting of leuprolide acetate in PLGA microspheres, that has been administered for several years to adults and children [32]. PLGA degrades through bulk erosion to produce lactic and glycolic acid. It is commercially available in a variety of molecular weights, copolymer compositions and polymer end groups (e.g. lauryl alcohol or free acid). Polyanhydrides are another group of polymers that have been approved for use in humans [33] and have been used to deliver proteins [34–36] and model antigens [37]. Unlike PLGA, they degrade by surface erosion, releasing the antigen entrapped at the microsphere surface [38].

Both PLGA and polyanhydrides are insoluble in water and require the use of organic solvents in the microencapsulation process. Typically, the polymer is dissolved in an organic solvent such as methylene chloride or ethyl acetate to facilitate microsphere fabrication. The antigen is then mixed by homogenization or sonication to form a fine dispersion of antigen in polymer/organic solvent (emulsion or suspension). The conventional processes for microsphere formation are solvent evaporation and solvent extraction (coacervation) methods. The solvent evaporation method has been used to prepare both PLGA [5,39] and polyanhydride [37] microspheres for vaccine applications. This process involves the suspension (solid antigen) or emulsion (aqueous antigen solution) of the antigen in the polymer-organic solvent solution. Microspheres are then formed by mixing this solution with water containing

an emulsifier (e.g. polyvinyl alcohol). Additional water is then added to facilitate removal of the organic solvent from the microspheres allowing them to harden. The final microspheres are dried to produce a free flowing powder that may be re-suspended immediately prior to injection.

The water-in-oil-in-water (W/O/W) double emulsion method is the most widely used method of antigen encapsulation in PLGA microspheres. Several reports have focused on the optimization of this process for the entrapment of proteins and subunit antigens (for a review see [40]). A recent study has utilized the W/O/W double emulsion to prepare a number of different PLGA microsphere formulations for a subunit vaccine [41]. In this study, an aqueous solution of the antigen, MN rgp120, was added to the polymer solution to form the primary emulsion. The volume of the aqueous solution relative to the volume of organic solvent was found to be a critical parameter that determined the release characteristics of the microspheres and the encapsulation efficiency (ratio of theoretical to experimental protein loading) of antigen. Similar results have been previously shown for other proteins [42]. The encapsulation efficiency was found to increase with increasing the

kinematic viscosity of the polymer–organic solvent solution. The kinematic viscosity of the polymer solution may be increased by decreasing operation temperature or increasing the polymer concentration in organic solvent. At low aqueous (antigen phase) to organic phase (polymer phase) volume ratios (aqueous:organic ≤ 0.1 ml/ml), 100% of the antigen was encapsulated in PLGA microspheres and the microspheres had a triphasic release: initial burst, lag phase with little or no release, and a second release phase [41]. This result has also been observed previously [42]. The lag phase was dependent upon the polymer degradation rate which, in turn, was dependent on polymer composition and molecular weight (Table 1). The lag phase between the initial and second release phases increased as the lactide content was increased from 50% to 100% at the same polymer molecular weight, or as the polymer molecular weight was increased at a constant lactide:glycolide ratio. In addition to a low aqueous phase volume, operation at low temperature (2–8°C) provided an increased encapsulation efficiency, a reduction in the initial burst, and possibly increased antigen stability against thermal inactivation. This study, as well as others [42], have shown that

Table 1

Correlation between PLGA properties and second burst of MN rgp120: Development of pulsatile antigen delivery systems^{a,b}

Inherent viscosity of polymer solution (dL/g)	Lactide:Glycolide ratio, polymer supplier	Second burst ^c time (days)
Dichloromethane ^b		
0.21	50:50, Boehringer Ingelheim	25
0.24	50:50, MTI	35
0.75	50:50, Medisorb Technologies International	40
0.17	75:25, BI	50
0.21	75:25, MTI	45
0.62	75:25, MTI	80
0.24 and 0.75 ^d	50:50, MTI	40
0.21 and 0.76 ^d	50:50, BI	40
0.17 and 0.67 ^d	75:25, BI	60
Ethyl acetate ^b		
0.75	50:50, MTI	40
0.61	65:35, MTI	45
0.71	65:35, MTI	60
0.62	75:25, MTI	70
0.22	100:0, MTI	190

^a Adapted from [41].^b Microspheres were prepared using 0.3 g PLGA/ml methylene chloride or ethyl acetate, 0.1 ml protein solution/ml organic solvent, and reduced temperature.^c Second burst from microspheres was usually observed over a 2–4 week period. The time listed is the start of release when the percent released was significant (> 10%/week).^d A 50:50 mass ratio of the low and high inherent viscosity PLGA was used to produce these microspheres.

increasing the polymer concentration in the first emulsion results in microspheres with a dense structure that requires extensive polymer hydrolysis prior to significant release of the entrapped antigen (e.g. triphasic release).

On the other hand, continuous protein release was observed at aqueous to organic phase ratios of greater than 0.2 with both low molecular weight polylactic acid (PLA) and 50:50 lactide:glycolide PLGA [41]. Alternatively, a continuous release of antigen may be achieved by using low molecular weight polymers ($< 15\text{--}20$ kDa) and high protein loading ($> 10\%$ w/w), but it is often difficult to achieve a low initial burst release using the W/O/W process under these formulation conditions [43]. Several continuous release systems for both PLGA [44] and polyanhydrides [45] were recently described in the literature. Unfortunately, most studies included only *in vitro* release data and did not demonstrate a continuous release *in vivo*. In particular, a PLGA formulation that was observed to have a continuous release *in vitro* resulted in a triphasic release *in vivo* [43]. This formulation also had a high initial burst (35%) that is often seen with high protein loading ($> 10\%$ w/w) PLGA formulations produced with a W/O/W process.

Another process for microencapsulation capable of providing a continuous protein release from PLGA formulations has recently been described [46]. This process involves the use of spray freeze drying to form the microspheres, thereby avoiding the second aqueous phase where the antigen may be extracted from the nascent microspheres [47]. The microspheres are formed by spraying the antigen-polymer solution into frozen ethanol layered with liquid nitrogen. The ethanol is then thawed and warmed to -70°C to extract the organic solvent from the microspheres. Microspheres made by this process were shown to have a continuous protein release both *in vitro* and *in vivo* [46]. Other approaches that have been utilized to produce continuous protein release from PLGA microspheres with a low initial burst include the co-encapsulation of low molecular weight polylactide oligomers [48] and the use of high protein loading ($> 10\%$ w/w) [49]. In the case of polylactide oligomer coencapsulation [48], BSA was released with approximately zero-order kinetics for up to 15 days *in vitro*, whereas human serum albumin (HSA) microspheres loaded at 12% w/w

released HSA continuously over 26 days *in vitro* without a significant initial burst release [49]. However, *in vivo* release studies were not performed in either of these studies. A mathematical model has recently been developed which focuses on the evolving porosity of PLGA microspheres during their degradation to predict the rate and release profile of macromolecules from PLGA microspheres [50].

