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Review article

Revisiting PLA/PLGA microspheres: an analysis of their potential in parenteral vaccination

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Abstract

Poly(lactide) and poly(lactide-co-glycolide) microspheres have been studied for controlled antigen delivery and immune response enhancement for more than a decade. Early developments of such vaccines were basically technology-driven, stemming from the well-established biocompatibility of these polymers in concert with their innate properties to tailor rates of bioerosion and release. More recently, other features have become equally or even more appealing, such as the adjuvancy of such microspheres and their ability to elicit cellular effector responses, so-called cytotoxic T-cell responses, in addition to antibody responses observed already in the very early studies. In this review, we intended to revisit microsphere-based vaccines designed for the parenteral route and attempted to outline major developmental issues, as well as to analyze immunological fundamentals and data associated with antigen delivery by microspheres.

Keywords: Vaccines; Adjuvants; Immunization; Immune responses; Antigen delivery; Microencapsulation

1. Introduction

A major milestone in the development of poly(lactide) and poly(lactide-co-glycolide) (PLA/PLGA) microspheres (MS) as antigen delivery system was, without any doubt, the World Health Organization (WHO) Special Programme for Vaccine Development, initiated in the late 1980s [1]. PLA/PLGA-MS were assumed to release encapsulated antigens in a continuous or pulsatile manner, thereby mimicking the repeated injections of conventional vaccination schedules. A primary target of such a single-injection vaccine was neonatal tetanus in Third World countries, which was believed to be preventable by prolonged tetanus-toxoid delivery after a single vaccination of pregnant women. Further, single-injection vaccines were desired for many other antigens to facilitate vaccination campaigns, particularly in the Third World with the often insufficient health-care logistics. Now, over 10 years later and certainly later than originally anticipated, first clinical feasibility trials, under the patronage of the WHO, with a single-injection tetanus vaccine might become reality. This would mark the end of arduous efforts of several academic and industrial groups world-wide to improve physical characteristics, antigenic stability and immunological performance of MS loaded with tetanus toxoid. Irrespective of the outcome of these humans trials, PLGA-MS are now considered of general potential for antigen delivery, being potentially relevant for conventional, synthetic and recombinant subunit antigens or DNA vaccines.

Early developments of PLGA-based vaccines were basically technology-driven, stemming from the well-established biocompatibility of these polymers in concert with their innate properties to tailor rates of bioerosion and release [2]. More recently, other features have become equally or even more appealing, such as the adjuvancy of PLGA-MS. This may appear quite intriguing for a polymer generally approved for slow-release injectables. A further most attractive quality of PLGA-MS is their ability to elicit cellular effector responses, so-called cytotoxic T-cell (CTL) responses, in addition to the antibody responses observed already in the very early studies [3–5]. This makes PLGA-MS particularly attractive to fight intracellular infections

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Abbreviations: MS, microspheres; QC, quality control; Ab, antibody; mAb, monoclonal antibody; APC, antigen presenting cells; Th1 and Th2, type 1 and 2 T-helper cells; CTL, cytotoxic T lymphocytes; MHC, major histocompatibility complex.

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through bacteria, viruses, and parasites, as well as for cancer vaccination and therapy. Apparently, microencapsulated antigen can enter into pathways of processing and presentation that induce immune responses otherwise restricted to endogenous antigens, i.e. antigens produced de novo within cells. As a consequence, PLGA-MS might even begin to acquire a role as basic tool in immunology, as this type of delivery system allows to carry various antigens, excipients and co-adjuvants in the very same dosage unit and to deliver them at the same time. Moreover, additional features of MS technology (e.g. particle size, surface properties, and vehicles for MS administration) can be varied so that they might affect the type and extent of immune response.

Much of this information began to emerge already in the early and mid 1990s and is extensively covered by several reviews [6–10]. However, one question that remains is why only few of the leading industrial players in the vaccine area became attracted by MS technology. Among other reasons, uncertain commercial potential and patentability have been frequently used arguments. We feel, however, that the scientific culture and possibly also ethical aspects should have been equally important determinants in these considerations. Two additional issues of recent vaccine developments need to be mentioned. Firstly, for many years vaccinology has been dominated by what has been called the rational structure-based design of synthetic antigens as the premier avenue of success. Under these premises, MS technology did not appeal to many vaccinologists primarily oriented toward molecular biology. In an essay-style paper, van Regenmortel [11] recently argued compellingly that immunogenicity is basically not amenable to molecular design, as it is triggered not only by molecular recognition, but also by extrinsic factors such as the individual gene repertoire, self-tolerance and a variety of cellular and regulatory mechanisms. Secondly, the advent of the encouraging prospects of DNA vaccines in the early 1990s [12] may have also contributed to the so far limited impact of MS technology on basic and applied vaccinology. However, as the constraints of naked DNA injections are progressively recognized [13], the quest to make DNA vaccines more efficient needs to be combined with a search for more efficacious DNA-delivery systems. MS technology in combination with biosensing to target professional antigen-presenting cells (APC, e.g. dendritic cells and macrophages) is currently considered a promising approach.

Under these aspects, MS technology provides a flexible platform for the delivery of various types of antigens to induce either immunogenicity or, for allergologic treatments, tolerance. In this review, we revisit PLGA-MS-based vaccines and endeavor to analyze their immunological performance. Moreover, we will hypothesize on perspectives how to design PLGA-MS as a versatile platform for antigen delivery. This review may not be appropriate for newcomers in the field. We attempted to make a synopsis of major developmental issues and immunological basics and data related to PLGA-MS for antigen delivery.

2. Quality assessment of microsphere-based vaccines

To advance the development of vaccines from discovery to marketed products, a range of pre-clinical quality controls (QC) need to be undertaken. These studies are directed towards defining the characteristics of the product, including the parameters which determine the pharmaceutical product quality, pre-clinical safety and biological action or potency in an appropriate animal model. This is important not only for establishing the biological properties of the material and evaluating its possible risks, but also for planning protocols for subsequent clinical studies from which safety and efficacy are usually evaluated. For a relevant design and interpretation of pre-clinical testing, the key concepts of immunological defence mechanisms must be understood. We therefore find it helpful to accommodate a brief reminder of the most crucial cellular and molecular steps involved in the generation of adaptive immune responses (Fig. 1). For a more extended overview, the reader is advised to consult immunological textbooks. The synopsis of Fig. 1 should be supplementary to the subsequent discussions on QC and the ensuing evaluation of the potential of microparticulate vaccines. Finally, the methods of

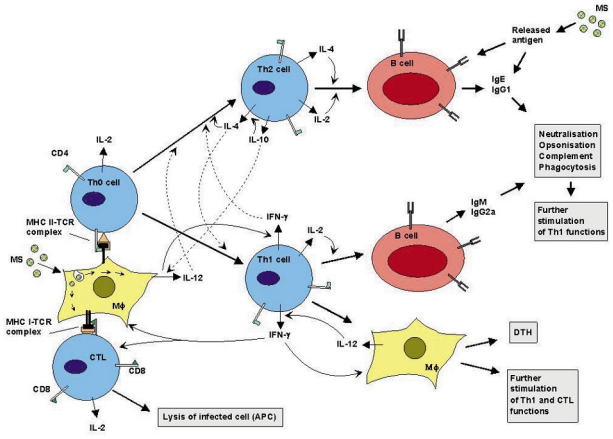

Fig. 1. Cellular and molecular interactions in the generation of adaptive immune responses. Microspheres (MS) deliver the encapsulated antigen to antigen presenting cells (e.g. macrophages or dendritic cells) via phagolysosomes or via cytosol, with the former mechanism supporting a MHC-class II-restricted immune response, and the latter a MHC-class I response. Maturation of $CD4^+$-T helper (Th) cells is a direct function of the types of cytokines secreted during the primary immune response. Typically, interleukin (IL)-4 and IL-10 (also IL-5 and IL-6) drive Th-cell polarization towards Th2 cells and related humoral mediated defence mechanism, whereas IL-12 and IFN-γ (also IL-18) support a Th1- or a cell-mediated response (solid curved feedback arrows). Th1- and Th2-associated cytokines tend to regulate reciprocally, as IFN-γ and IL-12 inhibit Th2-cell functions, while IL-4 and IL-10 inhibit Th1-cell functions (dashed curved feedback arrows). IL-2 is important for general T-cell proliferation without influencing Th-polarization. MS can release extracellular antigens which stimulate secondary immune responses through interactions with circulating or B-cell bound immunoglobulins. Very importantly, Th1-cells release IFN-γ, which co-stimulates Mϕ and cytotoxic T cells. Mϕ, macrophage; MHC, major histocompatibility complex; TCR, T-cell receptor; DTH, delayed type hypersensitivity; CTL, cytotoxic T lymphocyte.

Table 1
Methods for characterizing the most relevant pre-clinical and clinical properties of antigen-containing MS

Methods	Comments
In vitro	
Ag content in MS	No accurate methods are presently available. Antigenicity measurement is not necessarily required [21–23]
Ag release from MS in vitro	Kinetics are dependent on experimental set-up. Antigenicity measurement is not necessarily required [26]
MS size and morphology	Small MS can be more efficaciously phagocytosed by APC than large MS. The importance of morphology and surface properties are still speculative [17,30,79]
Injectability	For dosing consistency and safety, the dispersed MS must be injectable through the maximum permitted needle size, which depends on the animal species
Residual processing solvents and other components	Of special relevance for MS prepared by solvent evaporation and coacervation [31]
Sterility	Achieved by γ-radiation or aseptical procedures [33,80]
In vivo	
Safety	Absence of acute or sub-acute toxicity and allergic reactions
Administration modes	Route of administration, co-administered compounds, vehicle and animal species may influence immune responses and their interpretation. Control experiments are crucial
Immunogenicity	Induction of adaptive immune responses in vivo. An in vitro or in vivo neutralization test may be more relevant than simple Ab- or CTL quantitation
Efficacy	Adaptive protection against infectious pathogens in vivo: its determination generally requires a challenge test

QC-testing and their importance will be outlined (Table 1), and the relevance of pre-clinical data for the immunogenicity of antigen-containing MS discussed (Table 2).

2.1. Generation of immunity

Vertebrates resist infection by pathogenic organisms in several ways. In a first row of defence, innate mechanisms against infection exclude or kill infectious agents upon first contact. For those pathogens capable of establishing an infection, several early non-adaptive immune responses are crucial to moderate the spreading of the infective agent until an adaptive immune response can be elicited. Adaptive immunity takes several days to develop, as T- and B lymphocytes must encounter a specific antigen, proliferate and differentiate into effector cells (Fig. 1). T-cell dependent B-cell responses cannot be initiated until antigen-specific T cells (T-helper cells) had a chance to proliferate and differentiate. Moreover, the first appearance of a pathogen in an organism involves the recruitment of phagocytosing APC, such as macrophages and dendritic cells. These cells stimulate T cells through the interaction between antigen bound to a MHC-molecule and the T-cell receptor. Upon this contact, cytokines are secreted, which largely control the type of immune response to be generated: cellular or antibody(Ab)-mediated effector responses stimulated by T-helper (Th) type 1 (Th1) or Th2 cells, respectively.

