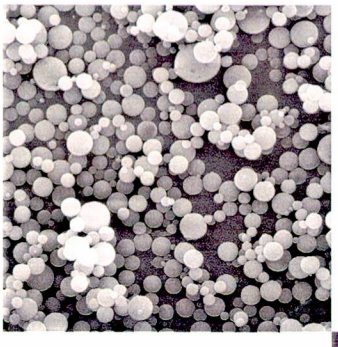
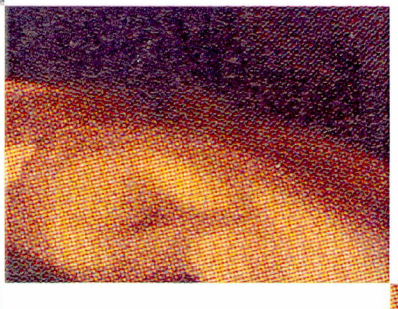
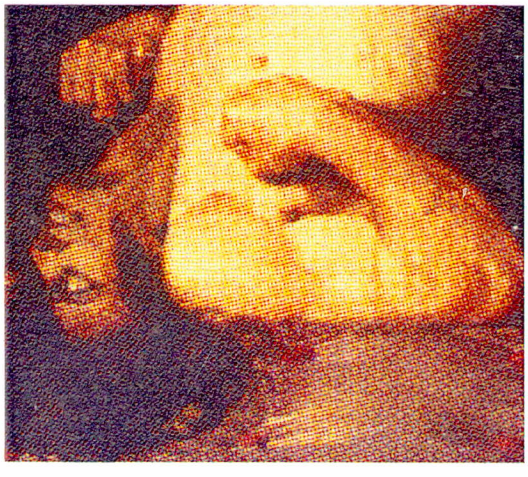
SINGLE-DOSE VACCINES



on engineering and quality assessment of PLA/PLGA microsphere-based vaccines

Pål Johansen





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ETH Dissertation No. 12764

SINGLE-DOSE VACCINES

on engineering and quality assessment of PLA/PLGA microsphere based vaccines

Dissertation ETH Zurich

submitted for the degree of
Doctor of Natural Sciences

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Zurich, 31.7.1998

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Preface

Despite continuous improvements in therapeutic and prophylactic health care, approximately 17 million deaths were caused by infectious diseases in 1996. To prevent further mortalities, political will and improved medical support are needed, and indeed the planning of preventative measures are placed high on the agenda of multinational organisations such as UNO, EU, the World Bank, and Red Cross. The WHO-governed *Expanded Programme on Immunisation* and *The Children's Vaccine Initiative* have improved world wide vaccination coverage significantly since their commencement in 1974 and 1990, respectively. However, much effort has been put on developing new or better vaccines, and a milestone would be the introduction of a single-dose vaccine which reduces the number of repeated administrations required for long term protection.

Biodegradable microspheres prepared with the polyester poly(lactide) and poly(lactide-co-glycolide) have demonstrated their potential in vaccine formulations. Investigations both in vitro and in vivo have revealed that antigens can be encapsulated in microspheres, released at a controlled rate over several months, and be efficiently presented to the immune system. Hence, a microsphere-based vaccine can be designed to mimic the conventional immunisation schedule in delivering both priming and booster doses. The overall objective of this doctoral study was to develop an efficient single-dose vaccine against tetanus. Major focus was on stabilising tetanus toxoid contained in microspheres.

In this doctoral study, progress towards the realisation of a single-dose vaccine based on poly(lactide) and poly(lactide-co-glycolide) will be demonstrated. Focusing on tetanus and diphtheria toxoids – a single-dose vaccine against tetanus being a priority project of WHO – different approaches to improve the toxoid encapsulation and stability in microspheres, the toxoid release from the microspheres, and their immunogenicity have been studied; this is reviewed in chapter 1. The absence of booster effects in animals so far, after a single injection of tetanus toxoid containing microspheres, has been ascribed to toxoid stability problems. Therefore, chapter 2 and 3 describe how excipients and polymers can be used to stabilise the toxoid during encapsulation and to improve the release performance from polyester microspheres. In chapter 4 and 5, the immunological evaluation of these stabilised vaccines is performed in mice and guinea pigs, and to summarise, the *co*-encapsulation of additives appears to be crucial to the vaccine's efficacy. This is further verified in chapter 6, which is an investigation on the physico-chemical, conformational and antigenic stability of tetanus and diphtheria toxoids in aqueous solutions stressed by elevated temperature and acidic moieties.

The frequently reported incomplete release of proteins from polyester microspheres is, in chapter 7, demonstrated to be partly due to the experimental set-up which very much influences the observed release. For example, toxoid adsorption to both glass and microspheres was significant.

The last chapter, chapter 8, describes an approach to improve protein encapsulation in microspheres by a thermodynamic tool. Thermodynamically expressed interactions between the formulation components were used to predict protein entrapment and release from poly(lactide) microspheres.

Presently, a scaling-up process of the developed technologies, and the preparation of sterile clinical samples for the first ever human trial with a single-dose vaccine against tetanus, is under

way. The initiation of the clinical trial signifies the importance of the reported results, is a natural epilogue of this pharmaceutical-biological thesis, and should eventually encompass an important progress towards the world wide introduction of single-dose vaccines: a type of vaccine, which would be of tremendous importance for vaccination programmes and routines, their compliance, and the immunisation efficacy in developing countries: «a giant leap for mankind».

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Nothing said above or below will more than hint at the number of and nature of my personal obligations to the many individuals whose suggestions, criticism and support in general have at one time or another sustained or directed my technical skills or intellectual development. Much time has elapsed since the rather amorphous ideas of this thesis began to take shape. A list of all those who may properly find some signs of their influence in its written result would be almost co-extensive with a list of my friends and acquaintances. Under the circumstances, I must restrict myself to the few most significant influences that even a faulty memory will never entirely suppress.

It was Bruno Gander who first introduced me to the art of applied pharmaceutical technology and who initiated the transformation of my conception of the nature of pharmacy in general, and of immunisation in special. Ever since that process began, he has, being my supervisor, been generous with ideas, criticism, and time - especially including the time required to read and suggest important changes in the draft of my manuscripts. Further, I could never have get to this point if not my *patriarch* Hans P. Merkle had not magnanimously allowed and encouraged me to pursue my own interests. These two gentlemen's open minds, but comprehensive, intelligent and complementary critics helped me turn amorphous ideas into a mature scientific work.

Furthermore, I am very indebted to friends, acquaintances, colleagues, and other 'resources', with whom I have collaborated, who have given me feedback of various kind and helped me sorting out fragments of ideas and hypothesis to a more developed and intelligible scientific material which finally became ripe for a doctoral thesis. The incomplete list of these persons include, in alphabetical order, Régine Audran, Giampietro Corradin, Reinhard Glück, Hans Hengartner, Nam-Trân Ho, Ying Men, Linda Moon, Wolfgang Sadée, Steven P. Schwendeman, Dorothea Sesardic, and last but not least Harjit Tamber.

My final acknowledgements, to my father and my wife, must be of a rather different sort - although the latter assisted me on linguistic and stylistic parts of the manuscript. In ways which I shall probably be the last to recognise, both have contributed intellectual ingredients to my work. But they have also done something more important. They have let the work go on and even encouraged my devotion to it. Anyone who has wrestled with a project like mine will recognise what it has occasionally cost them. Thanks a lot.

Zurich, Switzerland P.J.

Contents

Works and Projects

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Engineering of Antigen Loaded PLA/PLGA Microspheres for Single-Dose Immunisation

Assessment of Quality and Efficacy

ABBREVIATIONS

PLA, poly(lactic acid); PLGA, poly(lactic-*co*-glycolic acid); MS, microspheres; APC, antigen presenting cell; MHC, major histocompatibility complex; ER, endoplasmatic reticulum; mAb, monoclonal antibody; Ig, immunoglobulin

INCOMPLETE WORLD WIDE VACCINATION

The introduction of antibiotics and vaccines has greatly improved the global health situation. To-date, vaccines perhaps represent the most successful medical development ever but due to insufficient immunisation, epidemiological diseases still are the leading killer among all diseases. 17 million deaths in 1996, one third of the total number world wide, were due to infectious diseases [1]. The WHO-governed Expanded Programme on Immunisation (since 1974) and The Children's Vaccine Initiative (since 1990) have, however, improved the world wide vaccination coverage significantly, and within the year 2000, the WHO intends to eliminate or to eradicate several infectious diseases such as leprosy, poliomyelitis and neonatal tetanus [1]. Apart from increasing vaccination coverage, novel or improved vaccines would reduce infectious diseases. Here, a new era in vaccinology is developing which includes traditional antigens, recombinant protein antigens, sub-unit vaccines, and the use of genetic material (DNA or mRNA) where the induction of immune responses follow an *in vivo* expression of a protein antigen subsequent to the introduction of sequences encoding that protein [2,3]. Finally, and the main topic of this review, a major step toward a better vaccine and vaccination coverage would be the introduction of single-injection vaccines to reduce the number of repeated booster administrations required for long-term protection [4].

BIOCOMPATIBLE AND BIODEGRADABLE MICROSPHERES AS SINGLE-DOSE VACCINES: THE TECHNOLOGY

Polymers as vaccine carriers

The potential of biodegradable polymers for controlled delivery of drugs over a prolonged period has been described [5]. These delivery systems have also been applied in the development of new vaccines and adjuvants by encapsulation of antigens in polyester microspheres (MS) (Table I). Moreover, such polymeric MS have shown strong adjuvant properties and may replace or complement aluminium salts (alum), which have been used since 1926 [6] for antigen immune-potentiation. MS vaccines against epidemic diseases such as diphtheria, tetanus, malaria, hepatitis B, measles and HIV [7-12] are also under development for both parenteral and mucosal immunisation. These would be of utmost benefit for developing countries [13,14], where health conditions are poor and most individuals do not return for booster doses.

Control of antigen release at predetermined rates or pulses over a defined time period is mediated by the polymer type and physico-chemical properties [5,15,16]. By using different polymers with different degradation and release characteristics, one can mimic the conventional multiple vaccination schedule [4,17,18]. MS can be designed to release the antigen at fast or slow rates and in a continuous or pulsatile manner [19-21]. Further, MS surface properties as well as size may affect the interaction of particle with the antigen presenting cell [22,23] and must be considered with respect to how the antigen is recognised by the immune system. For illustration, gelatine MS have shown some interesting properties [24]. When cross-linked at low concentrations of glutaraldehyde, they swelled to a higher degree in aqueous solution, leading to an increase in the size of MS. The increased size caused a decrease in phagocytosis, whereas the antigen release rate increased when the concentration of cross-linking agent was low. The balance of the two factors, the MS' susceptibility to phagocytosis and the rate of antigen release, affected the efficacy of gelatine MS to induce antibody response [24].

Several polymers have been investigated for the purpose of engineering single-dose vaccine delivery systems [20,21,74,75], although only few are approved for parenteral use, e.g., polyester of lactic and glycolic acids (PLA and PLGA). These polymers have in common that, upon hydrolysis, they produce carboxyl acid moieties (monomers and oligomers). Ionised carboxylates are electrophiles and can react with protein nucleophiles such as amines, hydroxyl and sulphhydryl residues, which can be detrimental to antigen stability [76]. For small sub-unit antigens with little secondary and tertiary structure, the chemical stability may be most critical,

whereas high molecular weight antigens are sensitive to both physical and chemical changes. For illustration, antigen instability has been described as the major hurdle for the development of a single-dose MS formulation against tetanus [14]. The search for a long-term release system for antigens has, therefore, turned out to be a compromise between the sustained release from possibly hydrophobic polymers, which may be combative to antigens, and the use of more antigen-compatible hydrophilic polymers, which on the other hand are associated with faster release [77]. However, still no clear consensus has so far been constituted with regard to which type of release kinetics is optimal for achieving long-term immunity [14,31]. The optimum is presumably dependent on the antigen recognition pathway (see below). Therefore, still much effort in MS vaccine research is focused on polymer types and properties as well as their effect on antigen presentation and recognition.

Table I - Microsphere based single-dose delivery vaccines with clinical relevant antigens.

Antigen	Comment	Reference
Tetanus toxoid	Jackson *et al.*: oral vaccine	[8,20,25-41]
Diphtheria toxoid		[7,42-44]
Ricin toxoid		[45,46]
B. pertussis	Fimbriae	[47-49]
Cl. botulinum	Type A botulism made using a recombinant C fragment antigen	[50]
HIV-1	MN rgp120	[51-53]
Rabies	T_H and B-cell epitope from virus: Peptides 31D and V10c-31	[54]
Pb CS 252-260	Synthetic peptide form the *circumsporozoite* (CS) protein of *Plasmodium berghei* (pb)	[9]
Multiple antigen peptide, MAP	T_H cell epitopes: MAP P30P2 and MAP $(NANP)_6P2P30$	[9,20,55]
R5	B-cell epitope from the syncytial virus	[56]
HSD-DT	Birth control vaccine (diphtheria toxin conjugated)	[57-59]
SEB	Staphylococcal enterotoxin B	[60,61]
VEE	Venezuelan equine encephalomyelitis virus is a mosquito-borne arbovirus	[62]
E. coli	Enterotoxigenic E. coli fimbrial adhesion, CFA/I, which elicits anti-colonization immunity	[63-66]
Influenza	Formalin-inactivated influenza virus type A: A/H3N2	[67-71]
HBsAg	Recombinant Hepatitis B surface antigen	[10,72,73]

Microsphere preparation methods

For reasons of injectability and a possible targeting of antigen to antigen presenting cells, a MS vaccine should consist of dry particles of a few micrometers or smaller which are dispersible in aqueous solutions. Larger particles or aggregates of smaller particles may not be injectable through conventional needles, or may not be phagocytosed for antigen presentation. The latter may cause weaker immune responses, in particular against antigens which follow an intracellular recognition pathway (T-cell priming or MHC restriction). MS preparation methods, such as solvent evaporation, solvent extraction, coacervation and spray-drying, have previously been described [33,40,78-84] and reviewed [31,85,86] and are be presented here.

Criteria for MS-based single-dose vaccines

There are several requirements to be met for a MS-based single-dose vaccine (Table II). From a toxicological viewpoint, vaccine excipients such as polymers and additives must be biodegradable and histocompatible [87]. The MS should exert adjuvancy, i.e., enhanced immunogenicity, which is of particular importance for weakly immunogenic sub-unit antigens, and for immune responses restricted to the intracellular major histocompatibility complex (MHC) pathway. Here,

the MS must be of suitable size to permit uptake by antigen presenting cells such as dendritic cells or macrophages. Furthermore, the entrapment of antigen in the MS should be efficient and not compromise the antigenic stability, especially at slightly elevated temperatures; this is especially important in developing countries in order to obviate the need for cold transport and storage chain. A single-injection vaccine should of course be injectable with conventional needles and sterile, the latter being achieved by aseptic processing, gamma irradiation or electron beam radiation. However, radiation can, by formation of free radicals upon radiation [88], accelerate polymer degradation [89,90], alter the release kinetics [91,92], and may compromise the antigen integrity [26]. Finally, single-dose MS vaccines may produce long-term immunity, similar to conventional vaccination.

Table II - Selection criteria for MS-based single-dose vaccines.

Efficient antigen entrapment in MS	Sterility	Biodegradability
Stability of antigen, polymer and excipients	Adjuvancy	Histocompatibility
Injectability	Immunogenicity	

Polymer solvent selection

Almost all manufacturing technologies take use of organic polymer solvents for MS preparation. Although many solvents may be suitable for the microencapsulation process, several aspects such as polymer solubility and safety in humans (USP XXIII, 1995) are to be considered, and solvent residuals in MS have been reported [93]. The solvent should not chemically or physically affect antigen stability, and when pertinent, provide a stable W/O-emulsion during production without extended use of emulsifiers.