The final processing step may also have an impact on the release of antigen from polymer microspheres. In one study, freezing of the final microspheres either during lyophilization or vacuum drying resulted in cracking of the microspheres, while air drying of the microspheres at $2\text{--}8^{\circ}\text{C}$ minimized structural defects in the microspheres as well as reduced the initial burst [41]. However, several other studies have successfully utilized lyophilization of a frozen PLGA microsphere suspension without inducing structural defects in the microspheres.

4. Sterility and manufacturing considerations

Several parameters must be considered before the development of a production process for antigen-microspheres (for reviews see [16,39]). One major consideration is the method of producing a sterile microsphere product (for a review see [51]). Microspheres are too large to sterile filter, leaving the manufacturer with two options for producing a sterile product: aseptic processing throughout the microencapsulation process, or terminal sterilization. Terminal sterilization is usually achieved by gamma irradiation or electron beam irradiation. However, radiation can cause the formation of free-radicals, leading to significant polymer degradation with lactide/glycolides [52–54]. This polymer degradation can cause changes in the performance of antigen-microspheres, such as altered release kinetics [55–57]. The formation of free radicals as a result of irradiation can also have deleterious effects on the antigen, such as oxidation, denaturation and aggregation [58–60], which can lower the potency of the antigen [60]. As a result, the recommended method of attaining a sterile antigen-microsphere product is via aseptic processing conditions, as further described by Cleland [39] and Kline et al. [16].

Finally, since toxic organic solvents such as dichloromethane are often used to produce PLGA

microspheres, the levels of these solvents in the final product must be very low to meet acceptable residual solvent levels dictated by the FDA. For example, a commercial microsphere-based product, the Lupron Depot[®], contains < 50 ppm dichloromethane per dose. Another solvent that has been used to produce PLGA microspheres, ethyl acetate, is considered much safer than dichloromethane, as witnessed by its current use as a pharmaceutical additive to provide flavor (apple flavor). Ethyl acetate is less popular than dichloromethane for microsphere production, however, because lactide/glycolide polymers are less soluble in ethyl acetate and may be more difficult to remove from the final product. In any case, the FDA will likely require extensive testing to validate the safety of any final antigen-microsphere product.

5. Antigen stability during microsphere preparation and release

Vaccine stabilization during microencapsulation, storage and *in vivo* release is paramount to the success of single-immunization vaccines utilizing subunit antigens. A large volume of literature has focused on the stabilization of peptides and proteins under typical pharmaceutical conditions including lyophilization, freezing and drying, rehydration and incubation under physiological conditions (for reviews see [28,61–70]); however, far fewer studies

have focused on the stability of proteins or subunit antigens encapsulated in polymer microspheres (for a review see [71]).

Protein (or subunit antigen) stability in polymer microspheres is of great importance because the native form is often required to invoke a neutralizing antibody response, as well as to promote appropriate affinity maturation of antibodies. Therefore, it is recommended that the stability of the antigen be considered extensively prior to embarking on the development of microsphere formulations. Antigen is exposed to harsh conditions during the microencapsulation process and *in vivo* prior to release (see Table 2). Therefore, to maximize the probability of releasing intact antigen from PLGA microspheres, initial studies of antigen stability should be performed. Initial studies should include screening of stabilizers to prevent denaturation during encapsulation and incubation at physiological pH, ionic strength and temperature over the desired release time.

A recent study of two proteins demonstrated a rapid method for screening stable formulations for encapsulation [72]. These studies involved formulation of the protein in different excipients and buffers followed by emulsion of the protein solution in an organic solvent. After emulsification, the protein was extracted from the organic solvent by addition of excess buffer. The extracted protein was characterized by analytical methods that provided information

Table 2
Conditions during antigen encapsulation and release that may affect its stability

Condition to which antigen may be exposed	Typical effect on antigen	Method(s) to minimize effect on antigen stability	References
Organic solvent/water interface	Unfolding, aggregation	Stabilizers, surfactants	[72,73]
Shear	Unfolding, aggregation	Surfactants	–
Heat during encapsulation	Unfolding, aggregation	Operate at low temperature	[41,65]
Freezing and drying (lyophilization)	Unfolding, aggregation	Stabilizers (cryoprotectants and lyoprotectants)	[69,70]
Hydrophobic polymer surfaces during drying	Unfolding, aggregation	Surfactants, stabilizers	[72,73]
Incubation in aqueous environment at 37°C	Deamidation, oxidation	Chemical modification of protein	[61,66]
Low pH environment	Unfolding, iso-Asp formation	Make microspheres more porous, use partially insoluble buffering excipients	[75]
Silicone oil and heptane with coacervation method	Unfolding, aggregation	Stabilizers, surfactants, use solvent evaporation technique	–

on the physical and chemical state of the protein. These studies, and others [73], revealed that excipients such as mannitol, trehalose, gelatin and human serum albumin can provide stabilization of proteins during their emulsification in organic solvents. It was hypothesized that the effectiveness of mannitol and trehalose may be due to their ability to cause preferential hydration of the protein [63], resulting in a reduced exposure of the proteins to the water-organic solvent interface. This approach may be applicable to vaccine formulations in general, but each antigen must be tested in a variety of formulations to obtain a stable candidate formulation for encapsulation.

After selecting a stable formulation for encapsulation, antigen stability must be determined during the incubation of the microspheres *in vivo*. Porous PLGA microspheres typically become fully hydrated within a short period of time (minutes–hours) when placed in an aqueous environment, such as an intramuscular or subcutaneous injection site [74]. Microencapsulated antigen is therefore exposed to an aqueous environment for extended periods of time prior to its release. To adequately assess antigen stability during this incubation period, the antigen should be placed at conditions that mimic the physiological environment, including physiological ionic strength, pH and temperature (serum proteases or other macromolecules that can not readily diffuse into the PLGA microspheres need not be included). Under these conditions, proteins may undergo a number of deleterious chemical reactions and may form aggregates [28]. The most commonly observed degradation reactions are deamidation, oxidation and aggregation [61]. These reactions are highly antigen-dependent, and may or may not have a prominent influence on the immunogenicity of a particular antigen.

Recently a model protein was encapsulated in PLGA microspheres and its degradation in the microspheres was compared to its degradation in aqueous solution [75]. This study evaluated both the physical and chemical degradation of the protein. A comparison of the protein degradation in the microspheres and in the control solution revealed that the PLGA did not influence the rates of protein degradation. Furthermore, this study suggested that the pH within the PLGA microspheres was not significantly different from the bulk solution when the release

buffer was replenished daily. Under physiological conditions, the microspheres will be exposed to a large excess of buffer ions and salts as well as a continuous flow of fluid both in subcutaneous and muscular tissues. Therefore, if stability of the antigen can be achieved under these physiological conditions, it can be released *in vivo* in its native form at the desired time.

Other work on the stabilization of vaccine antigens during encapsulation and release from microspheres was recently reviewed [39,71]. Additional work was recently described for the stabilization of tetanus toxoid through the use of excipients [73] or covalent modification of the protein [76]. In some cases, the vaccine antigen may either be stable during encapsulation and release (e.g. MN rgp120), or may provide a sufficient immune response as a partially denatured protein [77,78]. However, most antigens will likely benefit from unique stabilization strategies to assure that they are encapsulated and released in their most immunogenic form.