Upon a second contact with a pathogen, immunological memory plays a crucial role. Immunological memory is indeed the basic rationale behind the concept of vaccination. However, the nature and origin of memory is still debated, with two models being pursued [14]. One model postulates the switching of cells involved in a primary response from an effector to a long-lasting memory state, which is maintained without continued stimulation by antigens. The second model envisions that memory cells need to be re-stimulated periodically. Knowledge of the relevance of either of the two models should be of utter importance for future vaccine developments. It has even been claimed that a persisting antigen might be counter-productive for generating T-cell memory, because it could push T cells into overdrive and ultimately trigger cell suicide [15]. If so, vaccines would have to be metabolized and eliminated rapidly for T-cell memory to develop. This view, however, is crossed by the steadily increasing Ab levels and maturation of Ab, as induced by certain MS formulations [16–20], which successfully induced immunity in test animals.

2.2. In vitro properties of microsphere-based vaccines

An important QC of MS-based vaccines (Table 1) is the quantification of the actual antigen load entrapped inside the particles. This has been intensely investigated, but still no simple method exists to estimate antigen content in MS [21–23]. Most methods impair antigenicity or result in inaccurate estimation of the protein content, difficulties which may hamper submission of new investigational drug (NID) applications. Further, the actual antigen content of MS might even affect the type of immune response to be induced (Table 2) [24]. High concentrations of protein antigens often induce strong Th2 primary responses, but poor affinity maturation during the secondary response. Conversely, low antigen loads in MS would require a higher amount of MS to be administered. This again might reduce the drainage of MS to secondary lymphatic tissues, although the effect on the immune response is unclear and might depend on how the organism fights infections.

Antigen release kinetics from MS is another important and critical QC aspect [25]. Intriguingly, in vitro antigen release not only depends on the properties of the actual MS formulations, but also on the experimental test system and assay method used [26]. Typically, protein adsorption to

Table 2
Hypothetical impact of relevant physico-chemical microsphere (MS) properties on the immunogenicity of microparticulate vaccines.

MS size	MS surface properties	Ag[a] content in MS	Ag[a] release kinetics from MS
MS typically < 10 μm: Uptake by APC, which enhances stimulation of primary and MHC-restricted responses (also CTL) [30]	**Hydrophobic MS**: recruit possibly more phagocytosing cells than hydrophilic MS (unpublished observations) [79,81]	**Ag type** and **amount** presented to $CD4^+$-cells during primary stimulation can determine functional phenotype (Th1 or Th2) [24]	**A short initial Ag burst release**, possibly followed by timely separated **pulses** of additional Ag doses, may be advantageous, if memory cells are long-lived [15]
MS typically >10 μm: Ag release to extracellular fluid for secondary Ab responses; Recruitment of APC for possibly direct Ag transfer into APC, or disintegration and subsequent uptake by APC	**Morphology** and **surface coatings** possibly influence interaction with phagocytosing cells [82]	**High Ag concentration** may produce Th2-responses, but poor maturation	**Continuous or pulsatile Ag release** may be appropriate, if memory cells need re-stimulation
		Low Ag content (high amounts of MS) may limit the transport rate to seoncary lymphatic tissue	

[a] Ag: antigen

surfaces in the release test system can mask some of the actual amount of antigen released and affect antigen stability [26]. This obviously sets hurdles to the QC of this type of antigen delivery system. Furthermore, different results will be produced depending on whether antigen protein or antigen activity, e.g. enzyme-linked immunosorbent assay (ELISA) activity, is measured [27,28]. However, to what extent antigenicity, i.e. the reactivity of antigen epitopes with complementary Ab paratopes, of encapsulated or released antigen is a criterion for the induction of immune responses is ambiguous, since MHC-restricted immune responses are driven by small peptides, derived from proteolytic processing and presentation of antigen by APC [29]. Consequently, the ultimate and only safe method to determine reactive antigen content and release from MS appears to be through direct measurement of immunogenicity (but not antigenicity) in vivo.

Physical characteristics of MS such as morphology, porosity and size distribution are investigated primarily as a QC of batch-to-batch consistency. Some of these properties can also influence antigen release kinetics, MS injectability and interaction with APC (Tables 1 and 2). Porous particles can produce a higher initial antigen release burst (so-called burst release) and a relatively continuous release of entrapped antigen as compared to dense particles, which are likely to give a low burst and a pulsatile release pattern. It has further been shown that smaller particles (typically below 10 μm) are more readily taken up by phagocytic APC than larger particles (typically above 10 μm) [30]. Therefore, small MS might stimulate more efficaciously primary responses or T-cell mediated immune response in general. On the other hand, larger particles can provide an extracellular depot for secondary immune responses by way of Ab- or B-cell stimulation. Furthermore, fragments of disintegrated MS or released aggregates of antigens may possibly even contribute to stimulation of T-cell mediated responses [17]. To what extent B cells can phagocytose MS and present the incorporated antigens, has not yet been investigated. Finally, the size, morphology and surface charge of the particles are, in addition to the dispersing vehicle, important for the injectability of MS. Typically, MS should readily pass injection needles with an inner diameter of 0.6 mm, when small animals have to be immunized by i.m. or s.c. routes.

Commonly, MS are fabricated by well-established technologies such as spray-drying, coacervation, or W/O/W-double emulsion solvent evaporation. Determination of residues of processing compounds such as residual polymer solvents (e.g. methylene chloride, ethyl esters) and polymer non-solvents (e.g. silicon oil, alkanes), and emulsion stabilizers, such as poly(vinyl alcohol) or surfactants is a further QC issue [31]. For some of these materials, maximum residual levels are defined by pharmacopoeias; for others, however, toxic effects must be excluded.

Finally, a parenteral MS-based vaccine must be sterile. As heat sterilization of the product in its container is not possible, due to the low glassy-to-rubbery state transition temperatures of PLA/PLGA, sterile products must be produced through γ-irradiation or aseptical processing in clean-room or isolator facilities. However, γ-irradiation is not permitted in several countries and can be detrimental to antigens [32] and polymers [33], due to chain scission, cross-linking and generation of living radicals [34]. On the other hand, antigen microencapsulation under aseptic conditions requires very laborious and costly validation work.

2.3. Animal testing of microsphere-based vaccines

Studies for determining pre-clinical safety and biological potency require appropriate animal models (Table 1). This is important not only for establishing biological activities of the product and evaluating its potential risks, but also for planning protocols for subsequent clinical studies from

which safety and efficacy in humans are eventually determined. Evaluation of new products at the pre-clinical stage will also guide the development of subsequent QC tests during production and post-licensure control of product consistency.

Immunogenicity is generally assessed in animals by monitoring the induction of Ab response. However, depending on the target infectious agent, the relevance of the type of immune response measured needs to be considered (Table 3). Is the mechanism known by which the infectious agent is eliminated? If so, is protection achieved by humoral, cellular or combined immune responses? Is the animal model adequate? Typically, different animal strains may show qualitative differences even within a species, BALB/c mice being a typical Th2 and C57BL/6 a typical Th1 strain [35]. Finally, does the vaccine formulation provide protection, and does the method applied to monitor immunogenicity correlate with the efficacy of the vaccine?

Ab are the important determinants in the protection against cytopathic bacterial and viral diseases, such as tetanus, diphtheria, cholera, hepatitis B, polio, influenza and rabies. However, determined Ab titres do not necessarily reflect vaccine efficacy, the prime vaccine prerequisite, which is commonly assessed by exposing immunized animals to a certain dose of the pathogenic material. It is noteworthy that only a dozen challenge experiments after immunization with antigen-loaded biodegradable MS have been published, although it is common view that such challenge tests are necessary to determine the protective potential of a vaccine, even when protection is provided predominantly by neutralizing Ab. For example, protection against vesicular stomatitis virus (VSV) is independent of immunoglobulin subclass, avidity and neutralizing activity [36]. By the same token, the quantitative hierarchy of CTL activity does not necessarily correlate with the ability to protect against infections, because of immuno-dominance of certain epitopes [37]. Discrepancy between protection and the level of neutralizing Ab has also been observed after immunization with diphtheria and pertussis antigens (D. Sesardic, personal communication). Very encouragingly though, there are data demonstrating correlation between Ab titre or neutralizing capacity and protection against some diseases (e.g. polio, measles and tetanus), allowing vaccine potency testing without a challenge test.

Various external factors, in addition to animal species, influence the development of immune responses. Among those, we consider the following of prime relevance for MS-based vaccines: (i) route of administration, (ii) number and interval of administration, (iii) amount of antigen and MS administered, (iv) co-administration of immuno-modulators such as cytokines or monophosphoryl lipid A, (v) the type of injection vehicle (e.g. saline, surfactants, vesicles or polymers). Moreover, the type of positive and negative control experiments and the quantification of the immune response and translation into numerical units are important developmental issues. Since most of these factors are not standarized, it is quite difficult to compare immunological data from independent investigations, and it is to hope that this situation will improve.

Table 3
The appropriateness of MS for vaccines probably depends on the infection site, infectious agent and the mechanism of how protective immunity is generated (microorganisms that have been used in MS-based immunizations are typed in bold)

Site of infection			
Intracellular		Extracellular	
Cytoplasm	Vesicles	Interstitial fluid	Epithelial surfaces
Typical organisms			
***Plasmodium* spp.** **HIV** *Rickettsia* spp. *Listeria monocytogenes*	***Mycobacterium tuberculosis*** ***Yersinia pestis*** *Salmonella typhimurium* *Leishmania* spp. *Listeria* spp.	***Clostridium tetani*** ***Corynebacterium diphtheriae*** ***Haemophilus influenzae* B** ***Staphylococcus aureus*** **Hepatitis B virus** **Influenza virus** **Measles virus** **HIV** Polio virus	***Escherichia coli*** ***Bordetella pertussis*** *Helicobacter pylori* *Vibrio cholerae* *Salmonella pneumoniae* *Candida albicans*
Protective immunity			
CTL Natural killer cells T-cell-dependent macrophage activation	Natural killer cells T-cell-dependent macrophage activation	Ab Neutralization Complement Phagocytosis	Ab, especially IgA Inflammatory cells

3. Evaluation of immunogenic properties of microsphere-based vaccines: a retrospective analyzis of published investigations

3.1. Microsphere-based vaccines for elicitation of humoral immune responses

3.1.1. Quantification of specific antibodies

When humoral immune responses are of interest, the types and quantities of antigen-specific Ab, the immunoglobulins, are generally determined by solid-phase immunoassay (SPIA). Specific Ab immunoassays provide a measure of Ab activity rather than readouts in gravimetric units. Frequently, Ab activity is expressed as *titre* or in *units* relative to a reference standard, e.g. international or ELISA units per millilitre (IU/ml) or (EU/ml). Sera of immunized animals contain mixtures of polyclonal Ab that possess different affinities to antigens. Typically, Ab detected by SPIA only cover those which remain stably bound and not the Ab total in the specimen. Consequently, Ab activity is the result of both affinity and concentration. Some of these aspects are further clarified below.