These are severe restrictions that rule out the selection of several polymer solvents. Some researchers made attempts to use more rationally organic solvent properties for finer tuning of MS manufacturing. For the solvent evaporation technique, successful drug entrapment was associated with a fast rate of precipitation of the polymer from the organic solvent and a high concentration of the polymer in the organic phase [95]. This was achieved by constructing a phase diagram for the polymer during MS formation based on the three-dimensional solubility parameter concept. A similar method has been used to optimise polymer solvent combinations for the coacervation process [81,82,96,97]. By the same token, thermodynamic tools were used in an attempt to control the efficiency of protein encapsulation in spray-dried MS [94,98]. Here, microencapsulation was studied taking quantitatively into account the molecular interactions between polymer, solvent and the aqueous protein phase (Fig. 1). Entrapment efficiency increased and burst release diminished when polymer-drug interactions were dominant over polymer-solvent and drug-solvent interactions. By this approach, the authors also constructed binary solvents, the properties of which correlated well with the theoretical model (Fig. 1), and which resulted in an efficient encapsulation of albumin in PLA MS.

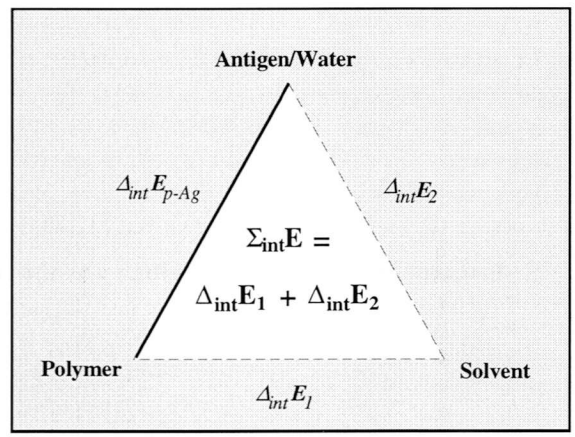

Figure 1 - Schematic model of intermolecular interactions taking place during the microencapsulation of an antigen into spray-dried polyester microspheres. Good product quality (efficient antigen encapsulation and low burst release) is expected if polymer-antigen/water ($\Delta_{int}E_{p\text{-}Ag}$) interactions dominate over the sum of polymer-solvent ($\Delta_{int}E_1$) and solvent-antigen/water ($\Delta_{int}E_2$) interactions [94].

Vaccine stability

Functional antigen stability is the first requirement for success, but often a major obstacle for formulation of biodegradable MS vaccines, and knowledge about the mechanisms associated with the loss of stability and related chemical and structural changes would help the rational development of antigen delivery systems. Degradation pathways for protein antigens can be separated into two classes, chemical and physical, which both by consequence affect the biological stability of the antigen. Chemical instability is any process involving changes in the protein primary structure via bond formation or cleavage, isomerisation, or via changes of the amino acid side group residues. Physical instability (non-covalent changes) refers to changes in the higher order protein structure (secondary and above) [99]. These latter include unfolding, aggregation, and precipitation, as well as adsorption to surfaces, and the consequence of this may be biological instability. Under certain circumstances, stabilisation strategies can be mutually exclusive. By biochemical means, proteins are most stable in solutions near their isoelectric pH, but physically least stable due to aggregation phenomena and adsorption on hydrophobic surfaces [100]. Moreover, in a molten-globule state, a protein is partially unfolded, has less tertiary structure and is much more reactive to most side reactions, leading to an irreversibly denaturated state [101,102].

During preparation, testing, storage, and biological application of antigen loaded biodegradable MS, the antigen is subjected to several processes which may be deleterious to its immunogenic function. MS are most often prepared from an emulsion, where an antigen dissolved in water is dispersed in an organic solution of the polymer. The surface-active antigen will diffuse towards the W/O-interface, where it may undergo structural changes [100]. Usually, the W/O-emulsion is homogenised by ultrasonication presumably producing a local temperature rise, strong mechanical forces on the antigen, and an increase in relative W/O-interface area. Homogenisation can also induce cavitation, which is often associated with protein inactivation. During MS preparation, solvent removal or MS hardening involve transport of solvent from the particle, which may cause structural perturbations of the antigen. Similarly, drying or lyophilisation of antigen containing MS have been shown to cause protein denaturation [103]. Finally, upon incubation in an aqueous medium (buffer or physiological fluid), water can enter the MS and cause hydration of the antigen. Indeed, proteins may be prone to moisture-induced aggregation, a phenomena reported to be more severe at slow partial hydration than when the antigen is dissolved [104,105]. Furthermore, dissolved antigen may be inactivated preferentially by hydrolysis and adsorption on surfaces. Eventually *in vivo*, local inflammation, enzymatic activity, and cellular interactions may further influence antigen stability and, hence, vaccine efficacy.

Stabilising additives in microspheres

In 1989, the WHO initiated a project aimed at developing a MS-based single-dose vaccine against neonatal tetanus, which still accounts for the death of about 450,000 children and 30-60,000 childbearing women and mothers who just have given birth [14]. Subsequently, technological tools were established, but the project long failed to be promoted to a clinical investigation phase, primarily due to an insufficient booster response in animals. This was ascribed to insufficient tetanus toxoid stability within the MS exposed to *in vivo* conditions over a prolonged time period.

One approach to overcome the problem of instability was to *co*-encapsulate putative additives known to have a stabilising effect on proteins (Table III). The use of additives such as salts, non-reducing sugars and polyols, minimise the free energy of the native protein by increasing the number of water molecules bound to the surface [106]. Salts may also have buffering effect and counteract the pH drop produced by polymer degradation. For example, acetate salts can scavenge liberated protons and create an acetate buffer along with further polymer degradation, diminishing the detrimental effect of low pH on protein stability. However, soluble salts may diffuse out of the polymer matrix through pores before the polymer starts degrading. Therefore, insoluble buffer salts are preferable, and indeed, calcium carbonates and phosphates have shown promising effects on tetanus toxoid loaded MS both *in vitro* [40], in mice [41], and, as a result of

excellent immune responses also in guinea pigs over 40 weeks (personal communication from Sesardic *et al.*, NIBSC, UK-Hertfordshire), clinical trials with tetanus toxoid loaded MS are presently under planning. By the same means, improved stability and controlled release of a human growth hormone (rhGH) was obtained by complexation with zinc salts prior to the microencapsulation [107,108]. This procedure reduced the aggregation of the protein significantly and facilitated a zero order release of intact rhGH over one month. However, it should be noted that the zinc salt was a carbonate which probably also raised the microclimate pH in the microspheres and by this means stabilised rhGH.

Table III - Additives used to stabilise proteins in polyester microspheres (MS). Abbreviations: BSA is bovine serum albumin; CA is carbonic anhydrase; CD is cyclodextrin; HPC is hydroxypropyl cellulose; OPV is Oral poliovirus vaccine; PEG is poly(ethylene glycol); PEO is poly(ethylene oxide); rhEPO is recombinant human erythropoietin; rhGH is recombinant human growth hormone Ttxd and Dtxd are tetanus and diphtheria toxoid, respectively;. a): Deuterium was not studied in OPV-containing MS.

Class of stabilisers	Additive	Stabilising principle	Protein	Reference
Salts	$CaCO_3$, $Ca_3(PO4)_2$, $CH_3COO^-Na^+$, $ZnCO_3$, Al_2O_3, $Al(OH)_3$	Buffering the pH drop inside the MS. Adsorbed peptide stabilised in the solid state. Additional adjuvance.	Ttxd, Dtxd, rhGH	[26,40,41,107,108, 113]
Surfactants	Poloxamer	Stabilisation by saturation of W/O- interphases. Reduction of MS water uptake by hydrophobic compounds.	Ttxd, rhEPO, CA	[34,40,114, 115]
Proteins	Gelatine, Albumin, IL-5, IL-6	Stabilise toxoid by electrostatic and hydrophobic interaction and may reduce adsorption of protein drugs to surfaces and interfaces by competition. Cytokine stimulate/support antigenicity.	Ttxd, Dtxd, rhEPO, CA, BSA	[27,40,44, 114-116]
Sugars, amino acids	Trehalose, Mannitol, Na-Glutamate, L-Arginin, CD	Trehalose enhance Ttxd loading. CD and L-Arginin stabilised rhEPO against aggregation.	Ttxd, Dtxd, rhEPO	[27,34,40,44, 114,115,117]
Fatty acids	Ethyl stearate, Caprylate	Reduction of MS water uptake and reported thermal stabilisation.	Ttxd	[40]
Alternative solvents	Heavy water (D_2O), Mineral oil	H-bonds strengthened by D_2O. Toxoid in oil-based cores entrapped within the MS.	OPV [a], Ttxd	[39,118,119]
Polymers	HPC, PEG, PEO	Double-walled particles prevent direct contact with PLGA MS. PEO assists refolding of molten-globule CA.	HBsAg Ttxd, CA	[34,115,120]

Large-size solutes such as sugars, polymers, polyols, anionic and non-ionic surfactants can stabilise the native state of proteins across the whole liquid exposed protein region by means of solvent exclusion [109] or by immobilisation of proteins. This is in agreement with data on the ELISA-antigenicity of tetanus toxoid which was partly preserved using additives such as poloxamers, trehalose and cyclodextrins [40,41]. Further, if protein denaturation occurs at the interface between water and polymer solution during MS preparation, surface active additives such as proteins and surfactants should counteract denaturation by reducing exposure and accumulation of protein antigens at the interface [34,40]. Moreover, stabilising effect of albumin (BSA and HSA) and gelatine on tetanus toxoid have been demonstrated [27,40,41,110]. Although HSA showed good stabilising properties, some authors [27] did not recommend its use, because HSA may become immunogenic by alterations of some of its epitopes upon MS preparation which could consequently lead to autoimmune reactions in humans. On the other hand, proteins can accelerate the degradation of polyesters by establishing an electric potential at the hydrophobic molecular interface between protein and polymer [111]. This may attract hydrogen ions, which directly affect polymer hydrolysis [112]. Furthermore, the hydrophilic character of most proteins can permit, when encapsulated, enhanced swelling of the polymer, causing increased antigen release rate of a more continuous pattern.

A further approach to improve the immune response to antigen loaded MS includes the encapsulation of both antigen and an adjuvant into PLA/PLGA MS. One study investigated the *co*-encapsulation of rgp120 and QS-21, an adjuvant of the saponin family, in PLGA MS [51]. The data showed improved stability of both rgp120 and QS-21 which both followed a similar release pattern despite of their different molecular weights. In contrast, investigations in mice on microencapsulated tetanus toxoid adsorbed on alum showed weaker immunogenicity than for both alum adsorbed tetanus toxoid and encapsulated tetanus toxoid without alum [26]. However, mice may be less sensitive to tetanus toxoid than, e.g., guinea pigs [14], or the doses used in mice were traditionally too high to allow differentiation in quantitative terms between different MS formulations and the alum formulation [8,41].

Quality Assessments of MS Vaccines

Estimation of antigen content

A important step in the quality control of antigen containing MS is the determination of the antigen content which may be defined as both the physical content of protein material within the MS and the content of antigenic reactive material. Several methods have been described, all of which have their *pros* and *cons* and may lead to different conclusions (Table IV). Only radio-labelled antigen may give a confident estimation of content in MS [34]. Radio-analysis is further beneficial since the antigen is determined directly in the solid state of the MS and not after additional processing of the MS, which itself can affect the estimated amount of antigen. On the other hand, labelling efficiency and radioactive quenching within the polymer may represent unknown variables affecting the analysis. Protein loaded MS may be dissolved in an organic solvent, typically dichloromethane, and the insoluble protein filtered and eluted into a buffer solution [83] or by extraction of protein upon addition of water to the organic polymer solution [37]. These methods have been reported to cause an underestimation of BSA, ovalbumin and lysozyme [44,121]. On the other hand, dichloromethane did not harm tetanus toxoid antigenicity during recovery of the antigen, as did polymer hydrolysis in a solution of sodium hydroxide and sodium dodecyl sulphate (SDS) at 50°C [122]. The same was observed for diphtheria toxoid [44], but again, polymer dissolution in dichloromethane caused an underestimation of toxoid loading in the MS as compared to polymer hydrolysis. Carbonic anhydrase in MS was analysed by loading the MS directly to the sampling zone of a SDS-PAGE gel, and the protein was extracted by an electric field [115]; only a semi-quantitative estimation of antigen content in MS was feasible. On the other hand, it was possible also to study aggregation upon preparation or release, as aggregated proteins were more strongly retarded in the gel than non-aggregated proteins. Furthermore, various analytical tools exists for estimation of antigen content in MS. These methods include either chemical assays (amino acid composition, nitrogen assay or protein assay), physical methods (spectrophotometry and radioactivity), immuno-chemistry (ELISA), or combinations of different methods, e.g., blotting and radio immuno assay (RIA). Each method, however, may itself influence the determination validity.

Table IV - Methods to assess antigen content in microspheres.

Estimation method	Comment
Polymer dissolution and protein elution	Underestimation of antigen, antigen maintains antigenicity.
Protein extraction	Underestimation of antigen, antigen maintains antigenicity.
Polymer hydrolysis	Good estimation of protein content, antigen loses antigenicity.
SDS-PAGE	Semi-quantitative method, aggregation may be detected.
Radioactive labelling	High estimation efficiency made from solid microspheres.

Generally, microencapsulation method, polymer type and formulation additives all may influence the estimation of antigen content inside MS [37]. Typically, differences in estimated antigen loadings were very notable for coacervated MS preparations [44]. This was presumably

associated with the presence of additives, especially amphiphilic additives, and residual solvents of both aqueous and organic nature [93], exerting a solubilising or emulsifying effect on the antigen extracted. Such a mixture of hydrophilic/hydrophobic compounds may also create new interfaces accessible to proteins, and therefore, be subject for further inactivation of the antigen.

In conclusion, it is of high importance to distinguish between the amount of protein and the amount of antigen and, hence, to interpret the obtained results with respect to the quality of the vaccine formulation. However, it appears that one method alone is not appropriate to assess different formulations. Hence, assessment of protein content from MS clearly requires optimisation and validation of the chosen method for each individual formulation type, and standard protocols are elusive.

Experimental design and evaluation of antigen release

Alum and W/O-emulsions provide a short-term depot of 8-10 days for antigens trapped at the injection site [123,124]. It has also been shown that tetanus toxoid was released from alum and IFA *in vitro* within the same time frame [125]. This may support the current assumption that antigen release from PLA/PLGA MS should follow a pulsatile pattern where each single dose is separated in time, but released within a short period, hence mimicking the doses of conventional vaccines. So far, the scientific basis for this rationale is still vague.

Development and quality assurance of MS vaccines also imply the characterisation of *in vitro* antigen release. One difficulty for rationally designing a pulsatile antigen release system is the frequently observed incomplete release from MS, as observed for albumin [126], carboxyanhydrase [115], interferon [127], and tetanus and diphtheria toxoids [27,40,122,125]. *In vitro* release experiments are commonly performed in glass or plastic tubes by dispersing the particles in a buffer and subsequent incubation at 37°C under agitation [94]. However, the experimental set-up exerts a great effect on the release of drugs from PLA MS [128,129]. In case of protein drugs and vaccines, a major problem may also arise from the acidic polymer degradation products, which can compromise the stability of the released protein, as can other interactions between polymer and protein as well. Typically, aggregation during release may cause loss of protein [76,114,130]. Thus, during *in vitro* release, continuous elimination of the mono- and oligomeric lactates and glycolates from the release medium by dialysis partly circumvented aggregation [131]. Further, protein adsorption on surfaces (e.g., on container for release test and on polymeric MS) may influence the release observed during a given release test. General mechanisms of and conformational changes upon protein adsorption on glass surfaces have been revealed [132] as well as has adsorption of BSA, IgG, calcitonin or tetanus toxoid on PLGA MS [25,133-135].

An evaluation of the in vitro release of tetanus toxoid from PLGA MS showed that toxoid adsorption on glassware and polymers was of high relevance as the measured amounts of released protein were greatly deceased by concomitant surface adsorption [125]. Importantly, particular attention should be paid to this process when release experiments are performed over a prolonged period of time, during which only minute amounts are released. As the release medium is generally replaced by fresh medium at regular intervals, low toxoid concentrations are maintained and, therefore, adsorption may become more important than release. Fig. 2 emphasises the practical importance of the *in vitro* release medium. In PBS, the maximum fraction of available tetanus toxoid varied between 30 and 80%, depending on the initial toxoid concentration. However, incubation of tetanus toxoid and MS in PBS containing small amounts of albumin significantly reduced the toxoid adsorption.