6. Potency of parenteral microsphere-based vaccines

The potency of several parenteral microsphere-based vaccines has been recently reviewed [5,39,79], as has the use of adjuvant-active particulates for vaccine delivery [16]. Of the papers describing parenteral vaccine delivery, tetanus toxoid (TT) has been the dominant antigen studied, most likely because it has been targeted as the first single-dose vaccine using polymer microspheres by the World Health Organization [80]. Table 3 summarizes some of the work with microencapsulated TT vaccine. Because TT is a relatively unstable antigen that aggregates easily [76,81], most of the recent work with microencapsulated TT has been directed at improving its stability during encapsulation and throughout release [73,76,81,82]. A recent study has investigated the use of a novel microencapsulation technique for tetanus toxoid in which the antigen is contained in oil encapsulated by a shell of PLGA [82]. The encapsulation of TT within an oil phase (mineral oil) was performed in an attempt to minimize contact of the antigen with water, thereby minimizing water-mediated antigen inactivation processes. Previous work with TT antigen has shown

Table 3

Examples of microsphere based single-dose vaccine systems with tetanus toxoid

Polymer(s)	Mean microsphere size	In vitro release profile	Animal and route	Comments	Ref.
Poly(D,L-lactide-co-glycolide-D-glucose) 55:45, 40 kDa	45 μm	Not reported for TT	Mice, s.c.	MS made by single emulsion method; TT antigenicity at least partially preserved; alum did not improve immune response; γ -irradiation induced a 90% loss of TT antigen detectable by ELISA; two doses needed to induce high antibody levels	[60]
PLGA 50:50, 100 kDa L-PLA, 3 kDa	9–55 μm (various)	Initial pulse, then continuous	Mice, s.c.	MS provided neutralizing antibody titers (NAT) > soluble TT; NATs increased over 24 weeks while total antibody remained constant or declined suggesting affinity maturation of antibody	[84]
PLGA 50:50, 3 and 100 kDa PLA, 3 and 50 kDa	6 and 60 μm 9 and 60 μm	Pulsed with high MW polymers; initial pulse then continuous with low MW polymers	Mice, s.c.	TT aggregation and partial loss of antigenicity was observed due to organic solvent and lyophilization; TT antigenicity enhanced by stabilizers; MS enhanced titers compared to soluble TT and were similar to alum-TT; titers from pulsed and continuous systems similar; reason for 'low' titers may be due to poor TT stability	[85]
PLGA 50:50, 12 kDa PLGA 75:25, 17 kDa PLA, 130 kDa	< 15 and > 20 μm < 15 and > 15 μm 32–70 μm	Pulsed release as a result of combination of formulations	Mice, s.c.	CTLs induced by one dose of combination of MS comparable to three doses TT-alum; combination of MS that gave pulsed release in vitro did not produce higher titers than any one preparation alone or one dose TT-alum; no boosting effect seen with additional doses of alum-TT (initial dose may have been too high)	[19]
PLGA 50:50, i.v. 0.33 dL/g PLGA 75:25, i.v. 0.80 dL/g	111 μm 128 μm	Pulses at 0, 21 days Pulses at 0, 53 days	Not applicable	TT entrapped in mineral oil core and surrounded by shell of PLGA; PLGA used as shell material determined onset of second TT pulse; > 92% of encapsulated TT released in active form	[82]
PLGA 50:50, i.v. 0.65 dL/g	50 μm	Initial pulse, then tapered	Not applicable	Gelatin and human serum albumin used to stabilize TT; active TT released for approximately 2 weeks	[73]

MS = microspheres; s.c. = subcutaneous injection; i.v. = inherent viscosity; lactic acid in polymers is D,L-lactic acid unless otherwise stated.

that it and diphtheria toxoid are sensitive to moisture-induced aggregation via formaldehyde-mediated cross-linking [76]. Using the oil-based PLGA microcapsules (OPM), Sanchez et al. observed an in vitro pulsed release of TT detected by ELISA at approximately 3 weeks (PLGA 50:50, inherent viscosity 0.33 dl/g) and 7 weeks (PLGA 75:25, inherent viscosity 0.80 dl/g), depending on polymer composition and molecular weight. Furthermore, greater than 90% of the TT released over a 63 day period was detectable by ELISA, suggesting that TT retained at least part of its structural integrity. No animal experiments were performed. In an earlier study, TT-containing microspheres were tailored to produce a priming antigen dose released over the first few days after injection followed by two 'boosting' doses released after 1 and 3 months, respectively, in order to mimic conventional vaccination schedules [83]. The pulsatile release pattern was

achieved by using fast releasing particles (i.e., PLGA 50:50) of a size in which many particles are susceptible to macrophage phagocytosis (1–15 μm) and which contain a relatively high TT loading (14.2 $\mu\text{g}/\text{mg}$ microspheres), combined with larger (10–60 μm) microspheres made up of slower degrading PLGA 75:25 with a relatively low TT loading (3.1 $\mu\text{g}/\text{mg}$). In vitro release studies revealed that the primary dose results from the burst release of both the PLGA 50:50 and PLGA 75:25 microspheres. The first 'booster' (second release phase), which came 3–5 weeks later, was due to release from the fast releasing PLGA 50:50 microspheres. The second booster was provided by the larger PLGA 75:25 microspheres and occurred approximately between weeks nine and eleven.

A continuous antigen release pattern for TT from PLA or PLGA microspheres has also been investigated [84,85]. In each of these studies, mice immun-

ized with microencapsulated TT produced enhanced anti-toxin neutralizing antibody titers compared to mice immunized with soluble TT for over 24 weeks. Neutralizing IgG antibody titers (evaluated by the toxin neutralization assay) increased with time, while total anti-toxin IgG (evaluated by enzyme-linked immunosorbant assay, or ELISA) remained approximately constant or decreased with time. The difference in the *in vivo* responses obtained by ELISA and neutralization tests was explained in terms of a progressive affinity maturation of the tetanus antibodies. This phenomenon correlates with *in vitro* release studies, which show an initial burst of antigen followed by a decreasing TT release rate over time. Furthermore, in one of these studies [84], TT-microspheres made of PLA (molecular weight 3000) with a small size and rapid release kinetics *in vitro* (average size 9 μm , 50% released within 10 days) were compared with TT-microspheres made of PLGA (50:50, molecular weight 100 000) with a larger size and slow release kinetics *in vitro* (average size 50 μm , 30% released at day 30). Mice receiving TT in the fast releasing PLA formulation produced higher toxin neutralizing antibody titers at earlier times (e.g., 4 weeks) than mice receiving the slow antigen releasing PLGA formulation. This behavior correlates with the faster initial *in vitro* release kinetics from these formulations, and the theoretical provision of an early high antigen load *in vivo*. A combination of fast and slow releasing microspheres was not evaluated. In these studies, however, a large percentage of encapsulated TT had lost its antigenicity prior to being released. The authors suggested that stabilizing TT for encapsulation in microspheres may, therefore, enhance the adjuvanticity of PLGA microspheres. In a subsequent study, gelatin or human serum albumin (both pharmaceutically acceptable excipients) were used as stabilizers for TT in PLGA microspheres [73]. Both excipients improved the stability of TT, however, gelatin was preferred as an excipient due to the possibility of generating an autoimmune response against HSA when it is delivered from PLGA microspheres. TT that was detected by an enzyme-linked immunosorbant assay (ELISA) was released over a two week period *in vitro* as an initial pulse followed by slow, tapered release (see Table 3).