In most immunization studies using MS, titre is frequently used, although difficult to compare from one study to another. For clarification, titre equals either the reciprocal of the highest dilution of the sample at which the Ab activity can still be detected ('end-point', values ± 2 or ± 3 standard deviations above background), or a mid-point in the titration plot where Ab activity is a percentage (e.g. Ab_{50} for 50% of the maximum detectable activity). The titration method is applicable to most assays, but results from different studies may not be directly comparable. Typically, the following parameters will affect the result of SPIA: (i) the purity of antigen used in a sandwich-type SPIA and its affinity to the Ab; (ii) the material of the titre plate on which the Ab is coated, as the adsorptive process is known to change the structure of the Ab and the availability of specific binding sites for antigen; (iii) the readout method of the SPIA, such as 'end-point', dilution at an optical density (OD) of 0.2, or Ab_{50}; (iv) precision of dilutions, as a titre of 1024, for example, is only a single twofold dilution apart from 2048; (v) single-point OD readings as compared to multiple point titration curve correlation.

Expression of Ab quantity as a percentage of functional Ab in a reference standard is an alternative method of expressing activity. In enzyme-based SPIA, so-called ELISA unit per millilitre (EU/ml) has been used by directly comparing the observed response level from test Ab to that produced by a reference standard Ab that has been assigned a quantity of 100 or 1000 U/ml. Thus, direct comparison of Ab levels after immunization appears to be the most reliable procedure when a reference standard is used, and when Ab levels are interpolated from a multiple-point dose-response curve.

The importance of using reference standard Ab in SPIA, whenever available, is further emphasized by the necessity of determining clinically functional Ab in immunized animals. Immunizing with a multideterminant antigen generally elicits a polyclonal Ab response against a variety of immunogenic epitopes. It appears, however, that not all the Ab produced are equivalent in their ability to elicit biologically relevant or clinically important effector functions. This is due to the immuno-dominance of certain antigens or epitopes that are not necessarily relevant for the target disease. Thus, an immunoassay should be better designed to quantitate Ab in a biological specimen that are not only immunoreactive with most immunogenic epitopes on the antigen, but are isotypes that have in vivo relevance as neutralizing Ab [38].

Reference systems are typically pools of serum containing high levels of Ab (defined isotypes) that meet certain requirements of performance. Reference standards generally have well-documented *potency* (U/ml), defined *specificity* with Ab that bind to all the immunoreactive and clinically relevant epitopes on the antigen of interest, and an *affinity* that approximates those possessed by the population of Ab in the test specimen. The best characterised standards are international or primary reference preparations that have been tested and aliquoted under the guidance of WHO International Laboratories for Biological Standards. Unfortunately, only few Ab standards with defined potency and specificity for known antigens have ever achieved the level of WHO-documentation, thus complicating direct comparison of Ab levels from independent immunization studies.

3.1.2. Antibody responses to microencapsulated antigens

Most animal studies on parenterally administered PLGA/PLA-microencapsulated antigens determined specific serum Ab levels as a measure for humoral response. Many reports used the model antigens bovine serum albumin (BSA) or ovalbumin (OVA), although most of the published investigations were on tetanus toxoid (TT). Other antigens of interest were of (i) bacterial origin: diphtheria toxoid (DT), pertussis toxoid (PT), staphylococcal enterotoxin B (SEB), *Escherichia coli*-detached fimbriae, subunit antigens from *Haemophilus influenzae* B (Hib), *Mycobacterium tuberculosis* and *Yersinia pestis*; (ii) viral origin: subunit antigens from human and simian immunodeficiency virus (HIV, SIV), hepatitis B virus surface antigen (HBsAg), influenza A virus, measles virus, parainfluenza virus, rabies virus nucleoprotein, Venezuelan equine encephalitis virus (VEE), respiratory syncytial virus (RSV); and (iii) parasitological origin: subunit antigens from *Plasmodium* species (malaria).

In the following, we shall present some of the most striking features of reported humoral responses. Firstly, the quantity and duration of Ab levels will be presented for some antigens considered only episodically (Table 4). In the second part, we attempt to analyze measured Ab responses to the most frequently tested antigens (BSA, OVA, TT, DT, HIV, *Plasmodium*-derived antigens) as a

Table 4
Humoral responses to microencapsulated antigens (Ag) studied episodically[a]

Antigen source/ preparation[b]	Injected dose (μg)	Ab level (titre or IU/ml)	Duration of Ab response (weeks)	Comments[c]	Ref.
B. pertussis/fimbrial proteins	20	10^4–10^5	>24	Alum was slightly superior to MS injected i.p.. Mice were protected against intranasal challenge	[83]
B. pertussis/PA, PT and FHA	50	10^4 (PA);10^5 (PT + FHA)	>15	Similar anti-FHA- and PT-titres with alum and MS, but higher anti-PA-titres with MS than with alum	[84]
Cl. botulinum/Fragment C	Not indicated	0.08–2.65 IU/ml	>8	70% of animals survived aerosol-challenge 12 weeks post-immunization with LD99 botulinum toxin A	[85]
Haemophilus influenzae (Hib)/Hib-tetanus toxoid conjugate	5	2–8 μg/ml anti-Hib antibody	>27	–	[52]
Y. pestis/F1 antigen	10	10^5–10^6	>6	One-μm sized MS were more protective than 8-μm sized MS	[42]
Y. pestis/F1- and V-antigens	0.47 (F1) + 3 (V)	10^4 (F1);4×10^3 (V)	>9	Administration routes were compared: i.m., i.n., i.t.; the i.m. route gave not necessarily the highest Ab titres, but the most sustained	[86]
Y. pestis/V-antigen	10–75	1400	>57	When IFN-γ was co-encapsulated, higher anti-V Ab levels were determined (1400) as compared to MS without IFN-γ (300)	[67]
E. coli/fimbriae	50	10^5	>17	Rabbits. Ab responses elicited by free and encapsulated antigen were comparable after single i.m. injection	[87]
Staphylococcal enterotoxin B (SEB)	50	10^5–10^6	>17	MS of 1–10 μm size gave a more rapid and stronger response than 10–110-μm sized MS. In vivo neutralization of free toxin (challenge doses: 5–25 μg i.v.) was observed in mice previously immunized with Ag-loaded MS, but not in those injected with free Ag	[43]
Staphylococcal enterotoxin B (SEB)	25	10^5–10^6	>13	MS showed strong booster effects. Mixture of MS of different sizes (1–10 + 20–50 μm) were superior to uniformly sized MS (either 1–10 μm or 20–50 μm)	[45]
Staphylococcal enterotoxin B (SEB)	2×100	2.5×10^4 U/ml	Not determined	Rhesus monkeys. Animals immunized twice with MS were protected against injected toxin; triple injection of alum-adsorbed Ag was superior in terms of Ab response and symptoms of illness	[88]
Staphylococcal enterotoxin B (SEB)	2×100	5×10^3 U/ml	Not determined	Rhesus monkeys. Different routes for priming/boosting: best regimen was by i.m. priming and i.t. boosting	[46]
Hepatitis B virus/HBsAg	20	10^1–10^2 IU/ml	>22	Guinea pigs. Single dose of encapsulated Ag was superior to single dose of alum-adsorbed Ag, but slightly inferior to two doses of alum-adsorbed Ag	[89]
Hepatitis B virus/HBsAg	30	10^2 IU/ml	>55	Single injection of MS produced a stepwise increase in Ab response, as opposed to three doses of alum-adsorbed Ag. No effect of MS size, admixture of alum and number of injection sites (1 or 2)	[44]
Hepatitis B virus/HBsAg	2×20	10–20 mIU/ml	>16	Guinea pigs. MS were superior to alum. Smaller sized MS (1–10 μm) produced a faster, and larger MS a longer lasting Ab response. Co-encapsulated MDP increased the response (to 48 mIU/ml)	[41]
Influenza A virus (H1N1 + H3N2)/HA	70 (protein)	$2–4 \times 10^4$	>10	Ab response increased with PLGA-hydrophilicity: PLGA85 : 15 < PLGA75 : 25 < PLGA65 : 35 < PLGA50 : 50. s.c.-priming/oral boost was superior to oral priming/s.c.-boost	[40]
Influenza A virus (H1N1 + H3N2)/ inactivated virus	15 (HA)	$2–10 \times 10^3$	>4	Haemagglutination inhibition antibody level was approx. 5-times higher with lamellar P(l)LA particles than with MS	[90]
Influenza A virus (H1N1)/HA	15 (HA)	$2–10 \times 10^3$	8	PLGA-MS were more efficient than PLGA-PEO-PLGA-MS. Best results when MS were suspended in Ag solution	[91]
Influenza A virus/ formalin inactivated virus	2×50	2×10^5	Not determined	Protection 4 weeks post-boosting in terms of cfu from washings of the nose, trachea and lungs. MS produced similar IgG-titres, but higher anti-HA-titres as compared to those induced with Ag solution	[92]
Parainfluenza virus type 3/purified V	2×100	3.2×10^3	>6	Hamsters. Almost complete protection of animals against a challenge dose of live virus. Neutralizing Ab. Sera showed reactivity against viral HN and F-protein	[93]
Rabies virus/ Nucleoprotein	10	10^3	Not determined	Only weak Ab response similar to Ag given in saline, but weaker than Ag in CFA (measured as single point 3 weeks after immunization). Good T-cell-response	[70]

Table 4 (*continued*)

Antigen source/ preparation[b]	Injected dose (μg)	Ab level (titre or IU/ml)	Duration of Ab response (weeks)	Comments[c]	Ref.
Respiratory syncytial virus/Synth. T-B-peptide	100	10^3–10^4	>8	PLGA50:50-MS gave highest titre at 3–4 weeks after immunization, PLA-MS at 6 weeks after immunization	[94]
Simian immunodeficiency virus/gradient-purified SIVmac251	$3 \times 100 + 2 \times 100 + 2 \times 500$	10^5–10^6	Not determined, 'short-lived'	Monkeys. 3 priming + 4 booster doses were required to elicit high and long-lasting Ab levels: 2 i.m.-booster doses (100 μg each) after 7 and 30 weeks, plus additional 4 intragastric or i.t.-doses ($2 \times 100 + 2 \times 500$ μg) after 52, 68, 72 and 75 weeks. Partial protection was achieved	[95]
Venezuelan equine encephalitis virus (VEE)/ formalinized	3–50	10^6	>9	MS induced 32-times higher Ab titres than free Ag. 100% and 75% protection with 50 and 12.5 μg doses in MS, respectively. Similar Ab responses with all Ag doses	[96]
VEE/formalinized	50	3×10^4	>9	Priming/boosting routes of s.c./i.t. or i.t./i.t. protected 100%, whereas the s.c./s.c. regimen protected only 90% of the animals	[47]
Ricin toxoid	10	OD of 0.6–0.8 at a dilution of 3.4×10^3	>52	PLGA50:50-MS induced an earlier Ab response (8 weeks) than PLA MS (17 weeks). Partial protection against an aerosol challenge with ricin was achieved	[39]

[a] Unless stated otherwise, the test animal species was mice, the route of administration was s.c., i.m. or i.p., and the determined antibody isotype was IgG.

[b] PA, Pertactin; PT, pertussis toxoid; FHA, filamentous haemagglutinin; F1, fraction 1 of the purified capsular protein-polysaccharide complex, which represents a 17.5 kDa subunit antigen; V, Secreted protein of 37 kDa; HBsAg, hepatitis B surface antigen; HA, haemagglutinin.