A further parameter of great importance for protein adsorption on glass and MS surfaces is the pH of the incubation solution [132,136]. This normally drops during degradation of polyester MS, possibly to different extents outside and inside the MS. Affinity normally increases with decreasing repulsion between the protein and the sorbent, and maximum adsorption is observed at the isoelectric point of the protein. The observed *in vitro* release of antigens from PLA/PLGA MS, therefore, depends on the specific evaluation conditions. As the antigen strongly adsorbs to hydrophobic surfaces, the significance of release data is limited, and attention should be paid to selecting appropriate experimental conditions.

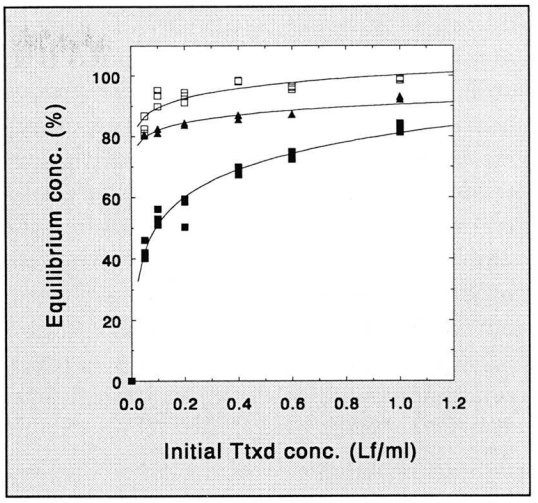

Figure 2 - Free tetanus toxoid in solution (equilibrium concentration) after incubation at 37°C with PLGA placebo MS containing trehalose (■) and with PLGA MS containing BSA and trehalose (▲) in PBS or incubation with PLGA placebo MS in PBS containing 0.2% BSA (□) (n = 3). The lines represent a logarithmic fit in order to visualise the trend and do not reflect a kinetic evaluation [125].

The analytical method applied to study release may make interpretation difficult [40]. Fig. 3 illustrates this for tetanus toxoid containing PLGA MS; ELISA and fluorimetry were the analytical methods, and trehalose and albumin were additives *co*-encapsulated with the toxoid. For MS without additives, a pulsatile release profile was obtained by fluorimetry, whereas a non-pulsatile and a largely incomplete release was observed by ELISA. When trehalose was *co*-encapsulated, a higher burst release (24 h) and a less significant second release pulse was observed by fluorimetry, whereas a non-pulsatile release was determined by ELISA. Here, trehalose caused some increase in the cumulative amount of toxoid released as compared to MS without trehalose. Finally, when albumin was *co*-encapsulated, a pulsatile and a more complete toxoid release was determined by ELISA. Supported by other studies in aqueous solutions [110], this was interpreted as an increased functional stability of the antigen in the presence of albumin.

Based on characteristics such as antigen content and release from MS, vaccine candidates may be selected for further investigations both *in vitro* and *in vivo*. However, the importance of antigenic and immunogenic reactivity and unique antigen release kinetics from MS is ambiguous, and it depends on the mechanism in which immunity finally is developed after immunisation. In the following, this is discussed further.

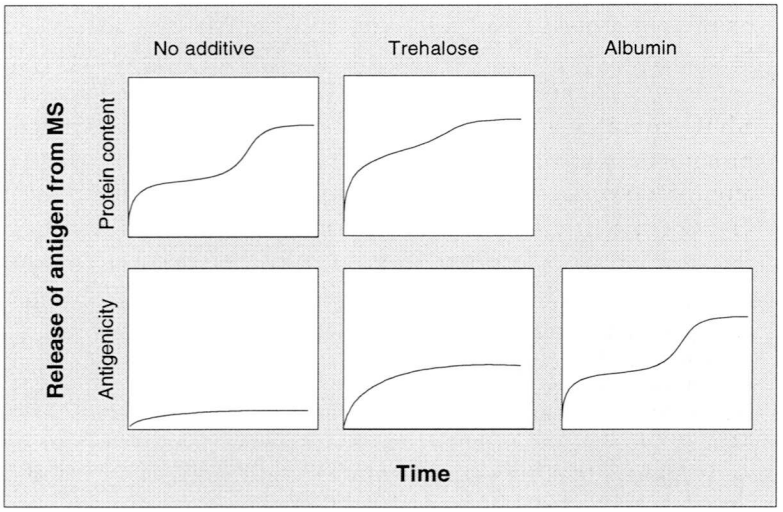

Figure 3 - Illustration of the effect of evaluation method and of co-encapsulated excipients on release kinetics of antigen loaded PLGA MS. By physical methods (e.g. spectroscopy), antigen release is described as protein content released, whereas by immuno chemical methods (e.g. ELISA), antigenicity is assayed. (See Ref. 40 and 110.)

Limitations to develop criteria for antigenicity of microencapsulated antigens

Antigens are molecules recognised specifically by T- and B-cells. By definition, two properties should be distinguished: *immunogenicity*, the capacity of an antigen to induce an immune response, and *antigenicity*, the capacity of the antigen to be recognised by specific receptors

expressed by T- and B-cells [137]. During encapsulation, storage, and release from MS, an antigen may undergo conformational changes (Fig. 4). As most antigens have several antigenic epitopes, conformational changes can lead to transformation (exposure and internalisation) of epitopes and, hence, alter the peripheral exposure and the affinity of the epitopes to antibodies (Ab), especially to mAb specific for a particular epitope as illustrated in Table V. In contrast, a more crude mixture of antibodies (polyclonal) could recognise the antigen despite intramolecular epitope transformation; mAb are often used as analytical tools to assess vaccine quality. When developing assays for antigens, consequences of structural alterations should be considered. A mAb assay, which recognises a single epitope and reacts with the antigen in its native state, may have limitations in as much as the antibody reacts with a single epitope only, although other may also exist. This may cause false negative interpretation of antigenicity and unnecessary rejection of candidate vaccines. For illustration, MS vaccines against tetanus, which did not show antigen content and release when determined by ELISA with mAb [40], did in fact show good immune responses in mice [41].

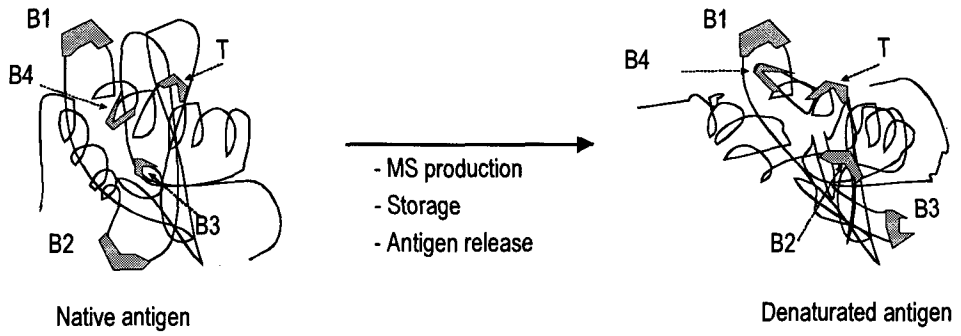

Figure 4 - Illustration of events leading to changes in antigen structure, and possibly function as denaturation may produce changes in antigenicity, i.e., binding affinity of the B-cell epitopes (B1-B4) to their respective antibodies. See also Table V.

Prone even more to controversy is the use of mAb in the evaluation of MS vaccines when the antigen is intended for targeting to antigen presenting cells (APC). This is the case for T-cell priming and vaccination against invasive pathogens. Here, the endocytosis of antigen loaded MS or the pinocytosis of antigen released from MS extracellularly is followed by antigen processing in the lysosome, during which the protein antigen is degraded to a linear peptide sequence of approx. 13-17 amino acids (Fig. 5). This peptide replaces the chaperon in the endogenous class II MHC complex and is finally expressed on the APC surface as a T-cell epitope. Now, T-cell receptors can recognise sequences of amino acids in the MHC groove, rather than protein shapes (conformational determinants) which are recognised by B-cell immunoglobulines (Ig). Considering that T- and B-cells normally recognise different epitopes, in search for MS vaccines for which reaction with mAb is used as the selection criterion (as in ELISA), again, potential vaccine candidates may be excluded because the antibodies recognise other epitopes than those responsible for induction and development of immunity.

Table V - Possible effects of antigen denaturation on binding affinity of monoclonal antibodies (mAb) to antigen B-cell epitopes (B). See also Fig. 4.

Antibody-epitope interaction	Affinity to native antigen	Affinity to denaturated antigen
mAb1 - B1	high	high
mAb2 - B2	high	low
mAb3 - B3	low	high
mAb4 - B4	low	low

Long-term immunity against micro-organisms of which elimination is mediated by antibody responses, can only be achieved by boosting the memory B-cell pool and neutralising IgG. This is the case for most non-invasive bacteria such as *C. diphtheriae, V. cholerae,* and *Cl. tetani.* MS vaccines may induce this by releasing the antigen, the conformation of which is recognised by B-cell receptors and circulating IgG. In this situation, an *in vitro* evaluation of the vaccine potential by using antibodies is relevant and an appropriate measure for functional and, hence, conformational stability of the antigen. However, mAb may have limitations in as much as antigens often have more than one immunogenic determinant (see Fig. 4). A conformational change causing the suppression of one epitope which under normal circumstances induces the production of neutralising IgG, may engender immunogenicity of other epitopes which were previously not accessible for reaction with T- and B-cell receptors and IgG due to the antigen's folded nature. Further, after T-cell priming, B-cell receptors and circulating Ig have low specific affinity. Therefore, more than one mAb, or even polyclonal antibodies or antiserum may be beneficial for detection of antigen loading or antigen release from MS vaccines, since other potentially immunogenic epitopes may be recognised and captured.

On the other hand, polyclonal antibodies may also capture epitopes which do not give rise to lymphocyte proliferation, production of neutralising Ig, and increased frequency of high affinity B-cell receptors. This may cause false positive results or an overestimation of antigen content encapsulated in or released from MS. Therefore, the *antigenicity criterion* in the design of MS-based vaccines has limitations which complicate the technological development of such vaccines. So far, animal experiments seem to be the only outcome of this conflict. However, to keep costs down and research on new vaccines delivery systems alive, new and safer methods need to be developed, which will allow selection of novel vaccines at an early stage in the development.

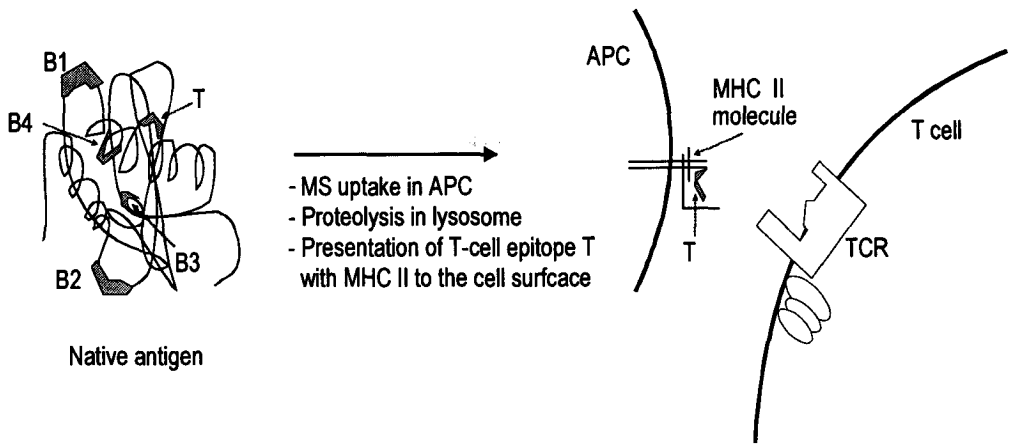

Figure 5 - Schematic illustration of cellular and molecular events during MHC class II restricted antigen presentation that cause loss in protein structure, antigen processing and exposure of the linear T-cell epitope (T), often buried within the antigen. (B1-B4 represent B-cell epitopes.)

Invasive pathogens such as *M. tuberculosis, M. leprae, L. monocytogenes,* many protozoa and most viruses, have in common that their elimination from the host organism is associated with immune responses where humoral immunity plays a limited role only (e.g., in aggregating pathogens and thereby, reducing the infectivity to neighbouring cells and tissues and increasing the likelihood of phagocytosis). Here, a cell-mediated and non-specific innate immune response is more important. Innate immune mechanisms include production of cytokines such as IL-2 and IFN-γ, which down-regulate bacterial or viral replication or stimulate phagocytic or cytotoxic cells in killing infected cells, while T-cells are alerted to viral and intracytoplasmic bacterial infections by cell surface MHC complexes. Contrasting the thymus dependent antibody response, cytotoxic T-cell responses are results of antigen determinant presentation with class I or class II MHC molecules to mature $CD8^+$ or $CD4^+$ T-cells. For MS-based vaccines, class I MHC antigen presentation pathways represent a major challenge in as much as the particles are normally endocytosed and degraded in the lysosome and not in the cytoplasm. Generally then,

the antigen would not associate with the endoplasmatic reticulum (ER) for reaction with class I MHC molecules. Still though, cytotoxic T-cell responses have been observed after immunisation of mice against malaria with a specific short epitope CTL peptide entrapped in MS [9]. The authors explained the response either due to delivery of exogenous antigen from the phagosome to the cytosol, which has been reported [138], or to direct binding of released antigen to class I MHC molecules on the cell surface. However, both mechanisms are somewhat speculative. Class I MHC molecules do not generally become loaded with peptides following processing of exogenous antigens [139]. They are unstable on the cell surface unless already occupied with peptide [140] and are not found in the compartments where class II MHC become loaded [141]. The binding of a free peptide to a membrane bound MHC complex implicates the replacement of another peptide already located in the MHC groove. The affinity of peptides to membrane MHC complexes are generally low and would require very high plasma concentrations of the peptide antigen (personal communication from D. Rognan), which is normally not obtained with conventional vaccine doses. Therefore, still much needs to be resolved with respect to the effect of cellular and molecular mechanisms on MS-based vaccination. However, other mechanisms and observations support the CTL response found after injecting malaria antigen-containing MS. If extracellularly released, antigen from MS is taken up in the cytosol of a cell by pinocytosis and its interaction with the ER may be feasible [142,143]. It has furthermore been suggested that only particulate antigens would be processed by phagocytic APCs for class I MHC presentation [144]. In line with this, the model antigens OVA and *E. coli* β-galactosidase primed CTL responses *in vivo* more efficiently than when solubilised [145]. In addition, lipid-encapsulated antigen [146], antigen on beads [147], or aggregated antigen [148-150] were effective in priming $CD8^+$ T-cells *in vivo*. Whether such associated or aggregated antigens are detectable *in vitro* is questionable, as far as the *antigenicity criterion* concerns, creating a Heisenbergian *uncertainty principle* that limits the value and *in vivo* correlation of immuno-chemical methods used for selecting MS vaccine candidates.

VACCINE DESIGN

Induction of immunological memory is described as the result of increased precursor frequencies of specific resting T- or B-cells [151] and is crucial to vaccine efficacy [152]. The exact biological mechanism required to establish and maintain immunological memory is, however, not well defined, which is unfortunate for researchers in vaccine development. Two mechanisms are described inducing memory that must be considered when designing new vaccines and vaccine delivery systems. First, immunological memory can be induced by a persistent, low-level *in vivo* stimulation by antigens. This drives the differentiation of B-cells into plasma cells which is of particular importance for the protection against non-invasive bacteria and cytopathic viruses [153]. Furthermore, memory cells are maintained in an activated state [154]. Second, if memory cells are long-lived, they can be committed without the requirement for continued stimulation by antigens [155]. Eventually, rational vaccine design relies on several other factors which preferably should be perceived and controlled, such as the biological mechanism for immunity which may differ between different pathogens, the molecular determinants for antigen recognition and induction of immune responses, in which way the vaccine is targeted to the effector site, and which adjuvants that are needed to obtain primary and secondary responses. Consequently, adjuvant is of crucial importance for the onset of the immune response but will, however, never correct the choice of the wrong epitope [156].