The encapsulation of other antigens, including model antigens such as BSA and ovalbumin, toxoids

such as diphtheria toxoid and staphylococcal enterotoxin B toxoid, birth control antigens, malarial antigens and viral antigens (including antigens from HIV-1) have also been encapsulated into polymer microspheres for single-dose vaccine development (for review see [5]). Table 4 summarizes some of these studies and highlights their most important findings. Recently, Cleland et al. have published a series of papers [41,86–88] describing their experiences with microencapsulated recombinant glycoprotein 120, strain MN (MN rgp120), a subunit protein in development as a prophylactic vaccine for human immunodeficiency virus type 1 (HIV-1), the virus that causes AIDS. In one study [87], they performed experiments to determine whether a continuous or pulsatile MN rgp120 release would provide a superior humoral immune response to the antigen. MN rgp120 was administered to guinea pigs with implantable osmotic pumps in either a continuous or pulsatile manner [87]. This study compared different delay times between immunizations as well as the use of the adjuvant, QS-21, in the primary and secondary immunizations. It was observed that a 2 week continuous administration of MN rgp120 was less effective in eliciting an immune response than pulsatile administration, wherein the secondary immunization was provided by either a continuous infusion or a bolus injection. In addition, they demonstrated that the adjuvant, while critical to obtaining a high initial antibody titer, was not required in the secondary immunization. Therefore, it was suggested that a pulsatile-release PLGA formulation of MN rgp120, with a solution of soluble QS-21 and MN rgp120 used to resuspend the microspheres, would be preferred for an MN rgp120 single-injection vaccine.

Additional studies of MN rgp120 in guinea pigs showed the importance of the adjuvant and immunization schedule on the peak immune response and the maintenance of long-lasting high antibody titers [88]. By using an adjuvant that provides a high initial antibody titer, the persistence of high levels of antibody was increased, resulting in greater antibody titers at later times. In addition, as the time interval between immunizations (primary and secondary) increased, the peak antibody titers were increased suggesting that an ideal pulsatile release formulation for MN rgp120 should have a long lag phase prior to the second release phase.

Table 4

Some examples of microsphere based single-dose vaccine systems with non-tetanus toxoid antigens

Antigen	Polymer(s)	Mean microsphere size	In vitro release profile	Animal and route	Comments	Ref.
BSA, γ -globulin, ribonuclease A	Ethylene-Vinyl Acetate (EVAc) (EVAc is non-degradable)	0.3 mm ³ pellets	Initial pulse, then continuous	Mice, s.c. implant	BSA antibody titers equivalent to two doses of antigen in CFA over 26 weeks; tolerance not induced by continuous release of various antigens	[17]
BSA	EVAc	5 mm i.d. by 3 mm thick pellet	Initial pulse, then continuous	Rabbits, s.c. implant	BSA antibody titers equivalent to two doses of antigen in CFA over 24 weeks; EVAc implant does not exert its adjuvant effect via inflammation	[151]
BSA	Poly(CTTH iminocarbonate)	0.5 mg pellet containing 50 μ g BSA	Continuous then tapered	Mice, s.c. implant	Tyrosine-based polymer gave rise to significantly higher titers than polymer without tyrosine; increased titers attributed to adjuvant-activity of di-tyrosine monomer	[94]
Ovalbumin	PLGA 50:50, 34 kDa	5.34 μ m	Not reported	Mice, i.p. primary, s.c. boost	Single emulsion method used; single dose MS superior to single dose in CFA over 12 weeks, similar to two doses in CFA	[152]
SEB	PLGA 50:50 (MW not disclosed)	1–10 μ m and 20–50 μ m	Not reported	Mice, i.p., oral, i.t.	Titers superior to soluble toxoid; mixture of MS with different sizes gave synergistic response; 3 oral doses gave rise to circulating and mucosal antibodies (gut, saliva, lung); oral and i.t. boosting with MS superior to soluble SEB	[109]
Diphtheria toxoid (DT)	PLA, 49 kDa	30–100 μ m	Initial pulse then continuous	Mice, s.c.	88% of encapsulated DT could be detected by ELISA; titers equal to three doses DT-CaPO ₄ (dosed on days 0, 30, and 60) over 75 days	[153]
MN rgp120	PLGA	20–100 μ m	Pulsed	Guinea pigs, s.c.	In vivo autoboot gave rise to enhanced titers including virus neutralizing titers; addition of soluble QS-21 to MS formulation resulted in dramatically enhanced titers; titers far superior with single-dose MS than with three doses on alum	[86]
HSD-DT	PLGA 65:35 PLA (MW not disclosed)	5–90 μ m	Continuous	Rats and monkeys, i.m.	Single dose PLA or PLGA MS equivalent to three doses on alum in rats; mixture of PLA and PLGA MS equivalent to three doses alum in monkeys	[77]
MN rgp120	PLGA 50:50, 34 kDa	0.37–0.50 μ m	Not reported	Mice, i.p., s.c., i.n.,	MS induced consistent HIV-specific CD4 ⁺ and CD8 ⁺ (CTL) T cell responses, indicating that gp120 fragments were presented on both MHC I and II molecules; levels of CTL induction were significantly higher than with soluble gp120 when given i.p., s.c., or i.n.; CTL activation demonstrated in vitro as well	[20]
Various peptide antigens	PLGA 50:50	0.45–0.60 μ m, 1.21–3.20 μ m, 6.24–32.1 μ m	Not reported	Mice, i.p., s.c.	High levels of CD8 ⁺ , MHC class I restricted, CTL activity induced by single or multiple injections of MS; induced CTLs could recognize virus-infected cells; small MS better than large at inducing CTLs	[21]

MS, microspheres; i.v., inherent viscosity; lactic acid in polymers is D,L-lactic acid.

Antigens: BSA, bovine serum albumin; SEB, staphylococcal enterotoxin B toxoid; HCG-DT, human chorionic gonadotrophin conjugated to DT.

Route of administration: s.c., subcutaneous; i.p., intraperitoneal; i.t., intratracheal instillation into lung; i.m., intramuscular; i.n., intranasal.