[c] i.n.: intranasal; i.t.: intratracheal; i.g.: intragastric (by intubation); MDP, muramyl dipeptide; F, fusion protein; HN, haemagglutinin–neuraminidase complex.

Table 5
Relevance of MS formulations and immunization parameters for the quality of humoral immune responses for the most studied PLA/PLGA-encapsulated antigens (Ag)

Parameters of presumably high relevance	Parameters of uncertain relevance	Parameters of presumably little relevance
Antigens: tetanus toxoid (TT) and diphtheria toxoid (DT)		
Release of Ag: fast releasing MS (e.g. those made with the more hydrophilic PLGA50:50 or with low molecular weight PLA/PLGA) induced a strong initial Ab response [2,16,17,19,20,55,59,97], Ab response peaked at 4–8 weeks post immunization [2,16,19,51]	**MS size**: for maximum Ab level and duration of Ab response [2,17,19,99]	**Specific polymer type**: MS with relatively fast Ag release seem to perform better at early time points than slow-release particles, but release kinetics can be varied by co-encapsulated additives, MS porosity, etc.; maximum plateau of Ab titre appears to be independent of polymer type [2,16,55]
MS size: smaller sized MS (<10 μm) induced a strong initial Ab response [2,16]	**Co-encapsulated additives**: potential toxoid stabilizers such as BSA, gelatine, sugars, polyoxamers, phosphate and carbonate mineral salts possibly improved slightly the immunogenicity of encapsulated Ag [49,56,98]	**Microencapsulation technology**
Neutralizing Ab: titres peaked at later time points than total Ab titres, which was ascribed to affinity maturation [17,19,20]	**Dose**: 0.2–20 Lf of TT; 3–75 Lf DT	**MS size distribution**: a broad particle-size distribution is certainly not disadvantageous
MS mixtures: mixtures of fast and slowly releasing MS (corresponding also to small and large particles) provided the most sustained responses [16,49,55]	**Form of TT** for encapsulation: fluid, lyophilized, alum-adsorbed [32]	**Surface characteristics**: hydrophobicity, smoothness
Second MS injection: provided very high boosting effects [32,49,50]	**Amount of administered MS**	**Reconstituting vehicle**: composition of the vehicle for dispersing the MS for injection, unless this exerts an adjuvant effect itself
Admixture of adjuvants to MS: admixed alum, IFA, vegetable oil, etc. potentiated the immunogenicity of encapsulated Ag [32,51,56,98]	**PLA/PLGA modifications**: ABA-triblock copolymers with a PEG and other polyester types [17,54,100] **Duration of release** **Animal model**: mice versus guinea pigs [101] **Admixture of alum to MS**: may produce lower secondary response, although primary response is greatly enhanced [55]	**Route of administration**: i.m. versus s.c.
Model antigens: bovine serum albumin (BSA) and ovalbumin (OVA)		
Immunogenicity: BSA-MS and OVA-MS induced slightly to substantially less Ab than positive controls (IFA, alum) [102–105]; Anti-BSA titres peaked between 3 and 8 weeks post immunization [72,106,107] and anti-OVA titres between 6 and 14 weeks post immunization [104,105,108,109]	**Dose**: 0.5–10 μg of BSA, and 25–500 μg of OVA in MS [72,103,107]]	**Surface adsorption capacity of MS** [113]
Release of Ag: the more hydrophilic and fast releasing PLGA 50:50 MS induced higher Ab titres than more hydrophobic type MS, including also poly(hydroxybutyrate) MS [106,110]	**MS mixtures**: comparable titres to individual MS and alum [112]	**Zeta potential** [113]
Admixture of adjuvants to MS: induced higher Ab levels [111]	**Polymer hydrophobicity** [102,113] **Co-encapsulated additives**: potential Ag stabilizers such as lecithin, poloxamer, bile salt, or ethoxylated monoglyceride increased slightly Ab production [109,114]	

Table 5 (*continued*)

Parameters of presumably high relevance	Parameters of uncertain relevance	Parameters of presumably little relevance
	PLA molecular weight [114]	
	PLGA 50:50 versus PLGA 75:25 MS [108,110,115]	
	Route of administration: s.c. versus i.p. [116]	
	MS size [105,112,117]	
	Adsorbed (on placebo MS) versus encapsulated Ag [72,108,112]	
	Antigen content in the MS: including related amount of MS administered [117]	
Antigen: HIV-derived		
Immunogenicity: Single dose of encapsulated Ag produced Ab titres that lasted over 1 year [60–62]	**Polymer type**: determined occurrence of a booster effect in vivo, with fast releasing MS (PLGA50:50) not providing such a boost, but with more slowly releasing PLGA65:35, PLGA75:25 or PLA providing boosts at increasingly later time points [61]	**Reconstituting vehicle**: composition of the vehicle for dispersing the MS for injection, unless this exerts an adjuvant effect itself [118]
Ag Dose: minimum 100 μg [60]	**Polymer type**: determined time point of maximal titres (12 weeks up to 1 year) [60,62]	**Route of administration**: s.c. versus i.m. [118]
Co-encapsulated adjuvant: Quillaja saponaria saponins ('QS-21', Stimulon) enhanced the Ab response [60], particularly the neutralizing Ab [61] **Admixture of adjuvant to MS**: Quillaja saponaria saponins ('QS-21', Stimulon) enhanced the Ab response [60,61]	**Release kinetics**: directly relevant for the kinetics of the Ab response [18,118]	
MS size: only affected the titre at early time points, but not at later stages [61]		
Antigen: Plasmodium-derived		
Imunogenicity: multiple antigen protein (MAP) type antigens were very immunogenic when encapsulated in PLA/PLGA-MS, i.e. comparable to MAP in IFA [4,30,63]	**Single polymer type MS**: produced slightly lower titres as multiple polymer MS mixtures [63]	
IgE-response: none with encapsulated antigens [63]	**Release kinetics**: relevant, to some extent, for the kinetics of the Ab response [2]	
	Adsorbed (on placebo MS) versus encapsulated Ag: protection achieved only with encapsulated Ag, but not with adsorbed Ag [4,18]	

function of MS characteristics across the different studies (Table 5).

For the episodically studied microencapsulated antigens, strong Ab responses were generally observed after a single administration (Table 4). The response was frequently comparable to that elicited by positive reference formulations, such as antigen in alum or Freund's adjuvant, given once or twice. Thus, the MS preparations proved to be highly suitable for a large variety of antigens, derived from various sources and having very different structural features. In some studies, the potency of the response was measured by SPIA using a reference serum of known neutralizing capacity, by in vitro or in vivo neutralization assays, or by measuring protection of animals after administration of a challenge dose of the fully active pathogen. The potency of the MS preparations was generally sufficient and at least equal to that of the positive reference formulations. Besides inducing strong and functional immune

responses, MS are of particular interest in releasing antigens over extended periods of time and, thereby, providing prolonged stimulation of the immune system. Consequently, most studies presented in Table 4 investigated the kinetics of the immune response after a single MS administration. High Ab levels were generally measured over several months and maintained until the study was terminated.

Other frequently studied parameters in immunization with microencapsulated antigens were the copolymer composition of PLA/PLGA, the size of the MS and their boosting potential when administered more than once by identical or different routes. The more hydrophilic PLGA50:50-MS were found to elicit faster and higher Ab titres than MS made of PLGAs with increasing lactide content or with pure PLA [39,40]. Similarly, smaller-sized MS (e.g. 1–5 μm) generally elicited a faster and sometimes more efficacious response than larger particles [41–43], although absence of size effects was also observed [17,44]. MS were also found useful for boosting [45]. In that respect, a regimen with priming by i.m. or s.c. routes and boosting by intratracheal route was found particularly efficient [46,47].

From our point of view, however, the studies presented in Table 4 do not permit identification of a larger common denominator regarding the potential of MS as delivery system for various antigens. Too manifold are the variables between the different experimental set-ups: antigen type, antigen purity, polymer type, antigen content in the MS, MS size and release kinetics, injected antigen dose (and amount of MS), animal model, vehicle for dispersing the MS for injection, route and schedule of administration, and Ab activity determination. For virtually all of the microencapsulated antigens tested, humoral responses were excellent and long-lived. The Ab had neutralizing capacity and the few challenge tests gave encouraging results.

As mentioned above, most immunization studies with PLA/PLGA-MS have used BSA, OVA, TT, DT, HIV, and antigens derived from *Plasmodium* spp. For those antigens, data on humoral responses are again difficult to compare due to the extreme variability of used materials, technologies, particle characteristics, animal models, immunization schedules and, last but not least, the modalities of determining Ab types and levels. Nonetheless, we attempted to analyze the available data and to divide formulation and immunization parameters into three categories with respect to their relevance for the quality of the humoral immune response: high, uncertain and little relevance (Table 5).

Without any doubt, tetanus toxoid (TT) represents the most frequently used microencapsulated antigen for parenteral immunization [2,48,49]. In most studies, single injection of TT-loaded MS provided high Ab titres or ELISA-Ab units (EAU) as well as high neutralizing Ab levels over several months, generally up to 1 year or more [16,50,51]. With fast-releasing MS, titres typically peaked at 4–8 weeks after immunization. Single injection of optimal MS formulations provided Ab titres comparable to those induced by two injections of alum-adsorbed TT [16,52], resulting in protection [53–55]. Analyzis of the isotype Ab revealed dominance of IgG_1 over IgG_{2a}, with the latter being similar to IgG_{2b} [16]; this pattern was consistent with that observed in the group injected with alum-adsorbed TT. Very interestingly, when neutralizing and ELISA-titres were compared, neutralizing Ab were higher at later time points [19,20,55]. This was ascribed to progressive affinity maturation. Admixture of known adjuvants, such as alum, enhanced substantially the immune response [32,51,56]. In summary, the results strongly suggest the feasibility of single-injection MS for tetanus vaccination, provided that relatively fast releasing MS are used and, ideally, mixed with a small amount of an adjuvant such as alum.

There are only few studies on microencapsulated diphtheria toxoid (DT). Nonetheless, they have been included in this section, because DT possesses a certain similarity with TT (both are derived from secreted bacterial toxins, which can be fully neutralized by Ab) and is commonly co-administered with TT in primary and secondary vaccinations (Table 5). Microencapsulated DT did not reveal cardinal differences in performance as compared to encapsulated TT. Similarly to TT, encapsulated DT induced, after single administration, Ab titres as high as alum-adsorbed DT injected twice or thrice [17,57,58]. MS-based vaccines would gain attractiveness when more than one antigen could be combined in a multivalent preparation. Two studies showed that bivalent MS formulations containing both TT and DT in the very same MS produced titres that were initially slightly lower than those observed with the individual monovalent formulations, but the titres became similar 30 weeks after immunization [55,59]. This opens interesting perspectives for future developments of multivalent single-injection MS preparations, a project which is ongoing in our laboratory.