In the case of polymeric MS as the antigen delivery system and adjuvant, physico-chemical properties, such as size, hydrophobicity, erosion type and rate, mainly determine the vaccine efficacy [22,23]. Small and hydrophobic MS may be better targeted to the intracellular MHC pathway. This applies for action against viral and intracellular bacterial infections, and for T-cell priming in general. Nevertheless, the induction of class I MHC restricted immune responses is still of capital challenge. In contrast, boosting of antibodies, expressed and secreted by the B-cells, is better encountered using large particles, which are not easily endocytosed and release freely antigen upon polymer degradation. Depending on how the polymer degrades, this can give

rise to either a continuous (upon surface erosion) or a pulsatile (upon bulk erosion) antigen release, the former of which supports the theory of persisting stimulation by antigens [154], the latter supporting the theory that memory cells are long-lived and can be committed without the requirement for continued stimulation [155] (see above). On the other hand, it has been criticised that neither continuous nor pulsatile release pattern take into account the phenomenon of affinity maturation [157], which occurs during the secondary response and is inversely related to the antigen dose administered. That is, high doses produce poor maturation as compared to low doses. Hence, in the interest of vaccine safety as well as specificity of immune protection, boosting doses of antigen from MS should be low. On the other hand, several micro-organisms, e.g., HIV and influenza viruses, can undergo frequent genetic mutations inducing chemical and physical alterations in lymphocyte receptors and Ig determinants. This can affect their affinity for antigens, and therefore, such antigen vaccines may be given at higher doses, which can counteract the lower affinity.

An important criterion in vaccine production is that the physico-chemical integrity and the biological activity of the antigen are preserved. Therefore, many investigators on MS-based vaccines have put much effort into the study of antigen stability [40,76,108, 110]. However, if the antigen is to be loaded on class II MHC molecules, one might be doing oneself a disservice focusing on antigen stability *per se*, since the protein antigen is degraded intracellularly to a small peptide before inducing any immune responses. Lifting the embargo on presentation of MS associated antigens on class I MHC molecules has yet to be clarified mechanistically. This is of chief interest since exogenous antigens on class I MHC may promote $CD8^+$ T cell responses to a broader range of bacterial, viral or even tumour antigens than is likely to be synthesised *de novo* within professional APCs [158]. Such access may follow phagocytic or macropinocytic capture of antigens [142,143]. In line with this concept, the conventional class I pathway may also induce more useful effector CTL because it seems unlikely that phagosomal processing would reproducibly generate the same profile of T-cell epitopes generated by the proteasome/TAP system in the post-Golgi pathway [158]. Finally, nucleic acid-based immunisation represents a special case since the vaccine (DNA or mRNA) is to targeted to the nucleus of APCs. This is additionally complicating the immunisation mechanism as compared to class II MHC restricted immunisation (targeting to endosomes) and class I MHC immunisation (targeting to the ER); for detailed information on nucleic acid vaccines, the reader is referred to other publications [3,159,160]. Hence, as far as vaccine stability is concerned, it is important to distinguish between physico-chemical and functional stability. Dependent on which presentation pathway the antigen takes (e.g., IgG, class I or class II MHC, or cell nucleus), vaccine stability must be defined and interpreted differently. Conformational changes and even chemical cleavage of the antigen may to a certain extent be favourable in one situation, but detrimental to vaccine efficacy in another.

To what extent long-term immunity is achievable with MS-based vaccines, it remains to be seen as more as the data of several ongoing projects become available and reviewed, but recent efforts with both conventional and sub-unit antigens show promising results with respect to the introduction of single-dose vaccines. A breakthrough in the developments of single-dose vaccines may significantly improve the global vaccination coverage, simplify immunisation programmes like those governed by the WHO, and be a giant leap for developing countries where health conditions are poor and most individuals do not return for their booster doses.

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2

Improving Stability and Release Kinetics of Microencapsulated Tetanus Toxoid by co-Encapsulation of Additives*

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* Pharmaceutical Research 15: 1103-1110 (1998). Associated paper: Audran et al. Enhanced *immunogenicity of microencapsulated tetanus toxoid with stabilizing agents*. Pharmaceutical Research 15: 1111-1116 (1998) (see chapter four).

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INTRODUCTION

The need for four to five vaccination sessions during the first two years of life is still a great hurdle in vaccination programs especially in developing countries [1-3]. Therefore, vaccine delivery systems have been proposed that are expected to deliver the necessary booster doses after a single injection [4,5], or which could be administered orally [6,7] or nasally [8].

In this respect, biodegradable microspheres (MS) made of poly(lactic acid) (PLA) or poly(lactic-*co*-glycolic acid) (PLGA) have been found particularly useful in animal models [9-11]. PLA/PLGA MS provide adjustable pulsatile antigen release patterns [12] and improve the immunogenicity of poorly immunogenic antigens [13]. In case of tetanus toxoid (Ttxd) loaded MS, antibody and T-cell responses were satisfactory, comparable to or even higher than those obtained with alum adsorbed toxoid. However, the expected booster effect in mice and monkey have not yet been achieved unambiguously. A potential drawback of PLA/PLGA MS is their production of acidic moieties during biodegradation. These components (lactic and glycolic acid, oligomers) are likely to affect the biological activity of proteins. Based on previous results, antigen instability is assumed to contribute to the often observed incomplete protein release and, consequently, to the lack of booster effect observed so far in animals after single parenteral administration [12].

Protein denaturation processes were ascribed to interactions with organic solvents or polymer residuals, or to aggregation of protein molecules [14,15]. Attempts to counteract loss of activity of microencapsulated proteins comprised varying the polymer hydrophilicity and molecular weight [16], altering the *in vitro* release testing by dialysis of the acidic polymer degradation products [17], and finally chemical modifications of the protein [18]. Surprisingly, few published studies have considered the *co*-encapsulation of stabilising additives [19,20], although protein stabilisation is very common in other pharmaceutical dosage forms [21,22]. These studies have shown that additives including proteins, lipids, polyols and surfactants, may stabilise biologically active proteins.

Here, we report on attempts to enhance encapsulation efficiency, preserve antigenicity, and optimise the release pattern of Ttxd from PLGA and PLA MS (see also associated paper, Audran *et al.*). Specifically, the effect of *co*-encapsulating various agents known for their protein stabilising properties were investigated: (i) trehalose, serum albumin and cyclodextrins as commonly known stabilisers for peptides and proteins; (ii) poloxamer L101 and L121 [23] and ethyl stearate as hydrophobic additives to lower the water content of the MS during release; (iii) calcium carbonate, calcium orthophosphate and sodium acetate as buffer salts to counteract the pH drop within the MS during hydrolytic polymer degradation; (iv) D_2O for strengthening the H-bonding in the toxoids [24]. Based on the *in vitro* results shown, the stabilisers albumin and trehalose may be clinically of the utmost advantage for MS as a single-dose vaccine delivery system.

MATERIALS AND METHODS

Materials

Aqueous solutions of tetanus toxoid (Ttxd), provided by WHO, were from Massachusetts Public Health Biological Laboratories, Boston, MA, (Ttxd, lot No. PSTtxd-20: 1400 Lf/ml, 42 mg/ml, or 333.3 Lf/mg protein nitrogen) and from Pasteur Mérieux, F-Lyon (Ttxd lot No. PTC 10005: 8500 Lf/ml, 26.3 mg/ml, or 323.2 Lf/mg protein nitrogen); concentrations were determined by the manufacturers. Poly(d,l-lactic-*co*-glycolic acid) (PLGA 50:50) with a M_W of approx. 12 kDa, and poly(d,l-lactic acid) (PLA) with a M_W of 129,7 kDa were purchased from Boehringer Ingelheim, D-Ingelheim (Resomer RG502 and RG502H, R206). Bovine serum albumin (BSA) for immuno enzyme assay was from Fluka, CH-Buchs, and human serum albumin (HSA) was from the Swiss Red Cross, CH-Bern. Horse anti-tetanus IgG (Ter21) and horse radish peroxidase conjugated anti-sheep tetanus IgG (SATS-PO) used in the enzyme-linked immunosorbent assay (ELISA), were from RIVM, NL-Bilthoven. Unless specified otherwise, all

other substances used were of pharmaceutical or analytical grade and purchased from commercial suppliers.

Preparation of microspheres

PLGA microspheres (MS) were prepared by spray-drying (Büchi 190, CH-Flawil) a dispersion of aqueous toxoid solution in a 5% (w/w) solution of PLGA in ethyl formate as described elsewhere [25]. The following additives were individually *co*-encapsulated: BSA, trehalose, calcium carbonate, calcium orthophosphate, sodium acetate, the poloxamers Synperonic PE/L121 and PE/L101 (ICI, Wilton, CT), α-cyclodextrin (α-CD), β-cyclodextrin (β-CD) and γ-hydroxypropylcyclodextrin (γ-HPCD) (Wacker-Chemie, CH-Liestal), ethyl stearate and caprylic acid. The additives were dissolved in either the organic or the aqueous phase. Those insoluble in either of the two phases were dispersed in the emulsion by magnetic stirring. PLA MS were manufactured by coacervation as described elsewhere [26]. Briefly, an aqueous phase containing Ttxd, BSA, and calcium carbonate was dispersed in 5% (w/w) PLA in dichloromethane. Coacervation was induced by adding silicone oil (1070 mPa.s; Fluka, CH-Buchs), and the hardening of the coacervate droplets took place in octamethylcyclotetrasiloxane (Abil K-4; Goldschmidt, D-Essen).

Determination of toxoid entrapment

Total protein content of the MS was determined by dissolving the loaded MS in dichloromethane, recovering the undissolved protein on a 0.2 µm regenerated cellulose filter (RC 58, Schleicher and Schuell, D-Dassel) and eluting the protein from the filter with 67 mM PBS of pH 7.4. The proteins (Ttxd and accompanying proteins from *Cl. tetani*) were assayed fluorometrically, whereas antigenically reactive Ttxd was determined by ELISA. Nominal loading means the theoretical amount of Ttxd encapsulated, and encapsulation efficiency means the experimentally determined amount of Ttxd encapsulated relative to the nominal loading. All determinations were made in triplicate.

In vitro release of toxoid

Toxoid release from 20 - 40 mg MS was conducted in 4 ml 67 mM PBS of pH 7.4 containing 0.02% sodium azide in rotating borosilicate vials at 37°C and assayed by ELISA or fluorimetry. During release, the pH of the release medium was monitored and kept constant. At regular intervals, 1 ml of the medium was replaced by fresh buffer and analysed by fluorimetry or by ELISA, after separation of the particles and supernatant by centrifugation at 3,500 rpm for 10 min. Release experiments were performed in triplicate. In an additional experiment, the pH-change of the incubation medium was studied as a function of buffer strength (10, 67 and 150 mM PBS) and amount of *co*-encapsulated calcium carbonate (0, 10, 20 and 30%, w/w); during this experiment, the release medium was not replaced.

Fluorimetrical assay of proteins

Ttxd encapsulated in or released from the MS were analysed fluorimetrically using excitation and emission wavelengths of 279 and 329 nm, respectively (Fluromax, Spex, Edison, NJ). When BSA was *co*-encapsulated, fluorimetry was not used, except in one experiment where total protein, i.e., Ttxd and BSA, remaining in the MS after 80 days release was detected.

The validation of the fluorescence method showed that the presence of the additives in the Ttxd solution did not influence the fluorescence intensity of Ttxd at various relevant additive concentrations (0.5 - 1.5 mg/ml) and incubation time (two weeks). Furthermore, during Ttxd release, only negligible shifts in the emission maximum were observed (329 nm ± 1 nm), which did not affect the emission intensity as Ttxd produced an emission plateau between 327 and 331 nm.

Enzyme-linked immunosorbent assay of tetanus toxoid

Flat-bottom 96-wells microtiter plates (Nunc-Immuno Plate Maxisorb, Nunc, DK-Roskilde) were filled with 100 µl of horse anti-tetanus IgG, 1 AU/ml, in 50 mM carbonate buffer pH 9.6,

and incubated at 4°C overnight. The plates were washed four times with 300 µl of 0.05% polyethylene sorbitan monolaurate (Tween 20) and 0.05% Na_2HPO_4 in water after each incubation step. After 1 h incubation at 37°C with 150 µl of 150 mM PBS pH 7.4 containing 0.5% BSA (PBS-BSA), the plates were incubated 2 h at 37°C with serial dilutions of standard and test solutions of Ttxd in PBS-BSA. Horse radish peroxidase conjugated anti-sheep tetanus IgG was added to each well in 100 µl of PBS-BSA, and plates incubated for another 2 h. Finally, 100 µl of 0.2 mg/ml peroxidase substrate 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (Sigma Chemical, St. Louis, MO) in 100 mM NaH_2PO_4 solution pH 4.0 was added to the plates, and the kinetics followed at 405 nm (Thermomax, Molecular Devices, Menlo Park, CA).

RESULTS

Microencapsulation of tetanus toxoid and of additives

The main purpose of this study was to screen a variety of formulation parameters to improve encapsulation efficiency, preserve antigenicity, and optimise *in vitro* release of encapsulated toxoid. The tested parameters were the type of toxoid, the nominal core loading, the type and amount of *co*-encapsulated additives, and the type of polymer. In the first series of preparations, low molecular weight PLGA 50:50 was selected because of its relatively short degradation time of 30 to 60 days. Thus, stability and *in vitro* release could be tested within a reasonable time frame. Various classes of potentially stabilising additives were selected, as specified above. The nominal amount of additives for *co*-encapsulation was between 1 and 20%, depending on the type of additive. We assumed that this concentration range should be sufficiently high for potential toxoid stabilisation, but not too excessive to compromise the formation of a release controlling PLGA matrix. The nominal toxoid loadings were between 0.4 and 3.5 Lf/mg MS (approx. 0.12 - 1.06%, w/w). Lower loading was were used to screen the effect of the various additives and higher values to optimise the more promising preparations.

For *co*-encapsulation, the additives albumin, trehalose, γ-HPCD, poloxamers, ethyl stearate, sodium acetate and D_2O were dissolved in either the aqueous toxoid solution (W) or the organic polymer solution (O), whereas α- and β-cyclodextrin and the calcium salts were finely suspended in the performed W/O-dispersion. Preliminary experiments showed substantial additive depots on the walls of the glassware, when the latter additives were introduced prior to ultrasonication. This resulted in low protein loadings (15 - 30%) of PTC (results not shown) suggesting that Ttxd was adsorbed on the additive particles. However, when the additive powders were introduced after W/O formation and dispersed by magnetic stirring, of Ttxd entrapment was significantly higher, i.e., higher than 70% for PTC (Table I).

The two types of methods used for measuring the toxoid content of the MS, i.e., fluorimetry and ELISA, showed a marked difference between protein and antigenically reactive toxoid. Depending on the formulation, protein encapsulation efficiencies (by fluorimetry) varied between 23 and 94% and those of ELISA responsive antigen between 0.2 and 43% (Tables I and II). This indicates substantial loss of ELISA-antigenicity during encapsulation or re-extraction of the toxoid from the MS. Without additives, the fluorimetrically determined encapsulation efficiency was 74% for PTC and 27 - 30% for PSTtxd-20. The corresponding ELISA values were 6.1 and 0.2% (Table I). *Co*-encapsulation of the additives influenced the encapsulation efficiency of PTC variably and PSTtxd-20 favourably.

When trehalose was *co*-encapsulated, the encapsulation efficiency in terms of protein content (fluorimetry) was over 90% for both Ttxd type (Table I). More interestingly, a substantial increase of ELISA-antigenicity was noted for the purified PSTtxd-20 (from 0.2 to 12.6%), but not for PTC. When BSA was used as an additive, loadings were determined only by ELISA. Encapsulation efficiency of ELISA responsive PSTtxd-20 increased from 0.2 to 10.8% with 1% (nominal) *co*-encapsulated BSA, and to approx. 30% at 5% BSA. Interestingly, comparable encapsulation efficiencies of approx. 30% of antigenic material were obtained for the two different nominal toxoid loadings, i.e., 0.7 and 3.5 Lf/mg corresponding to the determined loadings of 0.22 and

0.96 Lf/mg. As with trehalose, *co*-encapsulated BSA increased the antigen encapsulation efficiency more for PSTtxd-20 than for PTC. With the additive mixture trehalose/BSA, no further augmentation of antigen encapsulation efficiency was achieved.