To successfully develop PLGA vaccines for MN rgp120 as well as other subunit antigens, the second phase of release may need to vary in time from 1 to 6 months, or greater, and the protein released must

usually maintain its native conformation to elicit a neutralizing antibody response. As shown in Table 1, the timing of the second release phase is primarily dependent upon the polymer molecular weight and

the polymer composition (ratio of lactide to glycolide). By proper selection of the polymer, an *in vitro* autoboot of native MN rgp120 was achieved at times ranging from 1 to 6 months [41]. In each case, the peak antibody titers were increased by the addition of soluble MN rgp120 and QS-21 in the primary immunization. When the more rapid release formulations (i.e., 1 month autoboot) were administered as two separate immunizations greater than 2 months apart, the neutralizing titers achieved in guinea pigs [86] and baboons [89] were the highest titers achieved for MN rgp120 with any adjuvant or immunization schedule.

7. Combination preparations: Antigen-microspheres plus adjuvants

The mechanisms by which adjuvants work are diverse and still incompletely understood. However, it is known that adjuvants often exert their effects by direct immunostimulation (specific and non-specific), by providing a depot for antigen, or by enhancing the presentation or efficiency of presentation of antigen to immunocompetent cells. Many adjuvants possess one or more of these qualities. It is also not surprising that those adjuvants that possess more than one of these qualities are often relatively potent. An example of such an adjuvant is Freund's complete adjuvant (CFA), consisting of an aqueous solution of mycobacteria and antigen emulsified into paraffin oil using arlacel A as an emulsion stabilizer [90]. The three components of CFA (mycobacteria, paraffin oil and arlacel A) combine to cause a vigorous, non-specific inflammatory response at the site of injection while the emulsion serves as a depot for antigen. The inflammation is typically intense, often leading to abscess formation and pain. As a result, CFA is not used in humans and its use in animals is increasingly discouraged. However, because CFA combines at least two modes of adjuvanticity, it is not surprising that it has long been considered the gold-standard of adjuvants, and historically has been used as a tool for antibody production in animals. In an attempt to mimic the sustained adjuvant-action of CFA without the severe toxicity, a few groups have reported on the optimization of controlled release systems for vaccine

delivery by utilizing a combined microsphere-plus-adjuvant approach.

One study investigated the pulsatile delivery of QS-21, an adjuvant of the saponin family [91], from PLGA microspheres [86]. These studies revealed that the second phase of release for QS-21 occurred at the same time as MN rgp120 incorporated in the same PLGA formulation (coencapsulation of QS-21 and MN rgp120, or encapsulation of QS-21 alone). This result was unexpected because QS-21 is a much smaller molecule (~1200 Da) and was therefore expected to diffuse out of the microspheres more rapidly than MN rgp120 (~104 000 Da). However, it was subsequently determined that QS-21 was incorporated into microspheres as a micelle having a similar hydrodynamic diameter to MN rgp120 (~12 nm). In addition, QS-21 was found to be more stable in the microspheres than in solution at 37°C. This result was explained by its increased stability in the micellar form [92]. It may be possible to apply this approach to other surfactant-like adjuvants to achieve a pulsatile release from PLGA microspheres over long times *in vivo*. In many cases, however, the adjuvant may not be required in the secondary immunization, as observed for QS-21 and MN rgp120 [88].

For other adjuvants and adjuvant/antigen combinations, a continuous release from PLGA microspheres may be optimal. Continuous adjuvant release was observed for *N*-acetyl muramyl-L-alanyl-D-isoglutamine (muramyl dipeptide, abbreviated MDP). MDP is the minimal chemical structure from the mycobacteria cell walls used in CFA that still maintains all the adjuvant activity of the parent compound [12]. MDP is pyrogenic when administered as a bolus, however, its pyrogenicity can be significantly decreased by encapsulating it into polymer microspheres [18]. MDP has been encapsulated in a 65:35 lactide:glycolide PLGA (0.2 dl/g, Medisorb Technologies) using the double emulsion solvent evaporation method [88] and continuously released *in vitro* over a period of 46 days (unpublished data). The immune response to this type of continuous adjuvant release depot has not yet been assessed in animal models, but may provide a mechanism for improving existing vaccine adjuvants.

Finally, Stevens et al. have used microencapsulated nor-MDP (a non-pyrogenic derivative of MDP [12]) as an adjuvant for microencapsulated human

chorionic gonadotrophin conjugated to diphtheria toxoid (HCG-DT) in the development of a birth control vaccine [15]. High levels of HCG-specific antibody titers were achieved that lasted greater than one year when a combination of fast-, medium- and slow-degrading PLGA microspheres were used to co-deliver HCG-DT and nor-MDP to rabbits. The antibody titers obtained with antigen-microspheres plus adjuvant-microspheres were higher and lasted longer from a single injection than with any other of a wide variety of adjuvants that they evaluated.

The take home message from these studies, and others [93], is that the addition of soluble or microencapsulated adjuvant helps to produce greater antibody titers at early times, which can then be sustained for long periods of time by the continuous antigen stimulation from slow-release polymer microspheres.

8. Polymers with built-in adjuvants: Tyrosine-containing polymers

As discussed previously, antigens delivered via lactide/glycolide polymers often require the addition of an adjuvant to be maximally effective in initiating a protective immune response [15,86]. In addition, the use of PLGA for the delivery of antigens may be difficult to implement clinically and market due to the numerous patents that cover the delivery of proteins and vaccine antigens from PLGA polymers, including microspheres (for a review see [39]). In order to overcome these and other problems with PLGA microspheres, research has been directed toward the development of polymers designed specifically for vaccine delivery that would not require the addition of a soluble adjuvant. Toward this end, efforts have been made to develop polymers with adjuvants built directly into their polymeric backbone, to be released concomitantly with antigen as the polymer degrades.

There are many polymers with known adjuvant-activity (for a review see [16]). However, it was not until the mid-1980s that Kohn et al. described the synthesis of the first polymer with an adjuvant built into its polymeric backbone that was also capable of the controlled release of model antigens for long periods of time (weeks) [94]. They synthesized polyiminocarbonates based on L-tyrosine derivatives

[95] due to the inherent ability of L-tyrosine and many of its derivatives to stimulate a potent immune response to adsorbed antigens (for a review see [16]). N-benzyloxycarbonyl-L-tyrosyl-L-tyrosine hexyl ester (CTTH) [94], a dityrosine derivative, was used as the repeat unit of the polyiminocarbonate for immunization studies because it was found to have strong immunostimulating properties when used as an adsorbate for a model antigen, BSA. It was shown that vaccine release from poly(CTTH iminocarbonate) implants leads to enhanced levels of humoral immunity compared with release from a similar polyiminocarbonate which is not based on a tyrosine derivative, even though the two polymer implants were identical in size, shape, and exhibited identical *in vitro* antigen release rates [94] (see Table 4). Subsequently, Kline and Langer studied the use of tyrosine-based polyiminocarbonates of varying degrees of hydrophobicity in an attempt to enhance the adjuvanticity of the polymeric device [96]. Several iminocarbonate polymers, each made with a different dityrosine derivative, were used to release BSA from implant devices to immunize mice. The antibody titers obtained were invariably greater than multiple doses of soluble antigen and were, in many cases, comparable to titers obtained by immunization with BSA in CFA.