Ab responses to microencapsulated BSA and OVA, frequently used as model antigens, appeared to be slightly weaker as compared to those induced by the proteins adsorbed to alum or incorporated into Incomplete Freund's adjuvant (Table 5). Two striking differences were noted in the immunogenicity of these two proteins: (i) the dose of encapsulated protein necessary to induce a strong Ab response was approx. 1–10 μg for BSA, but as much as 25–500 μg for OVA; (ii) the Ab response peaked between weeks 3 and 8 after immunization with BSA, whereas for OVA, the peak occurred between weeks 8 and 14. In complete agreement with observations made with TT and DT, the most hydrophilic PLGA50:50-MS elicited the strongest Ab responses, and admixture of additional adjuvants further increased the titres. Most other parameters were of uncertain relevance, but certainly not in contradiction with the previous observation on TT and DT.

Antigens of chief interest are those derived from HIV (Table 5). The interest of using MS for this type of antigen lies probably more in the search for an efficacious adjuvant system capable to induce both Ab and CTL responses rather

than in the development of a single-injection delivery system (see Section 3.2). Single doses of up to 300 μg of HIV-derived recombinant or synthetic proteins in MS produced Ab levels lasting over more than one year. Some of the MS formulations appeared to boost the Ab response after approx. 6 weeks. Co-encapsulated or admixed adjuvant (Quillaja saponaria saponins, 'QS-21', Stimulon) enhanced the Ab response [60,61]. However, a physical mixture of antigen plus adjuvant induced, per se, high and long-lasting Ab responses [61]. Most encouragingly, high titres of neutralizing Ab were induced and maintained over at least 1 year [61,62].

A large group of intracellular infective agents, where both Ab and CTL responses are required (see Section 3.2), encompasses the *Plasmodium* species, with *P. falciparum* being the main causative agent of malaria in human. Although the importance of particular MS characteristics on the immunogenicity of encapsulated antigens has not yet been studied in much detail, the data on Ab titres and CTL responses are impressive. Typically, antigens encapsulated in MS induced Ab titres that were comparable to those elicited by the antigens mixed with IFA. Very interestingly, some of the studies used synthetic multiple antigen proteins, so-called MAP, which were composed of universal T-helper epitopes from TT and a specific B-cell epitope from *Plasmodium* spp. The synthetic constructs contained single B-cell epitopes that were composed of only 4–8 amino acids. The data confirm that MS are very potent delivery systems with in-built adjuvancy, being useful also for weakly immunogenic synthetic antigens.

Considering all the cited investigations on the various microencapsulated antigens, the MS technology appears most promising for a yet unrestricted variety of antigen types to elicit strong and long lasting Ab responses with neutralizing capacity. Tentatively, many studies suggest that fast releasing PLGA-MS are the best suited to induce high titres at early time points and combination with more slowly releasing MS types might help to maintain high Ab levels. Admixture of well-known adjuvants such alum or saponines may enhance the humoral response. Very encouragingly, there are several indications that antigen release from MS may promote Ab maturation with time. We consider the amount and quality of available data sufficient to promote clinical testing of MS technology-based new vaccines.

3.2. Microsphere-based vaccines for elicitation of cell-mediated immune responses

3.2.1. T-helper cell responses

As mentioned above, the initiation of a Th-response is essential for the development of a specific humoral immunity, i.e. clonal selection and specific Ab production. However, some studies with MS-based vaccines did not only investigate Ab responses, but also quantified cell-mediated responses, mainly Th-responses and cytokine production. T-cell proliferation assay is a classical method to estimate the extent of activation and proliferation of specific Th-cells. The assay is commonly performed with T cells from the draining lymph nodes (LN) or spleen (splenocytes) of animals previously immunized with an antigen. The level of T-cell proliferation is generally measured by [^{3}H]thymidine incorporation into proliferating T cells upon in vitro stimulation with the specific antigen. Counts from [^{3}H]thymidine (cpm) are then expressed directly or by a stimulation index (SI). The latter is defined as cpm-ratio of stimulated (with antigen) over non-stimulated T cells. Considering the difficulty to control cell-culture conditions during an assay and to avoid counts from non-specific proliferation, SI should be more accurate than the direct cpm-number to estimate the level of relative T-cell proliferative responses.

The selection of LN cells or splenocytes appears to depend on the immunization route. According to the limited data available, draining LN-cells were often chosen after s.c.-vaccination [16,63–65], while splenocytes were used after i.p. injection [66–69]. Ertl et al. reported that only T cells from draining LN, but not from spleen, were proliferative after s.c. injection of encapsulated rabies nucleoprotein antigen in mice [70]. However, the available data appear to be inconsistent. In our own studies, we have observed very strong and long-lasting T-cell proliferative responses with LN cells after s.c. injection of microencapsulated tetanus toxoid (TT) in mice [16]. Conversely, Walker and co-workers reported little or no induction of specific T cells from LN after s.c. injection of encapsulated TT in mice [71]. Nevertheless, in most studies on Th response, antigens contained in MS showed a considerable potential to induce specific T cells that maintained proliferative activity upon in vitro stimulation; the proliferative activity was generally largely superior to that observed with control formulations such as alum or Freund's adjuvants. Ertl and co-workers also reported that encapsulated rabies nucleoprotein antigen stimulated hybridoma T cells in vitro [70]. Interestingly, proliferation of T cells from LN was pronounced only in animals that had received the peptide encapsulated in low molecular weight PLGA50:50-MS, whereas animals injected with high molecular weight PLGA50:50- or PLGA85:15-MS did not produce proliferative T cells; this suggests that the antigen release kinetics, which varied with the MS types, influenced in fact the T-cell response. Differences in T-cell responses can further depend on the nature of the antigen. For example, T-cell proliferation was low 10 days after immunization of mice with TT entrapped in high molecular weight PLA-MS, but it was high when TT was administered in low molecular weight PLGA [16]; conversely, no such difference was observed with a small, synthetic *Plasmodium*-derived antigen incorporated into corresponding polymer-type MS [63].

The duration of T-cell proliferative responses can be maintained for a very long period of time. Strongly proliferative T cells were detectable for up to 45 weeks after a

single injection of TT contained in a mixture of three MS types, which were characterized by fast and slow antigen release and by small and large sizes [16]. This suggests that the small and fast release MS can facilitate particle uptake and antigen processing and presentation by APC (e.g. macrophages), while large particles may remain at the injection site and serve as an antigen depot. No clear correlation between the burst release of encapsulated antigens and the kinetics of T-cell proliferation has been reported.

A few studies investigated cytokine production of T cells. For example, immunization with microencapsulated HIV gp120 [66], synthetic peptides derived from mucin or OVA [64,65], or with recombinant protein from *M. tuberculosis* [68] generated high levels of interferon (IFN)-γ, low levels of interleukin (IL)-4, IL-10 or IL-5, suggesting a Th1-response profile. In contrast, alum is more Th2 promoting [66]. However, Iguarta and co-workers observed a Th2-response with microencapsulated BSA [72], suggesting that the antigen type also influences Th-cell differentiation towards a preferential Th1- or Th2-type.

3.2.2. Cytotoxic T-cell responses

Antigens encapsulated in or physically adsorbed on MS have been demonstrated to induce specific CTL responses, even though data are scarce. Short CTL epitopes derived from viruses and parasites were encapsulated in PLGA-MS and successfully primed specific CTL in mice [3,4,73,74]. Interestingly, specific CTL response was also obtained by injection of soluble CTL peptides mixed with placebo PLGA50:50-MS, whereas free peptides without MS or placebo MS alone failed to elicit specific CTL responses [3,4]. In addition, CTL activity induced by epitopes adsorbed on placebo MS was not affected by various MS types [4]. Thus, by speculation, peptide adsorbed on MS surfaces might bind directly to MHC-class I molecules on the surface of APC, which are recruited to the injection site. Furthermore, MS can act as immunostimulants by themselves, mediating the secretion of certain cytokines such as IFN-γ, to activate the immune system for specific CTL priming.

It is well established that CTL play a substantial role in host immune responses against infections caused by viruses, parasites, and certain intracellular bacteria, as well as against tumor cells. However, the common assumption that exogenous antigens are excluded from being processed within the cytosol/ER and transported to the MHC-class I molecules, has greatly limited the development of CTL vaccines with soluble protein antigens. Recent studies on MHC-class I-restricted antigen processing and presentation and in vivo CTL priming demonstrated that the particulate nature of antigens may activate certain APC such as macrophages or dendritic cells to mediate the presentation of exogenous antigens through MHC-class I molecules, hence to aid in the generation of CTL responses [75,76].

In the context of MS, preliminary studies on the mechanisms of processing and presentation of microencapsulated protein antigens through MHC-class I molecules have demonstrated that macrophages can internalize MS of small size (approx. < 10 μm) and then stimulate specific CTL in vitro [30]. Further, Moore and co-workers have reported that HIV-derived gp120 encapsulated in MS generated epitope-specific MHC-class I-restricted $CD8^+$-CTL responses after boosting [66]. These few investigations demonstrate the potential of PLGA-MS for targeting antigens via a MHC-class I-restricted pathway to induce CTL responses. Future studies will be required though to elucidate in more detail the cellular mechanisms involved in processing and presentation of exogenous antigens through MHC-class I molecules when delivery by PLGA-MS. In addition, MS formulations might be specifically designed and optimized to elicit predominantly a CTL response. Particle size, surface charge and hydrophobicity as well as antigen release characteristics should be some of the parameters to study.

4. Prospects and challenges in future vaccine development

Strategic decisions on the development of innovative vaccines generally consider market stronghold and socio-economic implications more than academic interests and challenges. Therefore, novel vaccines are primarily developed for so-called high-impact diseases. Although the scientific community participates in defining which diseases are of high impact, the preferences are very much set up by international and national health institutions such as the WHO, the U.S. National Institute of Health (NIH), the health commissions of the European Union or the G8-countries. Diseases such as AIDS, tuberculosis, malaria and cancer presently belong to the high-impact diseases. For 'non-high' impact diseases, new vaccines should be based on new concepts of immunization (e.g. DNA/RNA-vaccines), non-invasive immunization procedures (e.g. nasal, pulmonal, oral, powder-jet systems), or single-injection delivery systems. For genetic vaccines, several features appear to make them more attractive than protein or peptide vaccines. They might be more straightforward to manufacture, be relatively inexpensive and possibly less susceptible to instability than some of the subunit peptide and recombinant protein antigens. Since DNA mediates the production of endogenous antigen, which is liberated into the cytosol of transfected cells, it is expected that genetic vaccines might allow to accomplish better MHC-class I immune responses than exogenous protein or peptide antigens, which, after vaccination, are typically located in extracellular compartments or within phagolysosomes. However, as the constraints of naked DNA injections are progressively recognized [77], the quest to make DNA vaccines more efficient needs to be combined with a search for more efficacious DNA-delivery systems. Among others, MS technology is certainly a prime candidate to approach this aim.

The above-mentioned non-invasive immunization procedures, on the other hand, may be manageable by the users themselves and might be less associated with adverse effects related to either the antigen or the injection. Particularly nasal, pulmonal or oral vaccines would be most advantageous. Such vaccines do not have to be sterile, an aspect that simplifies greatly production, documentation for a new investigational drug (NID) application, and clinical-trial procedures. Finally, the possible reduction in the number of immunization sessions needed to achieve long-term protection by combining several vaccines in a single-administration vaccine delivery system would be of extreme advantage to improve vaccination coverage in developing countries. For some of these perspectives to come true, industrial commitment in the development of new vaccines must increase far beyond the present level.