Table I - Tetanus toxoid loadings of selected PLGA 50:50 (Resomer RG502) microsphere preparations as measured by fluorimetry and by ELISA. Nominal content and determined loading are relative to microsphere mass (w/w). a): HSA was used instead of BSA in view of using most promising preparations for clinical trials. n.d.: loading was not determined.

Additive		Determined toxoid loading (Lf/mg)		Encapsulation efficiency (%)	
Type	Nominal content (%)	Fluorimetry	ELISA	Fluorimetry	ELISA
Tetanus toxoid, PTC (8500 Lf/ml) Nominal loading: approx. 0.4 or 0.9 Lf/mg					
-	-	0.67	0.06	74.2	6.1
Trehalose	15	0.85	0.05	93.5	5.8
BSA	5	-	0.63	-	17.9
α-CD	10	0.69	0.03	75.7	3.3
β-CD	15	0.70	0.06	76.2	6.7
γ-HPCD	15	0.81	0.09	90.0	10.3
L 101	19	0.45	0.03	50.0	3.5
L 121	19	0.62	0.01	68.5	1.5
Ethyl stearate	15	0.54	0.06	59.9	6.3
$CaCO_3$	7	0.27	0.02	69.0	3.9
$CaCO_3$	15	0.63	n.d.	69.5	n.d.
$Ca_3(PO_4)_2$	15	0.11	0.01	27.5	2.5
Sodium acetate	15	0.31	0.04	34.8	4.2
Tetanus toxoid, PSTtdx-20 (1400 Lf/ml, column purified) Nominal loading: approx. 0.7 or 3.5 Lf/mg					
-	-	0.20	<0.01	27.0	<0.2
-	-	1.10	0.01	30.1	0.2
Trehalose	19	3.25	0.44	92.3	12.6
BSA	1	-	0.08	-	10.8
BSA	5	-	0.22	-	31.7
BSA	5	-	0.96	-	27.6
Trehalose/BSA	15/5	-	0.98	-	29.8
Trehalose/HSA [a)]	15/5	-	0.64	-	31.2
γ-HPCD	19	0.25	n.d.	33.8	n.d.
L 121	19	0.25	n.d.	33.8	n.d.
$CaCO_3$	15	0.18	n.d.	24.1	n.d.
$Ca_3(PO_4)_2$	15	0.17	n.d.	22.8	n.d.

Table II - Tetanus toxoid loadings of MS preparations made with a low MW hydrophilic PLGA 50:50 analogue (Resomer 502H) and with a high MW PLA (Resomer 206). Toxoid content was assayed by ELISA, nominal content and determined loading are relative to microsphere mass (w/w), and PLA MS were prepared by coacervation. a): HSA was used instead of BSA in view of using most promising preparations for clinical trials.

Polymer	Additive		Determined toxoid loading (Lf/mg)	Encapsulation efficiency (%)
	Type	Content (%)		
Tetanus toxoid, PTC (8500 Lf/ml), Nominal loading: 3.5 Lf/mg				
PLGA 50:50	-	-	0.74	21.2
PLGA 50:50	Trehalose	20	1.10	31.4
PLGA 50:50	BSA	5	1.09	31.4
PLGA 50:50	Trehalose/BSA	15/5	1.49	42.7
Tetanus toxoid, PSTtdx-20 (1400 Lf/ml), Nominal loading: 2.1 Lf/mg				
PLGA 50:50	Trehalose/BSA	15/5	0.84	40.2
PLA	$CaCO_3$/BSA	15/5	0.55	26.6
PLA	$CaCO_3$/HSA [a)]	10/5	0.57	27.5

Co-encapsulation of hydrophobic additives (poloxamer L101, L121 and ethyl stearate) generally lowered the entrapment of Ttxd (Table I). Similar observations were made with sodium

acetate. Calcium carbonate and orthophosphate, and α- and β-cyclodextrin had only minor effects. γ-HPCD, however, increased the encapsulation efficiency for both the fluorimetrically measured total protein (90% for PTC and 34% for PSTtxd-20) and ELISA responsive antigen (10% for PTC).

When using the hydrophilic PLGA 50:50 analogue RG502H (carrying free carboxylate end groups), the encapsulation efficiency of antigenic Ttxd (PTC) was substantially increased, i.e., from 6% for the standard PLGA 50:50 without additive to 21% for RG502H without additive, 31% (with trehalose or BSA) and 43% (with trehalose/BSA) (Table II). With PSTtdx-20, the additive mixture trehalose/BSA preserved 40% of the nominal ELISA-antigenicity. Hence, the combination of trehalose and BSA led to an additive effect on antigen encapsulation in hydrophilic PLGA 50:50. Incidentally, this additive effect was not observed with the standard PLGA 50:50. Thus, *co*-encapsulation of trehalose and BSA in RG502H MS produced the maximum antigen entrapment of all the 60 batches prepared in this study.

The more hydrophobic, high molecular weight PLA in combination with the additives $CaCO_3$/BSA and $CaCO_3$/HSA gave satisfactory ELISA responsive antigen encapsulation efficiencies of approx. 27% (Table II). Thus, the more hydrophobic PLA behaved similarly to standard PLGA 50:50 with respect to ELISA-antigenicity of encapsulated Ttxd.

Toxoid release

In vitro release of Ttxd (PTC and PSTtdx-20 preparations) was studied from various PLGA MS using both fluorimetry and ELISA. Both the total amount released and the release kinetics strongly depended on the *co*-encapsulated additive and the type of polymer used. The fast degrading PLGA 50:50 formulations generally showed either a triphasic release pattern with an initial burst, a dormant period of 2 - 4 weeks and a second release pulse, or a biphasic release composed of an initial burst and a subsequent sustained release phase.

For the PSTtxd-20 preparations containing nominal 3.5 Lf/mg MS and 19% trehalose, the ELISA responsive release showed a pronounced initial burst and a subsequent more constant release phase reaching approx. 0.21 Lf/mg MS (Fig. 1). A rather promising release pattern was obtained with the MS containing both additives BSA and trehalose (nominal 5 and 15%, respectively). The total PSTtdx-20 amount released was 0.5 Lf/mg MS corresponding to 50% of the determined dose. The release pattern again consisted of an initial burst (approx. 0.2 Lf/mg MS) followed by a constant release of the remaining 0.3 Lf/mg MS over 45 days.

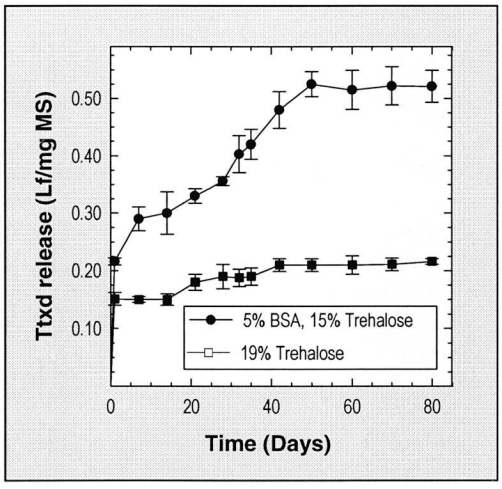

Figure 1 - Release of PSTtdx-20 from BSA and trehalose stabilised PLGA 50:50 MS as measured by ELISA. Nominal loading was 3.5 Lf/mg MS.

A biphasic release pattern of ELISA responsive PSTtxd-20 (Fig. 2) and PTC (Fig. 3) was observed with most of the MS preparations except those containing no additive or the additive BSA. With the low nominal core loading of PSTtdx-20 MS (all 0.7 Lf/mg), the amount of toxoid released was modest, but improved significantly by the use of the additives (Fig. 2). With nominal 1 and 5% *co*-encapsulated BSA, the release pattern became pulsatile. The dormant

period lasted from day 1 to day 21 or 28, and the second release pulse occurred between days 21 and 42 (with 1% *co*-encapsulated BSA) and between days 28 and 48 (with 5% BSA). Interestingly and supporting the previous results from the 3.5 Lf/mg MS, the preparation with 5% BSA released the highest total amount of ELISA responsive toxoid, i.e., 0.06 Lf/mg MS, corresponding to approx. 8.5% of the nominal or 27% of the detected toxoid loading. The *co*-encapsulated calcium carbonate and orthophosphate had only negligible effect on PSTtdx-20 release as compared to the MS free of additive (Fig. 2). A slight improvement in the total amount of released antigen was observed with *co*-encapsulated γ-HPCD, poloxamer L121 and trehalose. Nonetheless, the release kinetics of these preparations were unsatisfactory, since over 70% of the total antigen dose was released during the first 24 hours.

The ELISA responsive and fluorimetrically determined release of PTC toxoid was similar to that of PSTtdx-20 (Fig. 3). With *co*-encapsulated poloxamer L101, a substantial ELISA responsive burst release of 35 - 50% of the total dose was observed after 24 h. This release behaviour was also representative for the MS containing poloxamer L121, trehalose and γ-HPCD (data not shown). Conversely, MS without additive or with calcium carbonate yielded a lower burst (ELISA), followed by no or only a weak additional release. Similar profiles were obtained with *co*-encapsulated calcium phosphate and ethyl stearate (data not shown). When the PTC release from MS was assayed fluorimetrically rather than by ELISA, a three- to sixfold higher total release, with a pulsatile pattern was observed. Considering all formulations, burst release of protein was in the order of 2 - 30% of the total dose. In general, additives increased the burst, with trehalose and γ-HPCD exerting the most dominant effect.

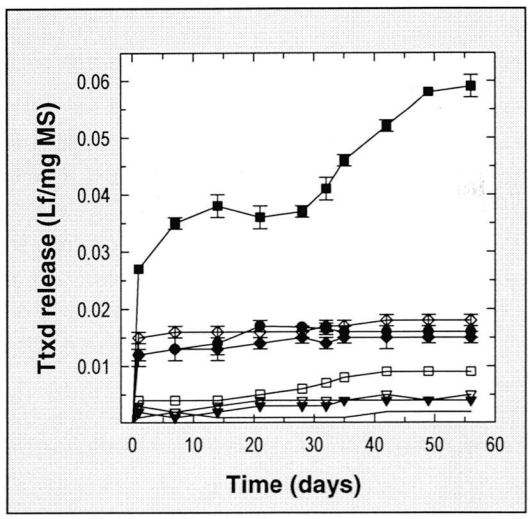

Figure 2 - Release of PSTtdx-20 from PLGA 50:50 MS with various stabilisers as measured with ELISA. Nominal loading of tetanus toxoid was 0.7 Lf/mg MS (■: BSA 5%; □: BSA 1%; ▼: $CaCO_3$ 15%; ▽: Ca3(PO4)2 15%; ●: γ-HPCD 19%; ◆ Poloxamer L101 19%; ◊: trehalose 19%; continuous line: no additive).

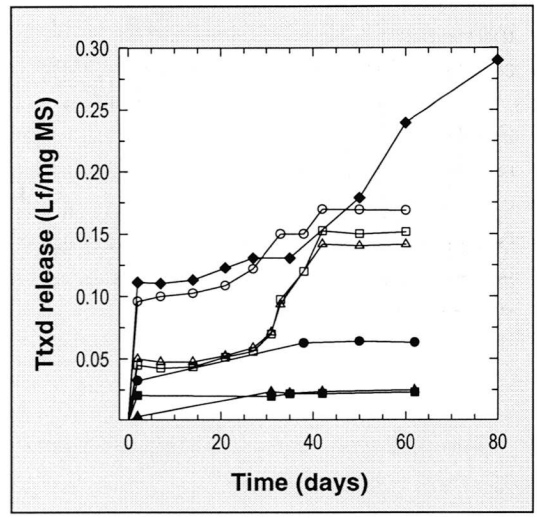

Figure 3 - Release of PTC from PLGA 50:50 MS as assayed by ELISA (filled symbols) and fluorimetry (open symbols). Nominal loadings were approx. 0.4 Lf/mg for the $CaCO_3$ containing MS and 0.9 Lf/mg for the other MS preparations. (▲, △: $CaCO_3$, 15%; ■, □: poloxamer L101 19%; ●, ○: no additive; ◆: BSA 5%)

For selected formulations, Table III illustrates the effects of type and amount of additive on the (i) burst release after 24 h, (ii) the total amount released, and (iii) the residual amount remaining in the remnant polymeric particles after 80 days. As the polymeric material was not always entirely degraded into water-soluble moieties within the time period of release (80 days for PLGA 50:50), the remnant polymeric particles were separated from the release medium, dried and dissolved in dichloromethane; residual protein was then recovered on a 0.2 μm cellulose filter and assayed. When no additive was *co*-encapsulated, both burst and total release were negligible. With nominal 3, 5 and 8% *co*-encapsulated BSA, comparable burst releases of about

13% of the total dose were observed, whereas the total amount released increased from 24 to 34% of the determined dose. In the remnant particles (after 80 days), the highest amount of total protein (fluorimetry) was found in the formulation without any additive. This was consistent with the lowest protein release until 80 days. However, the remaining protein was essentially non-antigenic in the ELISA. An important fraction of protein also remained in the particles containing trehalose, but here again, the measured protein was non-antigenic. More moderate fractions of protein remained in the MS containing BSA. Very importantly, however, a certain proportion of the protein maintained its ELISA-antigenicity, which appeared to be related directly to the nominal BSA content. Highest ELISA-antigenicity was observed in the particles produced with the additive mixture of BSA/trehalose.

MS prepared with the more hydrophilic PLGA 50:50 analogue RG502H (shown in Table II) released 21% (no additive), 47% (20% trehalose) or 36% (15% trehalose plus 5% BSA) of the determined ELISA responsive antigen dose within 24 h (PTC and PSTtxd-20; results not shown). While the toxoid preparations, without *co*-encapsulated albumin (no additives or trehalose only) showed no further release after 24 h, those containing trehalose plus albumin, exhibited a continuous release over 2 - 3 weeks. An opposite release behaviour was noticed with the PLA MS manufactured by coacervation and containing nominal 5% BSA and 15% $CaCO_3$. Only 3% of the determined Ttxd dose was released within the first 24 h (results not shown), whereafter the release was not further followed.

Table III - Tetanus toxoid release after 24 h and 80 days (ELISA), and amount of total protein (toxoid plus BSA; Fluorimetry) and antigenic toxoid (ELISA) residues remaining in the MS after 80 days. The table shows PSTtdx-20 loaded PLGA 50:50 MS (nominal 3.5 Lf/mg) with selected additives. Unreleased protein was measured after extraction of protein from remaining polymeric particles after 80 days. All values are in percent of determined protein content ± s.d. The preparations are not identical with those in Table II.