Although these studies had shown the benefits of using tyrosine-containing polymers for vaccine delivery, the polyiminocarbonates synthesized were low molecular weight, brittle polymers which could not be used to fabricate microspheres in a reproducible manner. In addition, the dityrosine iminocarbonates eroded much more slowly than they released antigen; typically, greater than 90% of the polymer remained at the injection site in mice after one year, a time when all antigen had been released [96].

To circumvent the problems with brittle and slowly-eroding poly(CTTH iminocarbonate), a new generation of tyrosine-containing polymers were sought which would allow the preparation of microspheres in a reproducible manner, and which would also exhibit increased erosion rates. Polyanhydride copolymers based on sebacic acid (SA) and 1,3-bis(carboxyphenoxy)propane (CPP) are versatile drug delivery vehicles, having been used for years to deliver a variety of drugs from proteins to low molecular weight chemotherapeutic agents [33,34]. CPP:SA copolymers are also currently used clinical-

ly to deliver BCNU locally within the brain to treat patients with brain tumors [33]. They have a history of safe use in humans and animals [33,97], and their erosion rate can be varied from hours to years depending on the ratio of SA to CPP in their backbone [98]. In addition, CPP:SA polymers are known for their ability to deliver drugs at the same rate as they erode [98]. These polymers were therefore modified for vaccine delivery by incorporating tyrosine directly into their backbone via an imide bond, resulting in tyrosine-containing poly-(anhydride-*co*-imides) [99]; see Fig. 1. Hanes et al.

synthesized poly(anhydride-*co*-imides) using various levels of three monomers: trimellitylimido-L-tyrosine (TMA-Tyr), SA and CPP. Various levels of CPP and SA were incorporated into the polymeric backbone to allow for long-term antigen delivery and to improve the polymer molecular weight and solubility in organic solvents, respectively. A systematic series of studies was performed to optimize terpolymer synthesis, resulting in polymers with weight average molecular weights in excess of 80 000 Da. Polymers with molecular weights in excess of 17 000 Da were synthesized that contained greater than 50% TMA-

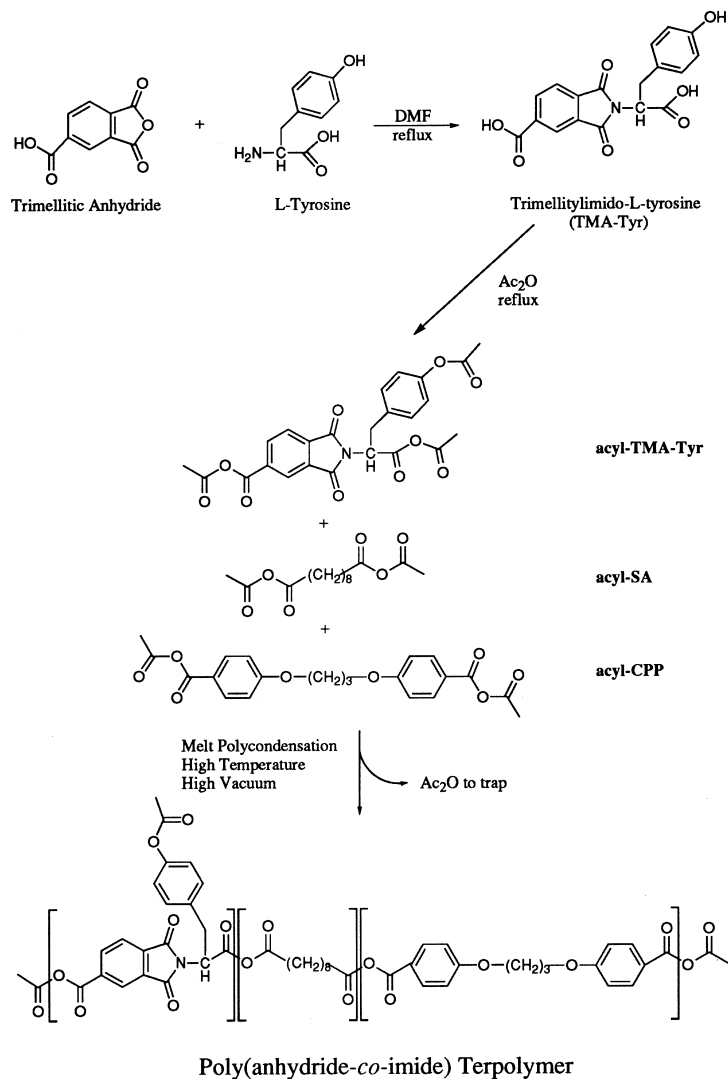


Fig. 1. Synthesis scheme of tyrosine-containing anhydride-*co*-imide terpolymers (Ac_2O = acetic anhydride). From [99] with permission.

Tyr in their backbone. The production of terpolymers with high molecular weights and various monomer ratios provided a series of polymers capable of a wide range of degradation and antigen release properties. Copolymers of TMA-Tyr, CPP and SA were shown to have a random distribution of the three monomers throughout their polymeric backbone. Furthermore, after an initial drop in M_w , the polymers showed good stability for long times (greater than 20 days) at room temperature in the solid state, making them potentially useful as carriers for antigens in regions of the world where refrigeration is difficult or expensive to maintain.

TMA-Tyr:SA:CPP terpolymers have subsequently been used to encapsulate BSA into injectable microspheres with high efficiency [100]. The microsphere surface morphologies were similar to those typically obtained using PLGA. Subsequently, Chiba et al. have shown that BSA release times from TMA-Tyr:SA:CPP terpolymers could be varied from a few days to greater than one month by varying the polymer composition (increased levels of CPP corresponded to prolonged protein delivery times) and the protein loading level (higher levels of protein in microspheres lead to increased rates of release) [101]. Release patterns of antigen consisted of an initial burst of protein followed by a slowly decreasing release rate over time, an antigen release profile that may mimic that due to natural infection [102]. This type of antigen release pattern has been found to be effective with several antigens in animal models (see Tables 3 and 4; for a review see [103]). Furthermore, polymer erosion and antigen release occurred simultaneously [38,101]. Initial biocompatibility studies of poly(TMA-Tyr:SA:CPP), with high percentages of all three monomers (40% TMA-Tyr, 30% SA, 30% CPP), in rats indicate that the terpolymers may also be safe [38].

Polyphosphates of a variety of dityrosine and serine-tyrosine monomers have also been synthesized and used as adsorbates for a soluble antigen extracted from *Schistosoma japonicum* [104]. The degradation rates of 11 mm i.d. \times 1.5 mm thick compression-molded polymer discs at physiological pH varied from less than a week to much longer, depending on the choice of blocking groups. Particulates with adsorbed antigen (mean particle diameter 10 μm , made using a colloidal mill prior to antigen adsorption) showed adjuvant activity com-

parable to CFA. No attempt was made to produce an antigen controlled release system, such as a microsphere.