Time will reveal which of these approaches will be applicable to MS technology. Future academic and industrial investment will certainly depend on the outcome of clinical tests (as frequently communicated by industrial representatives). It is noteworthy that more than 10 years of pre-clinical research has not yet ensued many published clinical trial with MS-based vaccines. To our knowledge, the only study published was one of 1994 on an oral *E. coli* vaccine, which reported quite promising results [78]. However, and very surprisingly, the study did not push MS technology towards clinical applications. Therefore, it is hoped that a clinical trial with a single-injection tetanus vaccine, which we are planning together with the WHO, will produce meaningful data with respect to further development of MS-based vaccines.

In our opinion, the foreseeable future of MS technology in vaccinology relies on two aspects: sustained antigen delivery and induction of CTL response. Firstly, it has been demonstrated for various antigens and in various animal models that MS can generate and stimulate immune responses over a prolonged time period. If immunological memory depends on persistent antigen stimulation rather than on long-lived memory cells, MS may prove superior to any multiple-dosing or -injection system, by providing a persistent antigen stimulation either through continuous release of antigen from the MS, or through sustained transport of MS to secondary lymphatic tissues. Consequently, this should reduce the need for boosting doses. Secondly, although not extensively studied, MS have proven to elicit strong and specific CTL responses. This feature is of particular advantage for vaccines directed toward infectious diseases of intracellular pathology, since the only adjuvant widely accepted for human application, alum, induces primarily Th2-responses (antibodies), but few Th1-responses (cellular mediators and effectors). Moreover, due to the potential in generating CTL, MS may slowly find applications in tumor therapy. It is reasonable to believe that this research branch will intensify, and we think that this may aid MS-based vaccination towards more extensive clinical evaluations.

More remote prospects for MS technology as a platform to establish future generations of PLA/PLGA-based microparticulate vaccine delivery systems are manifold. Co-entrapment of antigen cocktails, stabilizers, co-adjuvants and other additives in MS represents only one aspect. Additional aspects might be opened by addressing MS-based vaccines to specific targets, i.e. macrophages, dendritic cells, or other tissues or cells of the immune system. Identification of appropriate binding sites and sequences will be a prerequisite. Moreover, co-entrapment or coating with additives to affect the pathways of intracellular processing, trafficking and presentation of the antigen, currently under intense investigation in immunology, may further enlarge the potential of MS-based vaccines as to their efficiency and specificity. Thus, in addition to the present state of the art, MS technology appears to be versatile enough to allow the bioengineering of vaccine delivery systems of a complex nature and beyond our present anticipation. This potential has yet to be exploited and relies on tight transdisciplinarity between immunology, bioengineering and pharmaceutical technology, as well as industrial commitment.

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References

[1] B.R. Bloom, Vaccines for the third world, Nature 342 (1989) 115–120.
[2] C. Thomasin, G. Corradin, Y. Men, H.P. Merkle, B. Gander, Tetanus toxoid and synthetic malaria antigen containing poly(lactide)/poly (lactide-co-glycolide) microspheres: Importance of polymer degradation and antigen release for immune response, J. Control. Release 41 (1996) 131–145.
[3] C.D. Partidos, P. Vohra, D. Jones, G. Farrar, M.W. Steward, CTL responses induced by a single immunization with peptide encapsulated in biodegradable microparticles, J. Immunol. Methods 206 (1997) 143–151.
[4] Y. Men, H. Tamber, R. Audran, B. Gander, G. Corradin, Induction of a cytotoxic T lymphocyte response by immunization with a Malaria specific CTL peptide entrapped in biodegradable polymer microspheres, Vaccine 15 (1997) 1405–1412.
[5] D. O'Hagan, J.P. McGee, J. Holmgren, A.M. Mowat, A.M. Donachie, K.H. Mills, W. Gaisford, D. Rahman, S.J. Challacombe, Biodegradable microparticles for oral immunization, Vaccine 11 (1993) 149–154.
[6] G.F.A. Kersten, B. Gander, Biodegradable microspheres as vehicles for antigens, in: S.H.E. Kaufmann (Ed.), Concepts in Vaccine Development, Walter de Gruyter, 1996, pp. 265–302.
[7] T. Kissel, R. Koneberg, Injectable biodegradable MS for vaccine delivery, in: S. Cohen, H. Bernstein (Eds.), Microparticulate

Systems for the Delivery of Proteins and Vaccine, Marcel Dekker, New York, 1996, pp. 51–87.
[8] J. Hanes, J.L. Cleland, R. Langer, New advances in microsphere-based single-dose vaccines, Adv. Drug Del. Rev. 28 (1997) 97–119.
[9] D. O'Hagan, M. Singh, R.K. Gupta, Poly(lactide-co-glycolide) microparticles for the development of single- dose controlled-release vaccines, Adv. Drug Del. Rev. 32 (1998) 225–246.
[10] J.L. Cleland, Single-administration vaccines: Controlled-release technology to mimic repeated immunizations, Trends Biotechnol. 17 (1999) 25–29.
[11] M.H.V. van Regenmortel, Molecular design versus empirical discovery in peptide-based vaccines. Coming to terms with fuzzy recognition sites and ill-defined structure-function relationships in immunology, Vaccine 18 (1999) 216–221.
[12] D.C. Tang, M. DeVit, S.A. Johnston, Genetic immunization is a simple method for eliciting an immune response, Nature 365 (1992) 152–154.
[13] A.T. Kamath, C.G. Feng, M. Macdonald, H. Briscoe, W.J. Britton, Differential protective efficacy of DNA vaccines expressing secreted proteins of *Mycobacterium tuberculosis*, Infect. Immun. 67 (1999) 1702–1707.
[14] R. Ahmed, D. Gray, Immunological memory and protective immunity: understanding their relation, Science 272 (1996) 54–60.
[15] S.L. Swain, H. Hu, G. Huston, Class II-independent generation of CD4 memory T cells from effectors, Science 286 (1999) 1381–1383.
[16] Y. Men, C. Thomasin, H.P. Merkle, B. Gander, G. Corradin, A single administration of tetanus toxoid in biodegradable microspheres elicits T cell and antibody responses similar or superior to those obtained with aluminum hydroxide, Vaccine 13 (1995) 683–689.
[17] P. Johansen, L. Moon, H. Tamber, H.P. Merkle, B. Gander, D. Sesardic, Immunogenicity of single-dose diphtheria vaccines based on PLA/PLGA microspheres in guinea pigs, Vaccine 18 (1999) 209–215.
[18] J.L. Cleland, A. Lim, L. Barron, E.T. Duenas, M.F. Powell, Development of a single-shot subunit vaccine for HIV-I: Part 4. Optimizing microencapsulation and pulsatile release of MN rgp120 from biodegradable microspheres, J. Control. Release 47 (1997) 135–150.
[19] M.J. Alonso, R.K. Gupta, C. Min, G.R. Siber, R. Langer, Biodegradable microspheres as controlled-release tetanus toxoid delivery systems, Vaccine 12 (1994) 299–306.
[20] S. Cohen, M.J. Alonso, R. Langer, Novel approaches to controlled-release antigen delivery, Int. J. Technol. Assess. Health Care 10 (1994) 121–130.
[21] D.K. Xing, K. McLellan, M.J. Corbel, D. Sesardic, Estimation of antigenic tetanus toxoid extracted from biodegradable microspheres, Biologicals 24 (1996) 57–65.
[22] P. Johansen, H. Tamber, H.P. Merkle, B. Gander, Diphtheria and tetanus toxoid microencapsulation into conventional and end-group alkylated PLA/PLGAs, Eur. J. Pharm. Biopharm. 47 (1999) 193–201.
[23] R.K. Gupta, A.C. Chang, P. Griffin, R. Rivera, Y.Y. Guo, G.R. Siber, Determination of protein loading in biodegradable polymer microspheres containing tetanus toxoid, Vaccine 15 (1997) 672–678.
[24] S.L. Constant, K. Bottomly, Induction of Th1 and Th2 CD4+ T cell responses: the alternative approaches, Annu. Rev. Immunol. 15 (1997) 297–322.
[25] C.H. Niu, Y.Y. Chiu, FDA perspective on peptide formulation and stability issues, J. Pharm. Sci. 87 (1998) 1331–1334.
[26] P. Johansen, G. Corradin, H.P. Merkle, B. Gander, Release of tetanus toxoid from adjuvants and PLGA microspheres: how experimental set-up and surface adsorption fool the pattern, J. Control. Release 56 (1998) 209–217.
[27] D.-L. Xing, D.T. Crane, B. Bolgiano, M.J. Corbel, C. Jones, D. Sesardic, Physicochemical and immunological studies on the stability of free and microsphere-encapsulated tetanus toxoid in vitro, Vaccine 14 (1996) 1205–1213.
[28] P. Johansen, Y. Men, R. Audran, G. Corradin, H.P. Merkle, B. Gander, Improving stability and release kinetics of microencapsulated tetanus toxoid by co-encapsulation of additives, Pharm. Res. 15 (1998) 1103–1110.
[29] C. Watts, Capture and processing of exogenous antigens for presentation on MHC molecules, Annu. Rev. Immunol. 15 (1997) 821–850.
[30] Y. Men, R. Audran, C. Thomasin, G. Eberl, S. Demotz, H.P. Merkle, B. Gander, G. Corradin, MHC class I- and class II-restricted processing and presentation of microencapsulated antigens, Vaccine 17 (1999) 1047–1056.
[31] C. Thomasin, P. Johansen, R. Alder, R. Bemsel, G. Hottinger, H. Altorfer, A.D. Wright, G. Wehrli, H.P. Merkle, B. Gander, A contribution to overcoming the problem of residual solvents in biodegradable microspheres prepared by coacervation, Eur. J. Pharm. Biopharm. 42 (1996) 16–24.
[32] I. Esparza, T. Kissel, Parameters affecting the immunogenicity of microencapsulated tetanus toxoid, Vaccine 10 (1992) 714–720.
[33] M.B. Sintzel, A. Merkli, C. Tabataby, R. Gurny, Influence of irradiation sterilization on polymers used as drug carriers – a review, Drug Dev. Ind. Pharm. 23 (1997) 857–878.
[34] K. Mader, A. Domb, H.M. Schwarz, Gamma-sterilization induced radicals in biodegradable drug delivery systems, Appl. Radiat. Isot. 47 (1996) 1669–1674.
[35] J.D. Peterson, L.A. Herzenberg, K. Vasquez, C. Waltenbaugh, Glutathione levels in antigen-presenting cells modulate Th1 versus Th2 response patterns, Proc. Natl. Acad. Sci. USA 95 (1998) 3071–3076.
[36] M.F. Bachmann, U. Kalinke, A. Althage, G. Freer, C. Burkhart, H. Roost, M. Aguet, H. Hengartner, R.M. Zinkernagel, The role of antibody concentration and avidity in antiviral protection, Science 276 (1997) 2024–2027.
[37] A. Gallimore, T. Dumrese, H. Hengartner, R.M. Zinkernagel, H.G. Rammensee, Protective immunity does not correlate with the hierarchy of virus-specific cytotoxic T cell responses to naturally processed peptides, J. Exp. Med. 187 (1998) 1647–1657.
[38] J.E. Butler, R.G. Hamilton, Quantitation of specific antibodies: Methods of expression, standards, solid-phase considerations, and specific applications, in: J.E. Butler (Ed.), Immunochemistry of Solid-phase Immunoassays, CRC Press, Boca Raton, Fl, 1991, pp. 173–198.
[39] C. Yan, J.H. Resau, J. Hewetson, M. West, W.L. Rill, M. Kende, Characterization and morphological analysis of protein-loaded poly(lactide-co-glycolide) microparticles prepared by water-in-oil-in-water emulsion technique, J. Control. Release 32 (1994) 231–241.
[40] S.C. Chattaraj, A. Rathinavelu, S.K. Das, Biodegradable microparticles of influenza viral vaccine: comparison of the effects of routes of administration on the in vivo immune response in mice, J. Control. Release 58 (1999) 223–232.
[41] R.V. Nellore, P.G. Pande, D. Young, H.R. Bhagat, Evaluation of biodegradable microspheres as vaccine adjuvant for hepatitis B surface antigen, J. Parenter. Sci. Technol. 46 (1992) 176–180.
[42] K.M. Reddin, T.J. Easterbrook, S.M. Eley, P. Russell, V.A. Mobsby, D.H. Jones, G.H. Farrar, E.D. Williamson, A. Robinson, Comparison of the immunological and protective responses elicited by microencapsulated formulations of the F1 antigen from *Yersinia pestis*, Vaccine 16 (1998) 761–767.
[43] J.H. Eldridge, J.K. Staas, J.A. Meulbroek, T.R. Tice, R.M. Gilley, Biodegradable and biocompatible poly(DL-lactide-co-glycolide) microspheres as an adjuvant for staphylococcal enterotoxin B toxoid which enhances the level of toxin-neutralizing antibodies, Infect. Immun. 59 (1991) 2978–2986.
[44] M. Singh, X.M. Li, J.P. McGee, T. Zamb, W. Koff, C.Y. Wang, D.T. O'Hagan, Controlled release microparticles as a single dose hepatitis B vaccine: evaluation of immunogenicity in mice, Vaccine 15 (1997) 475–481.
[45] J.H. Eldridge, J.K. Staas, J.A. Meulbroek, J.R. McGhee, T.R. Tice,