	Tetanus toxoid release (%)		Protein residues in MS after 80 days (%)	
Additive in MS	Burst release (24 h, ELISA)	Cumulative release (80 d, ELISA)	Antigenic toxoid (ELISA)	Total protein: Ttxd, BSA (Fluorimetry)
none	0.2 ± 0.0	0.2 ± 0.0	< 0.01	26.4 ± 2.7
BSA 3%	13.0 ± 1.3	24.7 ± 0.8	0.22 ± 0.00	13.2 ± 0.2
BSA 5%	12.0 ± 0.5	28.1 ± 0.3	0.28 ± 0.04	7.4 ± 0.8
BSA 8%	13.1 ± 0.1	33.5 ± 0.9	0.43± 0.01	6.4 ± 1.1
Trehalose 20%	28.5 ± 2.2	40.7 ± 0.9	< 0.01	16.7 ± 1.0
Trehalose 15% + BSA 5%	22.5 ± 0.6	53.3 ± 2.9	0.56 ± 0.06	6.7 ± 0.5

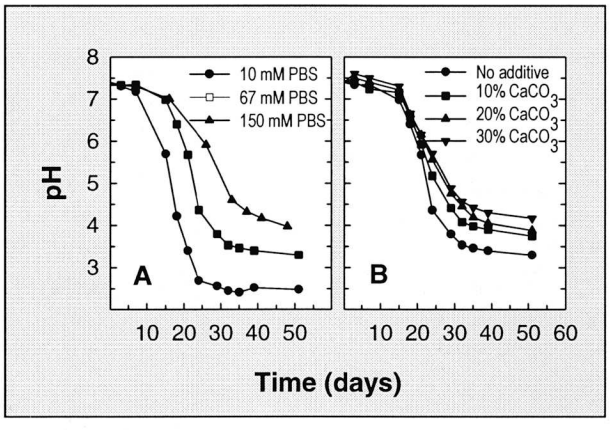

Figure 4 - Effect of buffer capacity of the release medium (A) and amount of $CaCO_3$ in 67 mM PBS (B) on pH as a function of time after incubation at 37°C.

pH neutralising capacity

The pH-stabilising capacity of the *co*-encapsulated calcium carbonate and orthophosphate was studied by pH-determination of the release media of increasing capacity during incubation of the corresponding PLGA 50:50 MS. When the phosphate concentration in the release medium was increased from 10 to 150 mM, the pH at the end of polymer degradation was increased from

approximately 2.5 to 4.0 and the onset of the pH-drop was delayed from day 10 to day 20 (Fig. 4A). By contrast, the *co*-encapsulated 10 to 30% of either of the calcium salts moderated the pH-drop of the incubation medium by maximum one pH-unit without significant delay of the pH-drop (Fig. 4B). For all formulations tested, the pH-drop started around day 16. From this it remains questionable whether *co*-encapsulation of calcium salts can enhance the stability of encapsulated toxoid, although a buffering of the micro-environment inside the MS cannot be excluded.

Discussion

Antigen instability is assumed to be one of the major causes responsible for the lack of booster effect observed thus far in animals after single parenteral administration of PLA/PLGA encapsulated toxoid. The physico-chemical and antigenic integrity of the toxoid within MS should depend on the encapsulation process, storage and release conditions. Here, we aimed at augmenting the stability of Ttxd in PLA/PLGA MS by *co*-encapsulation of various additives known for their stabilising effect on protein conformation and activity in aqueous solution [21,22], or which we assumed to exert such an effect. The *in vitro* results presented here demonstrate the stabilising effect of some of the selected additives on the encapsulated antigen. Most importantly, the data were confirmed in immunisation experiments in mice (see associated paper, Audran *et al.*) and in guinea pigs (Sesardic *et al.*, personal communication; independent testing). In the latter study, some stabilised formulations induced protective antibody titers for more than nine months after single injection; these titers were comparable to those induced by two Ttxd alum injections and superior to any titers determined so far in this animal model.

Amongst all additives, albumin was indeed the most powerful stabiliser and preserved up to 40% of the Ttxd ELISA activity, whereas hardly any ELISA-antigenicity was determined in the formulation without additive. This lack of antigenicity might have been caused either by the exposure of the toxoid to the organic solvents, or by the ultrasonication or the slightly increased temperature during spray-drying, or by the intensive water removal in the final drying of the MS. Especially the presence of an aqueous-organic interface can lead to preferential partitioning and aggregation of certain proteins [14]. Therefore, we can assume that increasing protein content saturates the W/O-interface and prevents the toxoid from direct exposure to the organic phase. On the other hand, the extraction procedure itself, using dichloromethane and a cellulose filter to collect suspended toxoid particles, may also lead to aggregation or similar denaturing phenomena [14,27,28].

Further, increasing nominal protein loadings lowered the fluorimetrically measured encapsulation efficiencies which is in agreement with previous results with microencapsulated BSA [25]. Conversely, increasing protein content improved the encapsulation efficiency of ELISA responsive toxoid. Hence, Ttxd stability greatly depended on the total protein content of the MS, which was varied by using standard or column purified Ttxd (26.3 vs. 4.2 mg/ml), or by encapsulation of different amounts of toxoid-solution (0.4 Lf/mg to 3.5 Lf/mg), or by *co*-encapsulation 1 - 8% (w/w) albumin.

The increased protein loading achieved by *co*-encapsulation trehalose or γ-HPCD might be attributed to preferential hydration of the toxoid. Nevertheless, in contrast to BSA, trehalose and γ-HPCD improved mainly protein content, but much less the ELISA-antigenicity. One may speculate that γ-HPCD accommodates amino acid side groups of the toxoid in its cavity and concomitantly interacts with the PLGA through Van der Waals forces and H-bonds. Thus, γ-HPCD might promote the interaction between polymer and protein and thereby increase the encapsulation efficiency of the protein. However, if γ-HPCD buries crucial epitope sites of the toxoid, the antigenicity might be lowered or lost completely. This may be one of the reasons for the large discrepancy between protein and antigen content. Incidentally, while cyclodextrins are known to enhance the fluorescence of weak fluorophores, this did not occur with Ttxd (PTC) solutions containing varying concentrations of γ-HPCD or trehalose (results not shown).

Deuterium oxide (D_2O) had no noticeable effect on protein and antigen loading although it has been demonstrated that D_2O could exert an important stabilising effect on a virus vaccine in an aqueous vaccine formulation when stored at ambient conditions or at increased temperature [24]. The expected strengthening of intra- and interproteinaceous H-bonding due to an additional neutron was ineffective here, either due to the absence of such a mechanism or to the very low D_2O content of the spray-dried MS. From our experience, approximately 0.5% residual water can be expected in MS after stringent vacuum drying (unpublished results).

With the hydrophilic PLGA 50:50 the entrapment of Ttxd was greatly improved, and most importantly, the antigenicity was preserved to a large extent. This indicates that H-bonds and polar interactions play an important role for toxoid encapsulation in PLGA, as both the hydrophilic additives and the hydrophilic polymer type increased the encapsulation efficiency of the protein antigen. Proteins in general form a hydrophobic core and expose their hydrophilic domains into the aqueous solution. We may assume that this facilitates hydrogen and polar interactions with the carboxylate end groups of the polymer. The hydrophilic form of PLGA 50:50 should consequently have a higher affinity to the exposed hydrophilic amino acid residues of the toxoids, thus ameliorating efficient entrapment into the MS.

Incomplete antigen release from PLGA and PLA MS may be caused by antigen instability, which may be related to the acidic micro-environment developing during polymer degradation or to physico-chemical interaction between protein and polymer [29]. Possible forms of instability include physico-chemical alterations as well as aggregation and adsorption. All these phenomena were indeed observed and reported in additional experiments [30,31]. During *in vitro* release from MS, the antigen is exposed to non-biological aqueous media and surfaces at 37°C. It has been shown that the solubility of Ttxd is lowered under such conditions, predominantly because of covalent, non-disulphide cross-linking [18]. The *co*-encapsulated additives were meant to counteract these phenomena.

If protein denaturation occurs indeed at the interface between water and polymer solution during MS preparation, amphiphilic additives such as albumin and poloxamers should counteract this denaturation by reducing the exposure and accumulation of protein antigens at the interface. For illustration, Synperonic F68 prevented totally Ttxd aggregation in aqueous solutions, although the protection was lost upon exposure to dichloromethane [10]. Further, large-size solutes such as sugars, polymers, polyols, anionic and non-ionic surfactants can stabilise the native state of proteins across the whole liquid exposed protein region [32]. This is in agreement with the ELISA-antigenicity preserving effect of the additives BSA, poloxamers and trehalose studied here. Moreover, a stabilising effect of 0.4% HSA and 0.2% gelatine on Ttxd have been reported [19]. In general, immobilisation of proteins by synthetic and natural macromolecules can increase the stability for processing and storage. Unfolded polypeptides will be much more prone to proteolysis than tightly packed globular proteins. Unfolded peptide chains may also aggregate to form inactive insoluble entities. However, refolding of individual chains may yield an incorrect, kinetically trapped conformation. Hence, for microencapsulating water-soluble proteins in PLGA/PLA MS, knowledge about physico-chemical alterations at the interface with the polymer solution would presumably facilitate adequate processing.

In addition to the microencapsulation process itself, the *in vitro* release is probably quite detrimental to the antigenicity of Ttxd. Indeed, the release kinetics showed a pulsatile pattern for protein and a continuous pattern for ELISA antigenic material (Fig. 3). This again suggests that the toxoid underwent alterations during release resulting in reduced antigenicity. However, BSA-containing formulations preserved more efficiently the toxoid stability as a second release pulse of antigenic material could be detected (Figs. 1-3). These findings agree with previous reports on protein stability [14]. During antigen release, the polymer degrades concomitantly thereby producing acidic moieties, which presumably create an acidic micro-environment inside the MS. The exposure to this increasing acidity may result in the loss of the Ttxd antigenicity. This would explain the observed very weak second toxoid release pulse and incomplete cumulative amount of toxoid released as measured by ELISA (Figs. 1-3). Hence, we expect both lactic and glycolic acids as well as their oligomers to alter the antigenicity of unreleased toxoids. In this context, it

was reported that dialysis of acidic PLGA degradation products during release resulted in a constant pH in the release medium and reduced chemical degradation of the model proteins carbonic anhydrase and bovine serum albumin [17]. Nonetheless, the environment around the MS might be less acidic and harmful to the toxoid *in vivo* than *in vitro*, as acids as well as toxoid will be forthwith removed by biological fluids or cells.

In the present study, albumin appeared to be the optimal stabiliser for Ttxd in PLGA MS. We can assume that two different mechanisms may have played a role: (i) protection of Ttxd from aggregation at the aqueous-organic interface by occupying this interface; (ii) protection of Ttxd from acidity induced aggregation or chemical alterations by acting as a proton scavenger. Both mechanisms will be promoted by the higher mobility and flexibility of the globular albumin (Mw 67 kDa) as compared to the cross-linked Ttxd (Mw 150 kDa). Moreover, albumin may function as an efficient sink for proton ions released during polymer hydrolysis because of its predominant amount present in the MS and its pI of approx. 4.7. Thus, acidity induced aggregation of Ttxd near or below its pI (approx. 5.1) or acid-catalysed chemical reactions such as proteolysis or Schiff base formation should be reduced for Ttxd.

Conclusion

In this study the entrapment efficiency and stability of tetanus toxoid in PLGA MS could be improved by *co*-encapsulation of additives. Hydrophilic substances such as trehalose and γ-HPCD significantly increased the incorporation efficiency of the protein vaccines, but did not itself contribute to stability improvement of either toxoid studied. However, BSA had a salient effect upon the antigenicity during the tetanus toxoid release, as for most BSA-containing formulations a distinct second release pulse was observed after 3-5 weeks time of incubation and a higher amount of antigenic material was released. Moreover, hydrophilic PLGA (Resomer RG502H) further improved the loading of antigenic toxoid. Finally, the present *in vitro* data were confirmed in immunisation experiments in mice (see associated paper, Audran *et al.*) and guinea pigs (Sesardic *et al.*, personal communication; independent testing).

Acknowledgements

This work was supported by the WHO global programme for vaccines and immunisation (GPV), Vaccine Research and Development, World Health Organisation, Geneva and by the Swiss National Foundation for Scientific Work. Further, the authors are grateful to Dr. Gideon Kersten for providing tetanus antibodies for the ELISA experiments and to Prof. Wolfgang Sadée for helpful discussions.

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3

Diphtheria and Tetanus Toxoid Microencapsulation into Conventional and End-Group Alkylated PLA/PLGAs*

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* European Journal of Pharmaceutics and Biopharmaceutics, 47: 193-201 (1999).
Associated paper: Johansen *et al.* Immunogenicity of single-dose diphtheria vaccines based on PLA/PLGA microspheres in guinea pigs. Vaccine, *in press* (see chapter five).

INTRODUCTION

In search of a single-dose vaccine delivery system, one of the most promising approaches has emerged to be the encapsulation of peptide or protein antigens in biodegradable microspheres (MS) of poly(lactic acid) (PLA), poly(glycolic acid) (PGA) or their *co*-polymers (PLGA) [1-5]. A potential drawback of PLA/PLGA MS, however, is their production of acidic moieties during polymer degradation which is likely to affect the antigenicity of the encapsulated antigens. On the other hand, protein instability such as aggregation [6,7] is assumed to contribute to the often observed incomplete *in vitro* protein release and, consequently, to the lack of booster effect observed so far in animals after single parenteral administration [8]. Attempts to counteract loss of activity of microencapsulated proteins comprised the use of different polymer types [9], chemical modification of the protein [10], and the *co*-encapsulation of stabilising additives [11,12]. Especially, albumin, trehalose and γ-hydroxypropylcyclodextrin enhanced tetanus toxoid loading in PLGA 50:50 MS [13]. The additives increased the content of antigenic (ELISA-responsive) toxoid and stabilised the toxoid during release. Further, in order to improve the immune response to tetanus toxoid, the antigen has been encapsulated as alum adsorbate [7]. On the other hand, the peptide growth hormone rhGH was stabilised in PLGA MS by complexation of rhGH with zinc carbonate [14,15]. This maintained the protein in its solid state until dissolution and release from the MS. Thus, protein aggregation, which preferentially takes place in the dissolved or the hydrated state, could be reduced.

A further approach to improve protein integrity in polymeric matrices consisted of the use of polymers with different properties. Block-*co*-polymerisation of polyesters or polyanhydrides with hydrophilic entities such poly(oxyethylene) produced more hydrophilic MS [9,16]. Enhanced hydration of polymeric matrices may create a better compatible microenvironment for proteins, though at the cost of a faster and more continuous release [9]. Further, tyrosine containing poly(anhydride-*co*-imides) were claimed to be of special interest for vaccines, as L-tyrosine has adjuvant properties [17]. Moreover, the model antigen bovine serum albumin (BSA) encapsulated in the more hydrophobic poly(hydroxybutyrate) induced promising immune responses [18]. By the same token, hydrophobic particles were phagocytosed more efficiently by macrophages [19]. Therefore, hydrophobic polymers may be of special interest for vaccine delivery systems not only to encapsulate lipophilic peptide antigens, but also to achieve more efficient antigen presentation. Conversely, the development of a long-term delivery system for proteins requires a well-tuned compromise between the sustained release properties provided by hydrophobic polymers and the higher affinity and often better stability of proteins in hydrophilic polymers.

Table I - Polymers used for the microe*ncapsul*ation of diphtheria and tetanus toxoids by spray-drying (SD) or coacervation (CO). Weight averaged molecular weight, M_w, values were determined by GPC using PS-standards (Ref. 8), and transition temperatures and crystallinity were determined by DSC. a): PLA end-groups were esterified with stearic chloride and stearyl alcohol. b): These temperatures were ascribed to the melting of the stearyl end-groups (43 and 52°C) and the melting of the crystalline l-PLA domains (~160 °C), whereas the other values represent glassy-to-rubbery state transitions of the amorphous polymer domains.

Polymer	Code	$\overline{M}_w$ (kDa)	Poly-dispersity	Transition temperatures (°C)	Crystallinity (%)	MS preparation method
PLGA 50:50	RG502	12	1.85	41	0	SD
PLGA 75:25	RG752	16	1.62	n.d.	0	SD
PLA	R202	14	1.90	45	0	SD
PLA	R206	130	2.15	55	0	CO
PLA [a)]	PLAst1	18	1.32	52, 67, ~160 [b)]	80	CO
PLA [a)]	PLAst2	10	1.27	43, 65, ~160 [b)]	70	CO

So far, most investigations on single-dose vaccine delivery systems have been done with the model antigens BSA [20] or ovalbumin [21], with tetanus toxoid [3,13], or sub-unit vaccines, e.g., against malaria [22,23] or HIV [4]. Many other antigens such as diphtheria toxoid are equally good candidates for such vaccine delivery systems [24,25]. Incidentally, an important increase in diphtheria incidence have recently been registered, especially in the former Soviet Union.

Consequently, a single-dose vaccine against tetanus and a single-dose combined vaccine against tetanus, diphtheria and pertussis have been given high priority by the WHO [26].