9. Mucosal immunization with antigen-microspheres

Microspheres have also shown potential as carriers for the delivery of vaccines to the mucosal surfaces of the body, especially the intestine and lung. Reviews covering the use of microspheres for oral vaccine delivery have been published recently [5,105,106]. Microspheres have received a great deal of attention as antigen carriers for oral administration due to their reported ability to protect the antigen from denaturation by bile salts, low pH and high levels of degradative enzymes in the gut and intestine [5,105,106]. Several groups have shown that microencapsulated vaccine can elicit production of secretory IgA, circulating antibody including IgG, or both much more efficiently than soluble antigen. A few groups have reported that microspheres of the appropriate size ($< 10 \mu\text{m}$) and surface characteristics (hydrophobic) are taken up from the GI tract by the Peyer's patches in the intestine (for a review see [107]). Microencapsulated vaccines generally result in greatly increased mucosal as well as systemic immune responses over soluble antigen [108,109], however, particulate uptake from the GI tract is still quite low with PLGA systems-typically less than a few percent [105,110].

Several studies involving the use of antigen-microspheres instilled into the lungs of animals as a liquid solution via an intratracheal tube have shown the effectiveness of pulmonary immunization with microspheres in eliciting a potent mucosal immunity [109,111–113]. However, although these studies have shown several potential benefits of pulmonary immunization with antigen-microspheres, it is obvious that intratracheal instillation is not an appropriate method of administration in humans. As a result, methods of producing an efficient aerosol of PLGA microspheres must be developed.

Because PLGA and other polymers degrade in water, dry powder formulations may have advantages over liquid formulations for microsphere aerosols. Recent studies have shown that dry-powder PLGA microspheres with exceptionally large aerosol

size (e.g. 5–15 μm) can provide an efficient means of macromolecule delivery via the lungs if the particles are made to be sufficiently light (i.e., porous particulates) [114,115]. Greater than 50% of the porous particles made it into the lung of rats in aerosolization studies [115]. Coating of the surfaces of PLGA microspheres with a surfactant molecule endogenous to the lung, dipalmitoyl phosphatidylcholine (DPPC), lead to further improved in vitro and in vivo aerosol fractions that make it into the deep lung [114]. Furthermore, they showed that insulin, a model macromolecule, was delivered to the systemic circulation for four days following aerosolization of insulin-PLGA microspheres into rat lungs. Insulin retained its biological activity as determined by its detection in rat serum using an enzyme-linked immunosorbant assay (ELISA), and as confirmed by serum glucose levels in treated animals which were reduced to approximately 25% of controls for 96 h following microsphere inhalation. Control rats received an identical dose of the same insulin-microspheres via subcutaneous injection (s.c.). The relative insulin bioavailability ($= \text{AUC}_{\text{inhaled}} / \text{AUC}_{\text{SC}} \times 100\%$) was 87.5%. By comparison, bioavailability (v. s.c. injection) of insulin administered to rats as an inhalation liquid aerosol using a similar aerosolization method was reported as 37.3% [116]. The longest previous sustained insulin release reported (6 h) was achieved using liposomes instilled into rat lungs as a liquid formulation, yielding an absolute insulin bioavailability of 30% [117].

10. Comment on animal models for single-step immunization

In testing any single-dose vaccine system versus multiple-injection protocols using standard adjuvants, it is important to use appropriate animal models and antigen/adjuvant doses. Ideally, an animal model that responds to the antigen in a similar manner to the human immune response, and, if possible, an animal model that can be used to demonstrate protection from infection with the human form of the pathogen should be used. This issue is particularly important for controlled release vaccines since some animal models (often times mice) have a very slow rate of antibody decay after only one or two immunizations with many antigens,

thereby masking any additional immunization from the continuous or pulsatile delivery of the antigen (for a review on antibody decay and animal models see [118]). Mice are acceptable assuming that antibody titers increase upon administration of additional doses of antigen (\pm adjuvant). One should be cautious, however, since mice will often produce high antibody levels following a single injection of antigen/adjuvant that are not elevated upon boosting [19,119]. Usually, studies must be performed in non-human primates prior to human clinical trials to demonstrate the safety and pharmacokinetics of the dosage form. These studies also provide additional information on the immune response to antigen.

11. Cytokine delivery for tumor vaccines

Cytokine administration can cause side-effects typically not considered acceptable with traditional vaccines. However, patients with cancer may be willing to accept these side effects in the context of a tumor vaccine. Vaccines and cytokine-therapy have been two of the most actively investigated areas in cancer immunotherapy. These two approaches developed rapidly in the past, but virtually without intersection [120]. Recent understanding that the generation of an anti-tumor immune response requires both tumor-specific antigens and appropriate costimulatory signals has led to strategies seeking to combine these two modes of therapy in an attempt to produce a potent tumor-specific systemic immunity.

The cloning of the gene for interleukin-2 (IL-2), and subsequent production of recombinant human IL-2, has opened the door to its use for cancer immunotherapy in humans. IL-2 has a number of activities associated with it, including the enhancement of T cell growth and T cell activation. IL-2 is also a known natural killer cell (NK) activation factor. Initial studies have utilized pharmacologic doses of IL-2 administered systemically [121] to treat patients with metastatic kidney cancer and melanoma. The response rate to systemic IL-2 in these patients has been roughly 20% [122]. However, systemic toxicity is often severe and most patients cannot finish the recommended course of therapy.

It has been argued [120] that the major conceptual problem with systemic cytokine administration is that lymphokines are known to maintain the spe-

cificity of immunologic responses locally, as a result of their paracrine function. That is, under normal physiologic conditions, appropriate lymphokines are produced in high concentrations local to the site of antigen where they serve as the necessary costimulatory signal for induction of an appropriate immune response. This paracrine function is ignored when cytokines are administered systemically. Furthermore, it is impossible to achieve high local concentrations of systemically-administered cytokines such as IL-2 without prohibitory toxicity.

A more promising approach that has been recently pursued to treat both systemic-(for review see Pardoll [120]) and brain-tumors [123–125] is the use of tumor cells genetically engineered to secrete high levels of cytokines. The first cytokine genes to be introduced into tumor cells were IL-2 [126,127] and IL-4 [128,129]. Since then, several cytokines have been introduced into tumor cells and their effect on tumor rejection and immunologic memory tested. The result is usually the production of a vigorous inflammatory response, specific to the cytokine used, that typically results not only in the complete destruction of the transduced tumor cells, but often in the induction of immunologic memory capable of protecting the treated animal against a subsequent tumor challenge at a distant site [120]. However, the use of genetically engineered cells for cytokine production is technically difficult, time-consuming and labor-intensive, and the product is often not reproducible.

Recently, microspheres have been used as a technically simple alternative to using transduced cells for local cytokine production [130]. The use of polymer microspheres mixed with irradiated tumor cells eliminates the need to transfect each individual patient's tumor cells with cytokine genes. In addition, cytokine-microspheres can provide a much more controlled and reproducible product, including a tight control over crucial parameters such as total cytokine dose and delivery period not possible with transduced cells. Furthermore, cytokines can be delivered for longer times from microspheres than from transduced and irradiated cells which are rapidly destroyed in vivo. Preliminary experiments have shown that cytokine-microspheres mixed with irradiated tumor cells are at least equally effective as cytokine-transduced tumor cells in B16-F10 systemic- [130] and metastatic-melanoma brain-tumor models [131].