R.M. Gilley, Biodegradable microspheres as a vaccine delivery system, Mol. Immunol. 28 (1991) 287–294.
[46] J. Tseng, J.L. Komisar, R.N. Trout, R.E. Hunt, J.-J. Chen, A.J. Johnson, L. Pitt, D.L. Ruble, Humoral immunity to aerosolized staphylococcal enterotoxin B (SEB), a superantigen, in monkeys vaccinated with SEB toxoid-containing microspheres, Infect. Immun. 63 (1995) 2880–2885.
[47] T.E. Greenway, J.H. Eldridge, G. Ludwig, J.K. Staas, J.F. Smith, R.M. Gilley, S.M. Michalek, Induction of protective immune responses against Venezuelan equine encephalitis (VEE) virus aerosol challenge with microencapsulated VEE virus vaccine, Vaccine 16 (1998) 1314–1323.
[48] R.K. Gupta, G.R. Siber, M.J. Alonso, R. Langer, Development of a single-dose tetanus toxoid based on controlled release from biodegradable and biocompatible polyester microspheres, in: H.S. Ginsberg, F. Brown, R.M. Chanock, R.A. Lerner (Eds.), Vaccine 93. Modern Approaches to New Vaccines Including Prevention of AIDS, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York, 1993, pp. 391–396.
[49] R. Audran, Y. Men, P. Johansen, B. Gander, G. Corradin, Enhanced immunogenicity of microencapsulated tetanus toxoid with stabilizing agents, Pharm. Res. 15 (1998) 1111–1116.
[50] R.K. Gupta, J. Alroy, M.J. Alonso, R. Langer, G.R. Siber, Chronic local tissue reactions, long term immunogenicity and immunologic priming of mice and guinea pigs to tetanus toxoid encapsulated in biodegradable polymer microspheres composed of poly lactide-co-glycolide polymers, Vaccine 15 (1997) 1716–1723.
[51] M. Tobio, S.P. Schwendeman, Y. Guo, J. McIver, R. Langer, M.J. Alonso, Improved immunogenicity of a core-coated tetanus toxoid delivery system, Vaccine 18 (1999) 618–622.
[52] R.K. Gupta, A.C. Chang, G.R. Siber, Biodegradable polymer microspheres as vaccine adjuvants and delivery systems, Dev. Biol. Stand. 92 (1998) 63–78.
[53] G.F. Kersten, D. Donders, A. Akkermans, E.C. Beuvery, Single shot with tetanus toxoid in biodegradable microspheres protects mice despite acid-induced denaturation of the antigen, Vaccine 14 (1996) 1627–1632.
[54] T. Kissel, R. Koneberg, A.K. Hilbert, K.D. Hungerer, Microencapsulation of antigens using biodegradable polyesters: facts and phantasies, Behring Inst. Mitteil. 98 (1997) 172–183.
[55] M. Singh, X.M. Li, H. Wang, J.P. McGee, T. Zamb, W. Koff, C.Y. Wang, D.T. O'Hagan, Immunogenicity and protection in small-animal models with controlled-release tetanus toxoid microparticles as a single-dose vaccine, Infect. Immun. 65 (1997) 1716–1721.
[56] M. Tobio, J. Nolley, Y. Guo, J. McIver, M.J. Alonso, A novel system based on a poloxamer/PLGA blend as a tetanus toxoid delivery vehicle, Pharm. Res. 16 (1999) 682–688.
[57] M. Singh, A. Singh, G.P. Talwar, Controlled delivery of diphtheria toxoid using biodegradable poly(D,L-lactide) microcapsules, Pharm. Res. 8 (1991) 958–961.
[58] M. Singh, O. Singh, A. Singh, G.P. Talwar, Immunogenicity studies on diphtheria toxoid loaded biodegradable microspheres, Int. J. Pharm. 85 (1992) R5–R8.
[59] M. Singh, X.M. Li, H. Wang, J.P. McGee, T. Zamb, W. Koff, C.Y. Wang, D.T. O'Hagan, Controlled release microparticles as a single dose diphtheria toxoid vaccine: immunogenicity in small animal models, Vaccine 16 (1998) 346–352.
[60] J.L. Cleland, M.F. Powell, A. Lim, L. Barron, P.W. Berman, D.J. Eastman, J.H. Nunberg, T. Wrin, J.C. Vennari, Development of a single-shot subunit vaccine for HIV-1, AIDS Res. Human Retroviruses 10 (1994) S21–S26.
[61] J.L. Cleland, A. Lim, A. Daugherty, L. Barron, N. Desjardin, E.T. Duenas, D.J. Eastman, J.C. Vennari, T. Wrin, P. Berman, K.K. Murthy, M.F. Powell, Development of a single-shot subunit vaccine for HIV-1. Programmable in vivo autoboost and long lasting neutralizing response, J. Pharm. Sci. 87 (1998) 1489–1495.
[62] J.L. Cleland, L. Barron, A. Daugherty, D. Eastman, C. Kensil, A. Lim, R.P. Weissburg, T. Wrin, J. Vennari, M.F. Powell, Development of a single-shot subunit vaccine for HIV-1. 3. Effect of adjuvant and immunization schedule on the duration of the humoral immune response to recombinant MN gp120, J. Pharm. Sci. 85 (1996) 1350–1357.
[63] Y. Men, B. Gander, H.P. Merkle, G. Corradin, Induction of sustained and elevated immune responses to weakly immunogenic synthetic malarial peptides by encapsulation in biodegradable polymer microspheres, Vaccine 14 (1996) 1442–1450.
[64] K.D. Newman, D.L. Sosnowski, G.S. Kwon, J. Samuel, Delivery of MUC1 mucin peptide by poly(D,L-lactic-co-glycolic acid) microspheres induces type 1 T helper immune responses, J. Pharm. Sci. 87 (1998) 1421–1427.
[65] K.D. Newman, J. Samuel, G. Kwon, Ovalbumin peptide encapsulated in poly(D,L lactic-co-glycolic acid) microspheres is capable of inducing a T helper type 1 immune response, J. Control. Release 54 (1998) 49–59.
[66] A. Moore, P. McGuirk, S. Adams, W.C. Jones, J.P. McGee, D.T. O'Hagan, K.H.G. Mills, Immunization with a soluble recombinant HIV protein entrapped in biodegradable microparticles induces HIV-specific CD8+ cytotoxic T lymphocytes and CD4+ Th1 cells, Vaccine 13 (1995) 1741–1749.
[67] K.F. Griffin, B.R. Conway, H.O. Alpar, E.D. Williamson, Immune responses to V antigen of Yersinia pestis co-encapsulated with IFN-gamma: effect of dose and formulation, Vaccine 16 (1998) 517–521.
[68] N. Venkataprasad, A.G. Coombes, M. Singh, M. Rohde, K. Wilkinson, F. Hudecz, S.S. Davis, H.M. Vordermeier, Induction of cellular immunity to a mycobacterial antigen adsorbed on lamellar particles of lactide polymers, Vaccine 17 (1999) 1814–1819.
[69] E.D. Williamson, G.J. Sharp, S.M. Eley, P.M. Vesey, T.C. Pepper, R.W. Titball, H.O. Alpar, Local and systemic immune response to a microencapsulated subunit vaccine for plague, Vaccine 14 (1996) 1613–1619.
[70] H.C.J. Ertl, I. Varga, Z.Q. Xiang, K. Kaiser, L. Stephens, L. Otvos Jr., Poly(DL-lactide-co-glycolide) microspheres as carriers for peptide vaccines, Vaccine 14 (1996) 879–885.
[71] K.B. Walker, D.K. Xing, D. Sesardic, M.J. Corbel, Modulation of the immune response to tetanus toxoid by polylactide-polyglycolide microspheres, Dev. Biol. Stand. 92 (1998) 259–267.
[72] M. Igartua, R.M. Hernandez, A. Esquisabel, A.R. Gascon, M.B. Calvo, J.L. Pedraz, Enhanced immune response after subcutaneous and oral immunization with biodegradable PLGA microspheres, J. Control. Release 56 (1998) 63–73.
[73] C.D. Partidos, P. Vohra, D.H. Jones, G.H. Farrar, M.W. Steward, Mucosal immunization with a measles virus CTL epitope encapsulated in biodegradable PLG microparticles, J. Immunol. Methods 195 (1996) 135–138.
[74] D.F. Nixon, C. Hioe, P.D. Chen, Z. Bian, P. Kuebler, M.L. Li, H. Qiu, X.M. Li, M. Singh, J. Richardson, P. McGee, T. Zamb, W. Koff, C.Y. Wang, D.T. O'Hagan, Synthetic peptides entrapped in microparticles can elicit cytotoxic T cell activity, Vaccine 14 (1996) 1523–1530.
[75] K.L. Rock, new foreign policy: MHC class I molecules monitor the outside world, Immunol. Today 17 (1996) 131–137.
[76] M. Jondal, R. Schirmbeck, J. Reimann, MHC class I-restricted CTL responses to exogenous antigens, Immunity 5 (1996) 295–302.
[77] R. Wang, D.L. Doolan, T.P. Le, R.C. Hedstrom, K.M. Coonan, Y. Charoenvit, T.R. Jones, P. Hobart, M. Margalith, J. Ng, W.R. Weiss, M.D. Sedegah, C. Taisne, J.A. Norman, S.L. Hoffman, Induction of antigen-specific cytotoxic T lymphocytes in humans by a malaria DNA vaccine, Science 282 (1998) 476–480.
[78] C.O. Tacket, R.H. Reid, E.C. Boedeker, G. Losonsky, J.P. Nataro, H. Bhagat, R. Edelman, Enteral immunization and challenge of volunteers given enterotoxigenic *E. coli* CFA/II encapsulated in biodegradable microspheres, Vaccine 12 (1994) 1270–1274.
[79] Y. Tabata, Y. Ikada, Macrophage phagocytosis of biodegradable