One purpose of this study was to examine whether previous successful concepts with tetanus toxoid and BSA microencapsulation could be transferred to diphtheria toxoid. In particular, we studied the effect of *co*-encapsulating albumin and trehalose with the toxoids into PLA/PLGA MS. The second purpose was to investigate the influence of polymer hydrophobicity of commercially available and novel more hydrophobic polymers on encapsulation and burst release of diphtheria and tetanus toxoids.

MATERIALS AND METHODS

Materials

Aqueous solutions of diphtheria (Dtxd) and tetanus (Ttxd) toxoids, provided by WHO, were from Pasteur Mérieux, F-Lyon (Dtxd, lot No. 386: 6500 Lf/ml and 24 mg/ml protein; Ttxd, lot No. 10005: 8500 Lf/ml and 26.3 mg/ml). All polymers used for antigen microencapsulation are described in Table I. Briefly, poly(d,l-lactic-*co*-glycolic acid) (PLGA; Resomer RG502 and RG752) and poly(d,l-lactic acid) (PLA; Resomer R202 and R206) were purchased from Boehringer Ingelheim, D-Ingelheim. The non-commercial poly(l-lactic acid) polymers, end-group substituted with stearyl moieties (PLAst1 and PLAst2), were a gift from G. Rafler, Frauenhofer IAP (D-Teltow). Bovine serum albumin (BSA) for immuno enzyme assay and d(+)-trehalose dihydrate were from Fluka, CH-Buchs. All other substances used were of pharmaceutical or analytical grade and purchased from Fluka, CH-Buchs.

Methods

Preparation of microspheres

PLA and PLGA microspheres (MS) were prepared by spray-drying (Büchi 190, CH-Flawil) a W/O-dispersion of aqueous toxoid solution in a 5% (w/w) PLGA solution in ethyl formate as described elsewhere [27]. In some cases indicated below, ethyl formate was replaced by dichloromethane. The potential stabilisers for the toxoids, i.e., BSA and trehalose, were *co*-encapsulated individually or concomitantly (Table II).

PLA and PLGA MS were also manufactured by coacervation as previously described [28]. Briefly, an aqueous phase containing the toxoid, BSA and trehalose, according to the experimental design in Table II, was dispersed in 2.5 - 5% (w/w) PLA in dichloromethane (the W/O-emulsion contained 5% aqueous phase). Coacervation was induced by adding silicone oil (274 or 1070 mPa.s; Fluka, CH-Buchs), and the hardening of the coacervate droplets took place in octamethylcyclotetrasiloxane (Abil K-4; Goldschmidt, D-Essen).

Table II - Experimental design to study the effect of co-encapsulated additives (factors A and B) on toxoid containing microspheres. The design was used for all polymers tested.

Experiment	Factor A: BSA (%)	Factor B: trehalose (%)
(1)	-	-
a	5	-
b	-	15
ab	5	15

Determination of toxoid and BSA content in the microspheres

Total Dtxd and BSA contents of the MS were determined by two methods. In *method A*, the loaded MS were dissolved in dichloromethane and the insoluble protein was recovered on a 0.2 µm regenerated cellulose filter (RC 58, Schleicher and Schuell, D-Dassel), wherefrom the protein was eluted with 67 mM physiological PBS of pH 7.4. In *method B*, the polymers were hydrolysed

by incubating the MS for 24 h at 37°C in 4 ml of 0.1 N NaOH with 0.05% polysorbate 20; this did not cause hydrolysis of the proteins. Ttxd was only studied after *method A* extraction.

The Dtxd and BSA content was assayed by reverse phase HPLC (Merck-Hitachi, D-Darmstadt) on a Vydac C_4 column (4 x 250 mm). The separation solvent was a mixture of 0.1% TFA in water (A) and 0.1% TFA in 95% acetonitrile (B). The initial A:B volume ratio of 3:1 changed along a linear gradient to 1:3 over 20 min at a flow rate of 1 ml/min. The antigenic response of Dtxd and Ttxd was determined by ELISA as described below.

In vitro release of toxoid and BSA

Toxoid release from 20 mg MS was conducted in 4 ml of 67 mM PBS of pH 7.4 containing 0.01% polysorbate 20 and 0.02% sodium azide in rotating (3 rpm) borosilicate vials at 37°C. At regular intervals, the vials were centrifuged at 3,500 rpm for 10 min to obtain a particle free supernatant. Then, 1 ml of the medium was withdrawn, assayed by HPLC and ELISA, and replaced by fresh buffer.

ELISA of Dtxd and Ttxd

The amount of encapsulated and released Dtxd and Ttxd was measured by enzyme-linked immunosorbent assay. Briefly, flat-bottom 96-well microtiter plates (Nunc-Immuno Plate Maxisorb, Nunc, DK-Roskilde) were filled with 100 µl of 1 AU/ml of horse anti-diphtheria IgG or horse anti-tetanus IgG (RIVM, NL-Bilthoven) in 0.05 M carbonate buffer of pH 9.6, and incubated at 4°C overnight. The plates were washed four times with 300 µl of 0.05% polysorbate 20 and 0.05% Na_2HPO_4 in water after each incubation step. After 1 h incubation at 37°C with 150 µl of 0.15 M PBS of pH 7.4 containing 0.5% BSA (PBS-BSA), the plates were incubated at 37°C for 2 h with serial dilutions of standard and test solutions of Dtxd or Ttxd. Horse radish peroxidase (HRP) conjugated sheep anti-diphtheria IgG or HRP-conjugated sheep anti-tetanus IgG (both from RIVM, NL-Bilthoven) was added to each well in 100 µl of PBS-BSA, and plates were incubated at 37°C for another 2 h. Finally, 100 µl of 0.2 mg/ml peroxidase substrate 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) (Sigma Chemical, St. Louis, MO) in 0.1M NaH_2PO_4 of pH 4.0 was added to the plates, and the kinetics followed at 405 nm (Thermomax, Molecular Devices, Menlo Park, CA).

Statistical analysis

All data were statistically evaluated by analysis of variance (ANOVA, Fischer) and the means compared by Student's *t* test.

RESULTS

Dtxd, Ttxd and BSA contents in the MS

First, the importance of the extraction method for the determination of antigen content in the MS was studied. Then, the effects of the polymer type, additive type, and polymer solvent on the loading efficiency, burst release and antigenic stability of encapsulated Dtxd and Ttxd were examined by using a factorial design (Table II).

Importance of extraction method on measured antigen content

Two different extraction methods were applied to determine microencapsulated Dtxd and BSA, i.e., extraction by solvents *(method A)* and through polymer hydrolysis *(method B)*. In contrast to the extraction by dichloromethane/water, polymer hydrolysis with sodium hydroxide/polysorbate destroyed the antigenicity of the encapsulated toxoid; here, only HPLC was applied to assay protein content.

For the spray-dried preparations, extraction method A indicated significantly higher (α = 0.01) Dtxd content in ten out of eleven MS batches than method B (up to 40% higher, results not shown). This difference increased with increasing polymer hydrophobicity. For the coacervated MS, however, a partly opposite behaviour was found (Fig. 1). For PLA R206 and for PLAst1 (18 kDa) MS, method B indicated significantly higher (α = 0.05) toxoid content than

method A. The difference was in the range of 20-70%, and the extreme case corresponded to R206 MS without co-encapsulated additives. Conversely, in coacervated PLAst2 (10 kDa) MS, the determined Dtxd content was statistically independent of the extraction method.

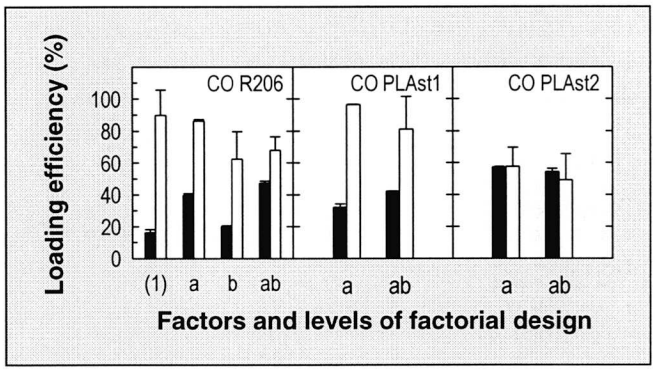

Figure 1 - Effect of extraction method on diphtheria toxoid content in three different PLA MS prepared by coacervation. Method A (extraction of protein in dichloromethane and PBS): filled bars; method B (hydrolysis of polymer in NaOH): open bars. See Tab. I for polymer specification and Tab. II for experimental design.

Effect of polymer type and co-encapsulated additives on Dtxd and Ttxd loadings

Table III shows the Dtxd loadings of spray-dried and coacervated MS when extracted by method A and measured by HPLC and ELISA. For the spray-dried MS, the loading efficiency decreased significantly (α=0.001) with increasing lactide-to-glycolide ratio of the polymer, and a significant (α=0.05) interaction between polymer type and additive was observed. The encapsulation efficiency of ELISA-responsive Dtxd was 27-64% for RG502, 15-43% for RG752, and 5-18% for R202. The percentages of Dtxd analysed by HPLC were generally 5-15% higher than those determined by ELISA. Similarly, the coacervated RG502-MS contained higher (α=0.001) Dtxd-loadings (60-76%) than the R206-MS (11.6-40.2%) or the end-group modified PLAs (29-57%). Further, PLAst2 (10 kDa) encapsulated Dtxd more efficiently (α=0.001) than PLAst1 (18 kDa). These results demonstrate that polymer hydrophobicity plays a crucial role in microencapsulation of Dtxd. Clearly, the more hydrophilic polymers (low M_w or high glycolide:lactide ratio) encapsulated more efficiently. The results are in agreement with previous observations in our laboratory [13], where the end-group uncapped PLGA 50:50 (with free carboxylate end-groups) gave higher loading efficiencies for both Dtxd and Ttxd as compared to the standard PLGA 50:50 (esterified end-groups).

Table III - Diphtheria toxoid loadings of selected spray-dried and coacervated PLA/PLGA microspheres according to the experimental design in Table II. The toxoid was assayed by ELISA and HPLC after dissolving the MS in dichloromethane and extracting the protein in PBS (method A). Nominal loading was 4 Lf/mg. (n=3). Level a was 5% BSA; level b was 15% trehalose. RG502 microspheres were prepared by both spray-drying and coacervation to examine the effect of preparation method.

Experiment	Loading efficiency (%)							
Spray-dried microspheres	RG502		RG752		R202			
	ELISA	HPLC	ELISA	HPLC	ELISA	HPLC		
(1)	63.7±2.5	74.9±0.6	43.2±1.8	55.0±9.3	16.1±2.5	32.4±5.2		
a	59.0±3.6	71.9±7.8	32.7±3.9	39.7±3.7	18.2±1.9	20.6±0.3		
b	54.9±2.1	56.1±0.9	19.7±2.5	28.9±1.0	4.2±0.1	10.3±0.7		
ab	27.0±2.7	44.9±5.0	14.8±0.0	12.0±3.7	5.3±0.0	11.0±0.4		
Coacervated microspheres	RG502		R206		PLAst1		PLAst2	
	ELISA	HPLC	ELISA	HPLC	ELISA	HPLC	ELISA	HPLC
(1)	-	-	11.6±1.5	16.2±2.0	-	-	-	-
a	60.1±4.1	45.2±4.1	34.4±1.0	39.7±1.4	28.5±3.8	32.2±2.1	56.7±2.9	57.2±0.3
b	-	-	12.8±0.2	20.3±0.4	-	-	-	-
ab	76.4±3.3	58.4±2.0	40.2±3.1	47.3±1.4	34.9±1.3	42.1±0.2	52.9±1.8	54.4±2.0

Table III further demonstrates that the additives BSA (levels a and ab) and trehalose (levels b and ab) had a significant (α=0.001) influence on Dtxd loading. After spray-drying, the highest Dtxd loadings were obtained without co-encapsulated additive (level 1). Trehalose reduced Dtxd loading more than did BSA. The statistically shown interaction between additive and polymer type effects revealed that the lowering of encapsulation progressed in the order of RG502, RG752 and R202 MS and was more pronounced when determined by HPLC than by ELISA. In contrast, during coacervation, BSA and trehalose improved slightly the Dtxd encapsulation efficiency in RG502 and R206 MS. For coacervating PLAst1 and PLAst2, BSA was required to prevent massive aggregation and precipitation of Dtxd in the W/O-emulsion prior to coacervation. Hence, this type of MS could only be produced in the presence of BSA. With PLAst1 and PLAst2, co-encapsulated trehalose caused only relatively minor changes in loading efficiency as compared to BSA alone.

Tetanus toxoid (Ttxd) encapsulation in RG502, RG752 and R202 by spray-drying was performed only in the presence of BSA or BSA and trehalose. Ttxd encapsulation followed a comparable pattern to Dtxd (Table IV). The Ttxd entrapment efficiency generally decreased in the order of RG502, RG752 and R202, except for the R202 MS containing the additive mixture of BSA and trehalose (level ab). Further, the encapsulation efficiency of Ttxd was lower (11-33%) than that of Dtxd (18-59%) when BSA alone was co-encapsulated (α=0.001). When both trehalose and BSA were co-encapsulated, there was no difference between Ttxd and Dtxd encapsulation in RG502 and RG752, but a relatively large difference in R202 (21% for Ttxd and 5% for Dtxd).

Effect of polymer solvent on Dtxd encapsulation

Microspheres made from RG752 and R202 produced unsatisfactorily low protein loadings (see above). This result is in agreement with observations during the preparation of the W/O-emulsion to be spray-dried. Indeed, proteins (Dtxd and BSA) appeared to precipitate upon ultrasonication, causing an incomplete entrapment in the MS. The exchange of the polymer solvent ethyl formate for dichloromethane produced a much more stable emulsion resulting in a higher (α=0.001) encapsulation efficiency (Fig. 2). This effect was very pronounced for both the R202 and the RG752 MS. When dichloromethane was used instead of ethyl formate, the encapsulation of Dtxd and BSA in R202 was improved from approx. 20 and 15% to 56 and 84%, respectively, whereas for RG752 MS, it increased from approx. 8 to 35-40% for both proteins (R202 and RG752 were studied at level a and ab, respectively).

Quality of BSA co-encapsulation

Obviously, BSA can exert a stabilising effect on the toxoid only if it is sufficiently *co*-encapsulated in the MS. We evaluated this by developing a HPLC method for simultaneous Dtxd and BSA quantification. The encapsulation efficiencies of BSA and of Dtxd in coacervated MS made of different polymers at levels a and ab are shown in Fig. 3. Typically, BSA was encapsulated to a higher extent than Dtxd, and the additional trehalose had no consistent effect on BSA loading. With Dtxd, the highest loading efficiencies were obtained in MS produced with the most hydrophilic and low molecular weight polymers (RG502 and PLAst2). For spray-dried MS, there were only minor differences in BSA and Dtxd loading efficiencies (results not shown).

Table IV - Tetanus toxoid loading and burst release (2 days) of spray-dried PLA/PLGA microspheres according to the experimental design in Table II. Toxoid loading was measured by ELISA after dissolving MS in dichloromethane and extracting the protein in PBS (method A). Nominal toxoid loading was 5 Lf/mg. (n=3)

Experiment	ELISA-responsive loading efficiency (%)			ELISA-responsive burst release (%)		
	RG502	RG752	R202	RG502	RG752	R202
a	33.5±4.9	23.4±5.8	11.5±6.1	17.2±6.0	53.0±4.4	73.1±1.6
ab	27.9±4.1	14.9±1.0	21.3±1.1	39.8±3.4	67.8±3.4	70.3±13.1

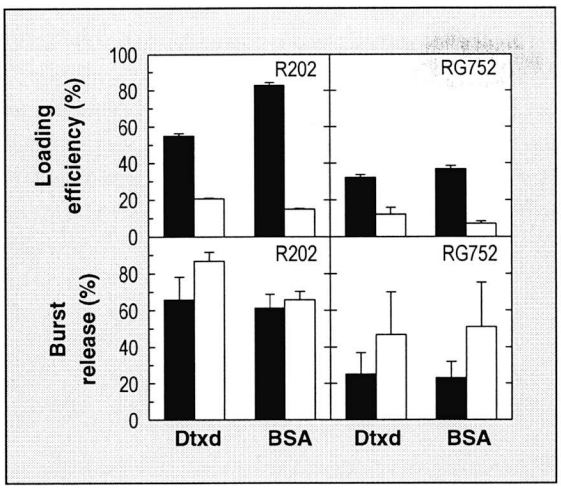

Figure 2 - Effect of polymer solvent on Dtxd and BSA content (top) and burst release (bottom) from spray-dried PLA R202 (level a) and PLGA RG752 (level ab) MS. Ethyl formate: open bars; dichloromethane: filled bars. The proteins were extracted from the MS by method A and assayed by HPLC. See Tab. I for polymer specification.