12. Challenges and future directions

12.1. Importance of release pattern for specific antigens (continuous and pulsatile)

Although a great deal of progress toward the development of polymeric controlled release systems as vaccine carriers has been made, several challenges remain. Further research directed toward understanding the ideal release kinetics for various antigens is needed to improve antigen-microsphere design. There is still no consensus on what type of release profile is best for all antigens. Several groups have shown efficacy with a burst of antigen followed by continuous antigen release (see Tables 3 and 4), while others have shown that a pulsatile antigen release is optimal for their particular antigen [87]. Studies utilizing controlled release osmotic pumps, like those described for MN rpg120 [88], are recommended before initiating the design of a PLGA microsphere system for a new antigen.

12.2. Improving the stability of liposomal vaccine preparations

Another area of future research is the use of microencapsulation technology to enhance liposome effectiveness by improving their in vivo stability following parenteral or oral administration. Liposomes containing antigens have been touted as suitable for parenteral vaccine delivery due to their propensity to be phagocytosed by macrophages in vivo [132,133]. However, their rapid phagocytosis is both a blessing and a curse when single-dose vaccines are desired. Rapid liposome phagocytosis results in a fast and efficient immune response to encapsulated antigen, however, it also leads to the absence of the long-term antigen depot [134] necessary to sustain the immune response for long times. In one approach to enhancing liposome stability in vivo, a model antigen (BSA) was loaded into liposomes that were subsequently encapsulated into a polyphosphazene microsphere matrix [135], thereby protecting them from rapid destruction in vivo. The negatively charged polyphosphazene microsphere matrix was further stabilized by coating it with positively-charged poly(L-lysine). Fifty days after injection into rats, no radio-labeled bovine serum albumin (BSA) was detected at the injection site when liposomes alone were used as the carrier. On

the other hand, almost 50% of the model vaccine was recovered on day 50 when the liposomal formulation was microencapsulated before injection. The increased antigen retention time correlated with the higher and longer lasting anti-BSA antibody titers seen with the microencapsulated liposome (MEL) formulation. At their maximal levels, antibody titers were 3–4 times higher with MELs than with non-encapsulated liposomes, and 2–3 times higher than with BSA in CFA. Liposomes polymerized via their lipophilic tails have also been developed in an attempt to stabilize antigen-liposomes for transit through the GI tract for mucosal immunization (for a review of microencapsulated liposomes and polymerized liposomes see [136]).

12.3. *Improving efficiency of oral immunization*

Another significant challenge is the further development of the mucosal route for vaccine administration [105]. Of particular concern is the poor efficiency of antigen-microsphere uptake in the gastrointestinal (GI) tract; typically less than a few percent of particulate systems such as microspheres are transported from the lumen into the Peyer's patches. Future vaccine systems for oral administration may be developed using novel strategies such as bioadhesive polymers. An appropriately designed bioadhesive polymer may, for example, adhere to the lumen of the intestine, preferably near the Peyer's patches, thereby increasing the bioavailability of vaccine through enhanced particle uptake or increased uptake of released vaccine [137]. The molecular and physicochemical properties found to be important in influencing the bioadhesive performance of polymers in the GI tract have been previously reviewed [138]. Alternatively, microspheres made of non-bioadhesive polymers may be linked to specific targeting ligands (e.g., anti-epithelial cell antibodies [139,140] or lectins [141]) to bring about an intimate and extended contact between vaccine vehicle and intestinal wall. In another approach, vaccine delivery systems may be designed which target and stimulate receptors on M cells to increase particle uptake by mechanisms such as phagocytosis [142]. M cells are located on the dome epithelium of the Peyer's patches where they function as gatekeepers to the mucosal immune system, delivering antigens across the epithelium to lymphocytes and macrophages and exporting secretory antibodies for mucosal defense.

The discovery of a molecular component unique to the membranes of M cells, which may then serve as a receptor, may lead to the possibility of targeting vaccine vehicles directly to M cells. This approach seems feasible since certain pathogenic bacteria and viruses are known to adhere selectively to M cells [143–148].

Recently, polymerized liposomes bearing functional lectin molecules were used to enhance liposomal particulate uptake by the Peyer's patches in the GI tract of mice [141]. Significantly higher levels of lectin-modified polymerized liposomes (up to 10.5% efficiency) were taken up from mice intestines *in vivo* compared to unmodified, 'lectin-free' polymerized liposome controls (3.2%). Peyer's patch targeting correlated with increased levels of particulate uptake from the GI tract, suggesting that lectin-modification promotes liposome binding to, and uptake into, the Peyer's patches.

Systems which target sites other than the GI tract may also prove useful and more efficient for the mucosal delivery of vaccines in the future. Among these types of systems are nasal [149] and intravaginal [150] delivery systems which make use of bioadhesive polymer microspheres, and polymer microspheres administered to the lung [109,111–113]. Among these options, pulmonary immunization with PLGA microspheres instilled into the lungs as a liquid formulation has shown considerable promise in inducing high levels of mucosal protection [109,111–113]. To make pulmonary microsphere delivery possible in humans, recent studies have been aimed at producing a microsphere formulation that can be readily inhaled using inexpensive, handheld dry-powder inhalers (e.g., Spinhaler[®] inhalation device from Fisons) [114,115]. They have shown that porous PLGA microspheres can be efficiently aerosolized as a dry powder into the deep lung of animals where they can sustain the release of macromolecules for days [114,115]. However, safety concerns with degradable polymer systems in the lung are yet to be addressed in this relatively new field of vaccine delivery.

12.4. *Achieving cellular immunity with microsphere preparations*

The ability to achieve cellular immunity with microencapsulated antigen has opened up a very exciting new field of research for microsphere-based

vaccines. Further research in this area will no doubt focus on the mechanisms of exogenous antigen presentation via MHC class I molecules and on achieving enhanced CTL stimulation [24].

13. Conclusions

Due to their many unique properties—including their ability to provide a long-term depot for antigen, to target macrophage phagocytosis leading to enhanced humoral and cellular immunity, and to their potential stability under warm, dry conditions—microsphere-based vaccines may eventually meet the challenge of providing immunity in a single-dose, a goal considered the ‘holy-grail’ of vaccine delivery. Poly(lactide/glycolide) microspheres are effective adjuvants for antigens delivered via the parenteral route, especially when antigen stability and ideal antigen release kinetics are achieved through judicious choice of the antigen-microsphere formulation method and materials. Microsphere-based vaccines have also shown a good deal of potential for immunization via the oral and pulmonary routes. Strategies for improved uptake of microspheres from the intestine, and for improved methods of microsphere aerosolization (such as the use of large, light particles), should make mucosal immunization with microspheres more effective in the future. The continued development of new polymers designed specifically for vaccine delivery, whose rights may be acquired by industrial vaccine manufacturers, may enhance industry’s willingness to get involved in the production of microsphere-based single-dose vaccines in addition to potentially yielding improved antigen delivery systems.

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