microspheres composed of L-lactic acid/glycolic acid homo- and copolymers, J. Biomed. Mater. Res. 22 (1988) 837–858.
[80] A. Merkli, J. Heller, C. Tabatabay, R. Gurny, Gamma sterilization of a semi-solid poly(ortho ester) designed for controlled drug delivery-validation and radiation effects, Pharm. Res. 11 (1994) 1485–1491.
[81] Y. Tabata, Y. Ikada, Macrophage activation through phagocytosis of muramyl dipeptide encapsulated in gelatin microspheres, J. Pharm. Pharmacol. 39 (1987) 698–704.
[82] C. Evora, I. Soriano, R.A. Rogers, K.N. Shakesheff, J. Hanes, R. Langer, Relating the phagocytosis of microparticles by alveolar macrophages to surface chemistry: the effect of 1,2-dipalmitoylphosphatidylcholine, J. Control. Release 51 (1998) 143–152.
[83] D.H. Jones, B.W. McBride, H. Jeffery, D.T. O'Hagan, A. Robinson, G.H. Farrar, Protection of mice from *Bordetella pertussis* respiratory infection using microencapsulated pertussis fimbriae, Vaccine 13 (1995) 675–681.
[84] R. Shahin, M. Leef, J. Eldridge, M. Hudson, R. Gilley, Adjuvanticity and protective immunity elicited by *Bordetella pertussis* antigens encapsulated in poly(D,L-lactide-co-glycolide) microspheres, Infect. Immun. 63 (1995) 1195–1200.
[85] R.L. Whalen, D.J. Dempsey, L.M. Thompson, K. Bucknell, R. Kunitomo, Y. Okazaki, H. Harasaki, Microencapsulated vaccines to provide prolonged immunity with a single administration, ASAIO J. 42 (1996) M649–M654.
[86] J.E. Eyles, I.D. Spiers, E.D. Williamson, H.O. Alpar, Analysis of local and systemic immunological responses after intra- tracheal, intra-nasal and intra-muscular administration of microsphere co-encapsulated *Yersinia pestis* subunit vaccines, Vaccine 16 (1998) 2000–2009.
[87] R. Edelman, R.G. Russell, G. Losonsky, B.D. Tall, C.O. Tacket, M.M. Levine, D.H. Lewis, Immunization of rabbits with enterotoxigenic *E. coli* colonization factor antigen (CFA/I) encapsulated in biodegradable microspheres of poly (lactide-co-glycolide, Vaccine 11 (1993) 155–158.
[88] J. Tseng, J.L. Komisar, J.-J. Chen, R.E. Hunt, A.J. Johnson, L. Pitt, J. Rivera, D.L. Ruble, R. Trout, A. Vega, Immunity and responses of circulating leukocytes and lymphocytes in monkeys to aerosolized staphylococcal enterotoxin B, Infect. Immun. 61 (1993) 391–398.
[89] H.K. Lee, J.H. Park, K.C. Kwon, Double-walled microparticles for single shot vaccine, J. Control. Release 44 (1997) 283–293.
[90] A.G.A. Coombes, D. Major, J.M. Wood, D.J. Hockley, P.D. Minor, S.S. Davis, Resorbable lamellar particles of polylactide as adjuvants for influenza virus vaccines, Biomaterials 19 (1998) 1073–1081.
[91] A.K. Hilbert, U. Fritzsche, T. Kissel, Biodegradable microspheres containing influenza A vaccine: immune response in mice, Vaccine 17 (1999) 1065–1073.
[92] Z. Moldoveanu, M. Novak, W.Q. Huang, R.M. Gilley, J.K. Staas, D. Schafer, R.W. Compans, J. Mestecky, Oral immunization with influenza virus in biodegradable microspheres, J. Infect. Dis. 167 (1993) 84–90.
[93] R. Ray, M. Novak, J.D. Duncan, Y. Matsuoka, R.W. Compans, Microencapsulated human parainfluenza virus induces a protective immune response, J. Infect. Dis. 167 (1993) 752–755.
[94] C.D. Partidos, D.M. Shaw, B. Gander, H.P. Merkle, C.R. Howard, M.W. Steward, Induction of antibody responses to a synthetic peptide entrapped in biodegradable microparticles, Vaccine Res. 3 (1994) 203–209.
[95] P.A. Marx, R.W. Compans, A. Gettie, J.K. Staas, R.M. Gilley, M.J. Mulligan, G.V. Yamshchikov, D. Chen, J.H. Eldridge, Protection against vaginal SIV transmission with microencapsulated vaccine, Science 260 (1993) 1323–1327.
[96] T.E. Greenway, J.H. Eldridge, G. Ludwig, J.K. Staas, J.F. Smiths, R.M. Gilley, S.M. Michalek, Enhancement of protective immune responses to Venezuelan equine encephalitis (VEE) virus with microencapsulated vaccine, Vaccine 13 (1995) 1411–1420.
[97] M.J. Alonso, S. Cohen, T.G. Park, R.K. Gupta, G.R. Siber, R. Langer, Determinants of release rate of tetanus vaccine from polyester microspheres, Pharm. Res. 10 (1993) 945–953.
[98] A. Sanchez, B. Villamayor, Y. Guo, J. McIver, M.J. Alonso, Formulation strategies for the stabilization of tetanus toxoid in poly(lactide-co-glycolide) microspheres, Int. J. Pharm. 185 (1999) 255–266.
[99] M. Singh, D. Thassu, G.P. Talwar, Vaccine delivery systems: Effect of microsphere size on the immune response, Proc. Int. Symp. Control. Release Bioact. Mater. 21 (1994) 881–882.
[100] T. Kissel, Y.X. Li, C. Volland, S. Gorich, R. Koneberg, Parenteral protein delivery systems using biodegradable polyesters of ABA block structure, containing hydrophobic poly(lactide-co-glycolide) A blocks and hydrophilic poly(ethylene oxide) B blocks, J. Control. Release 39 (1996) 315–326.
[101] R.K. Gupta, P. Griffin Jr., R. Rivera, G.R. Siber, Development of an animal model to assess the immunogenicity of single-dose tetanus and diphtheria vaccines based on controlled release from biodegradable polymer microspheres, Dev. Biol. Stand. 92 (1998) 277–287.
[102] B.R. Conway, J.E. Eyles, H.O. Alpar, A comparative study on the immune responses to antigens in PLA and PHB microspheres, J. Control. Release 49 (1997) 1–9.
[103] A.G.A. Coombes, E.C. Lavelle, S.S. Davis, Biodegradable lamellar particles of poly(lactide) induce sustained immune responses to a single dose of adsorbed protein, Vaccine 17 (1999) 2410–2422.
[104] J.P. McGee, S.S. Davis, D.T. O'Hagan, The immunogenicity of a model protein entrapped in poly(lactide-co-glycolide) microparticles prepared by a novel phase separation technique, J. Control. Release 31 (1994) 55–60.
[105] R. Nakaoka, Y. Tabata, Y. Ikada, Adjuvant effect of biodegradable poly(DL-lactic acid) granules capable for antigen release following intraperitoneal injection, Vaccine 14 (1996) 1671–1676.
[106] B.R. Conway, J.E. Eyles, H.O. Alpar, Immune response to antigen in microspheres of different polymers, Proc. Int. Symp. Control. Release Bioact. Mater. 23 (1996) 335–336.
[107] J.L. Pedraz, M. Igartua, R.M. Hernandez, A. Esquisabel, A.R. Gascon, B. Calvo, Long-term immune response in mice following subcutaneous administration of BSA-PLGA microspheres, Proc. Int. Symp. Control. Release Bioact. Mater. 24 (1997) 879–880.
[108] A.G.A. Coombes, E.C. Lavelle, P.G. Jenkins, S.S. Davis, Single dose, polymeric, microparticle-based vaccines: The influence of formulation conditions on the magnitude and duration of the immune response to a protein antigen, Vaccine 14 (1996) 1429–1438.
[109] H. Rafati, E.C. Lavelle, A.G. Coombes, S. Stolnik, J. Holland, S.S. Davis, The immune response to a model antigen associated with PLG microparticles prepared using different surfactants, Vaccine 15 (1997) 1888–1897.
[110] J. Hampl, M. Dittrich, J. Franz, S. Reschova, J. Stepanek, Adjuvant activity of linear aliphatic polyester and branched aliphatic oligoester microspheres, Int. J. Pharm. 144 (1996) 107–114.
[111] D. O'Hagan, H. Jeffery, M.J. Roberts, J.P. McGee, S.S. Davis, Controlled release microparticles for vaccine development, Vaccine 9 (1991) 768–771.
[112] D.T. O'Hagan, H. Jeffery, S.S. Davis, Long-term antibody responses in mice following subcutaneous immunization with ovalbumin entrapped in biodegradable microparticles, Vaccine 11 (1993) 965–969.
[113] H.O. Alpar, A.J. Almeida, Identification of some of the physicochemical characteristics of microspheres which influence the induction of the immune response following mucosal delivery, Eur. J. Pharm. Biopharm. 40 (1994) 198–202.
[114] H.O. Alpar, J. Akbuga, J.E. Eyles, E.D. Williamson, Immunological responses following oral and intra-muscular administration of PLA microspheres manufactured using surfactants with adjuvant properties, Proc. Int. Symp. Control. Release Bioact. Mater. 24 (1997) 785–786.
[115] E.C. Lavelle, P.G. Jenkins, J.E. Harris, Oral immunization of rain-

bow trout with antigen microencapsulated in poly(DL-lactide-co-glycolide) microparticles, Vaccine 15 (1997) 1070–1078.
[116] R. Nakaoka, Y. Tabata, Y. Ikada, Antibody production by administration of biodegradable granules incorporating antigen through different injection routes, J. Control. Release 40 (1996) 11–21.
[117] T. Uchida, S. Martin, T.P. Foster, R.C. Wardley, S. Grimm, Dose and load studies for subcutaneous and oral delivery of poly(lactide-co-glycolide) microspheres containing ovalbumin, Pharm. Res. 11 (1994) 1009–1015.
[118] M. Singh, J.P. McGee, X.M. Li, W. Koff, T. Zamb, C.Y. Wang, D.T. O'Hagan, Biodegradable microparticles with an entrapped branched octameric peptide as a controlled-release HIV-1 vaccine, J. Pharm. Sci. 86 (1997) 1229–1233.