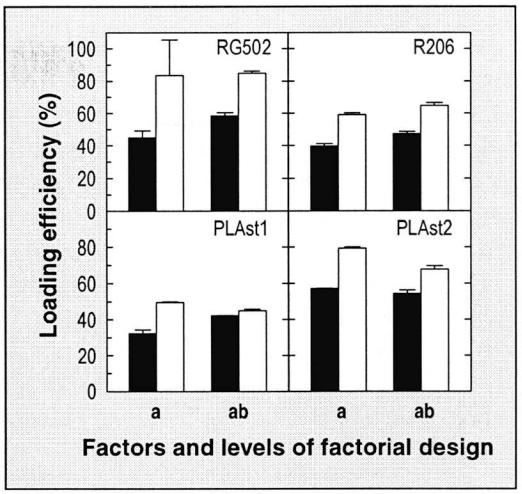

Figure 3 - Loading efficiency of encapsulated diphtheria toxoid (filled bars) and BSA (open bars) in selected coacervated MS (experimental levels a and ab according to Tab. II). The proteins were extracted from the MS by method A and assayed by HPLC.

Protein release from microspheres

The spray-dried MS generally gave a more pronounced burst release than coacervated MS. Interestingly, the burst release increased from 38 to 52 and 71% with the more hydrophobic spray-dried polymers (RG502 < RG752 < R202) at the experimental level (1) (data not shown). Fig. 4 illustrates the release of Dtxd from selected MS after 2 and 30 days. Comparison between spray-dried and coacervated RG502 MS showed that the spray-dried MS produced a burst release of 50% at level a and 85% at level ab, whereas the coacervated MS released only 12% and 33% at the corresponding levels (α=0.001). When comparing burst releases from the coacervated hydrophobic PLAst1, PLAst2 and the less hydrophobic RG502 MS, quite similar data were obtained at both experimental levels (a and ab), except for the PLAst2 MS at the experimental level a. For all MS, only minor increments in cumulative release were observed between days 2 and 30.

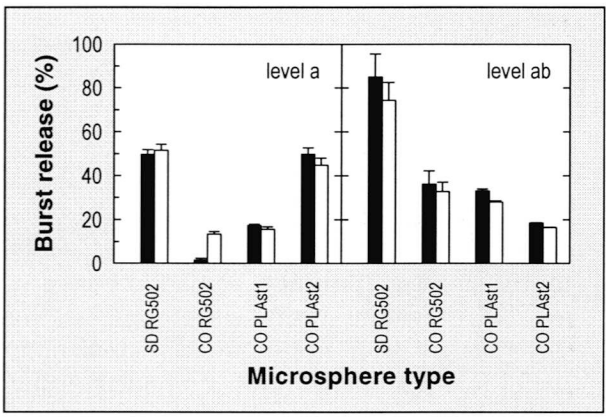

Figure 4 - Release of diphtheria toxoid from spray-dried (SD) and coacervated (CO) PLGA RG502 and PLAst1 and PLAst2 MS after 2 days (filled bars) and 30 days (open bars). (Left) Experiments at level a (co-encapsulated BSA). (Right) Experiments at level ab (co-encapsulated BSA and trehalose). Release was determined by HPLC.

The burst release (2 days) of Dtxd and BSA from spray-dried R202 and RG752 MS, produced with either ethyl formate or dichloromethane as polymer solvents, is shown in Fig. 2. MS prepared with dichloromethane released approx. 30% less Dtxd within 2 days than the MS made

with ethyl formate (α=0.001). Furthermore, the release of Dtxd from R202 MS (65-85%) was significantly higher (α=0.001) than from RG752 MS (25-50%). On the contrary, the type of solvent for preparing MS had only negligible effect on the burst release of BSA. Nonetheless, the effect of polymer type on the BSA burst release was similar to that observed for Dtxd.

Ttxd release from spray-dried PLA/PLGA MS followed an analogous pattern to Dtxd. The increase in burst release from 17 and 40 to 73 and 70% with increasing polymer hydrophobicity was very important (α=0.01) (Tab. 4). In addition, the burst release was higher (α=0.005) when both trehalose and BSA were co-encapsulated (level ab) than when only BSA was co-encapsulated (level a).

Discussion

Single-dose vaccines against bacterial, viral and parasitic diseases would be highly desirable for developing countries [29]. Several attempts have been made to use biodegradable polymeric MS as delivery system and adjuvant [8,22,23,30,31]. Here, we compared Dtxd and Ttxd in microencapsulation and studied the technological feasibility of using hydrophobic or high-molecular weight PLAs.

The results with spray-dried MS clearly demonstrate the effect of polymer properties on toxoid encapsulation. An increasing amount of glycolide monomers enhanced the encapsulation efficiency of both Dtxd and Ttxd. Thus, the efficiency of toxoid entrapment is probably not only a result of processing conditions, but much more one of molecular interactions. Incidentally, the inherent viscosities of the three polymers RG502, RG752 and R202 are comparable. Our hypothesis that H-bonds and polar interactions play a crucial role in the encapsulation of drugs and antigens in PLA/PLGA is supported by the following aspects. With decreasing polymer hydrophilicity (RG502 > RG752 > R202), lower H-bonding and polar interactions between the polymer and the hydrophilic protein can be expected. Proteins generally form a hydrophobic core and expose their hydrophilic domains into the aqueous environment, which possibly facilitates hydrogen and polar interactions with the polymer. In this context, the α-methyl group of the lactate may sterically hinder H-bond interactions between the carbonyl oxygen and protein residues. Furthermore, the polymer-solvent interaction energy increases in the order of RG502<RG752<R202 (unpublished results). As shown previously, the interactions between drug and polymer should become more important through weakening their individual interactions with the polymer solvent [27].

Comparing Dtxd and Ttxd, based on ELISA-antigenicity, Dtxd was more efficiently encapsulated than Ttxd (Tab. 3 and 4). However, the present investigation only allows us to speculate on the mechanisms behind this. Because of the lower molecular weight, Dtxd may be more flexible to adapt a conformation suitable for interactions with the polymer. Differences in conformational behaviour between Dtxd and Ttxd have previously been observed in our laboratory [32]. Dtxd underwent more readily than Ttxd conformational changes upon exposure to different environmental stress factors.

The two encapsulation methods of spray-drying and coacervation yielded encapsulation efficiencies that followed similar trends when process parameters were modified. With both methods, the entrapment was better in PLGA than in PLA and also better in low than in high molecular weight polymers.

PLA, containing stearyl end-groups, i.e., PLAst1 (18 kDa) and PLAst2 (10 kDa), represent a novel material for antigen microencapsulation. These hydrophobic polymers appear potentially useful for encapsulation of hydrophobic peptide antigens as well as for a more sustained release of peptides and proteins. The reason for the higher Dtxd encapsulation in PLAst2 as compared to PLAst1 is unclear. Other experiments in our laboratory revealed a higher fraction of amorphous phase, i.e., 30%, in the 10 kDa PLAst2, which should be favourable for accommodating the protein. On the other hand, for the encapsulation of Dtxd into these polymers, addition of BSA to the aqueous phase was a prerequisite to prevent Dtxd precipitation in the W/O-emulsion made prior to coacervation. BSA has been reported to adsorb on such

W/O-interfaces [33]. Previous observations in our laboratory (unpublished results) showed that BSA itself did not precipitate in W/O emulsions with PLAst1 and PLAst2. Therefore, preferential adsorption of BSA to the interface may prevent the interaction of the toxoid with the polymer or with the polymer solvent, thereby reducing the chance of toxoid precipitation.

In a previous study on Ttxd MS, co-encapsulation of BSA and trehalose was shown to improve the antigenic stability of the toxoid [13]. The antigenically reactive Ttxd released within 80 days amounted up to 50% of the actual dose. After that time, approx. 0.5% (relative to the actual dose) of unreleased antigenic Ttxd could still be extracted from the polymer mass. Thus, the mass balance of antigenic material amounted to approx. 50%. This result was consistent with the loss of Ttxd antigenicity measured in aqueous solutions exposed to 37 °C [32]. However, subsequent experiments revealed that the cumulative release of antigenic Ttxd may increase up to 75% when the release test is done in media containing BSA [38]. In the present study, Dtxd released over 30 days remained ELISA-antigenic both with and without co-encapsulated additives. During *in vitro* release, the antigen is exposed to non-biological aqueous media and surfaces at 37°C. It has been shown that the solubility of toxoids may drop under such conditions, predominantly because of covalent non-disulphide cross-linking [10]. As the isoelectric properties of Dtxd and Ttxd are similar, we may speculate that the number of accessible amino groups (e.g., lysine) might be relevant for cross-linking, aggregation, solubility and consequently also the antigenicity of the toxoid. The fewer free amino groups for Dtxd than for Ttxd [10] may account for the superior antigenic stability of Dtxd as compared with Ttxd. As a consequence, Dtxd may not require co-encapsulation of stabilising additives in MS delivery systems. Further, unfolded proteins are more prone to proteolysis than tightly packed globular conformations. Unfolded peptide chains may also aggregate to form inactive insoluble entities. Thus, the lower molecular weight of Dtxd, its relatively few free amino groups, and its reported conformational flexibility [32] may render this toxoid an excellent candidate for antigen microencapsulation.

A critical step in the quality control of antigen-containing MS is the determination of the antigen content (protein and antigenic material). Several methods have been used, all having their *pros* and *cons* [34-36]. All major variables, e.g., preparation method, polymer type and formulation additives, influence the assayed amount of protein and antigenic material. Amongst the two methods used here, toxoid extraction by hydrolysis in sodium hydroxide/polysorbate (method B) led to complete loss of Dtxd antigenicity, in agreement with a previous report [36]. On the other hand, extraction method B generally resulted in higher protein contents than extraction method A (dissolution in dichloromethane, and protein collected on filter). The differences in Dtxd loadings determined by the two extraction methods were most notable for the coacervated preparations. We assume that several parameters may contribute to this result. It may be related to the additives, especially the surface-active BSA. The lower Dtxd content determined by method A for coacervated MS may also be due to residual solvents such as water, silicon oil and octamethylcyclotetrasiloxane [28], which may solubilise or emulsify the toxoid extracted in dichloromethane. Such a mixture of co-encapsulated additives and solvent residues may also create new interfaces accessible to proteins. So, general protocols to assay protein content in MS may not be useful, but have to be established case by case.

The HPLC-method indicated generally higher, i.e. mean of 7% with an extreme of 18%, Dtxd loadings than ELISA. This suggests that HPLC may overestimate the amount of intact toxoid. This overestimation was particularly pronounced for the spray-dried preparations made without co-encapsulated additive (level 1) and those made with the RG502 (all levels). For three particular preparations, however, the ELISA-value was higher by 6 to 18% than the value obtained by HPLC. For the RG752-level ab preparation, this result is classified as outlier, while it appears significant for the coacervated RG502 batches. At present, we cannot provide explanations for these observed differences.

Another challenging obstacle in the development of sustained release dosage forms for proteins is the preservation of protein integrity at the site of injection, where the protein is hydrated at elevated temperature. The frequently observed incomplete protein and antigen release from PLA/PLGA MS may be ascribed to protein aggregation, adsorption or other antigen

inactivation. These phenomena may be caused by the acidic microenvironment developing during polymer degradation or to physicochemical interactions between protein and polymer. Physico-chemical alterations have been shown to occur with tetanus toxoid exposed to stress conditions relevant in microencapsulation [32]. Co-encapsulation of BSA and, to a minor extent, trehalose improved significantly the antigenic stability of Ttxd during preparation by spray-drying and during *in vitro* release [13]. The *in vitro* results were confirmed by enhanced immune response in mice [37] and guinea pigs (personal communication from D. Sesardic and A Sasiak, NIBSC London) with Ttxd MS containing stabilising additives. By contrast, the results of the present study reveal that the co-encapsulation of BSA and trehalose may not be necessary for Dtxd microencapsulation, as the antigenicity of this toxoid was preserved to an acceptable extent during processing and release. Nevertheless, whether or not the additives affect the immune response of Dtxd containing MS in animals is presently examined.

Acknowledgements

This work was supported in part by the WHO Global Programme for Vaccines and Immunisation (GPV), Vaccine Research and Development, World Health Organisation, CH-Geneva (WHO MIM/I5/181/225), and by the Swiss National Science Foundation, CH-Bern (SNF 31-37440.93). The authors are grateful to Dr. Gideon Kersten, RIVM, NL-Bilthoven, for providing diphtheria antibodies for the ELISA experiments and to Dr. Gerald Rafler, Frauenhofer IAP, D-Teltow, for providing the distearylated PLAs.

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4

Enhanced Immunogenicity of Microencapsulated Tetanus Toxoid with Stabilising Agents*

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* Pharmaceutical Research 15: 1111-1116 (1998). Associated paper: Johansen et al., Improving stability and release kinetics of microencapsulated tetanus toxoid by co-encapsulation of additives. Pharmaceutical Research 15: 1103-1110 (1998) (see chapter two).

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INTRODUCTION

Biodegradable microspheres (MS) of poly(d,l-lactide-*co*-glycolide) (PLGA) and poly(d,l-lactide) (PLA), currently used as a parenteral drug delivery system, are also effective for vaccination [1,2]. After injection, MS are degraded by non-enzymatic hydrolysis to lactic and glycolic acids, and they induce only a minimal inflammatory response [3]. *In vitro*, MS have the ability to release the entrapped peptides or proteins over extended time periods at a pulsatile rate, which might be useful for mimicking conventional immunisation schedules. Over the past few years, immunostimulating properties of MS have been studied using a variety of proteins [4-12], peptides [4,13-17], inactivated viruses [18,19], and proteins in association with adjuvant or cytokines [20,21], showing that specific antibody, helper and/or cytotoxic T cell responses were readily induced.

A single-dose vaccine has recently been developed with tetanus toxoid (TT) encapsulated in controlled release PLA/PLGA MS. *In vitro*, TT was released in a marked pulsatile pattern coinciding with the degradation kinetics of the polymers used [4]. In mice, a single injection of these TT-MS elicited strong and long-lasting antibody and T cell responses, which were comparable to those induced by alum-adsorbed TT, although no significant second increase of antibody titers was observed [4,7]. It was speculated that the absence of boosting effect on the primary antibody levels may be related to antigen instability within the microspheres after exposure to the physiological environment. Polymer degradation is thought to cause accumulation of acidic metabolites in the core of MS and the acidic environment could catalyse TT degradation or aggregation. Further, formation of pores in the MS might facilitate penetration of proteases from the extracellular fluids and thereby promote antigen degradation. Thus, it is important to stabilise the antigen in MS to enhance prolonged antigen delivery.

In the present study, various additives were *co*-encapsulated together with TT into fast-releasing MS, and the effect on TT immunogenicity was determined in mice by measuring antibody responses. The putative stabilisers were chosen from a previous comparative *in vitro* analysis of the antigenicity of encapsulated and released TT (see associated paper). These included (i) the water insoluble pH-buffering salts, calcium carbonate and calcium phosphate, expected to neutralise the acidification in the core of MS during polymer degradation, (ii) the hydrophobic poloxamers L121 and L101, expected to reduce the water uptake into the MS and thereby preventing a hypothetical protease uptake, and (iii) the commonly known protein stabilising agents cyclodextrins, trehalose and bovine serum albumin (BSA). The MS formulations selected for *in vivo* testing all showed *in vitro* higher ELISA-responsive TT encapsulation and release than the TT-MS formulation without *co*-encapsulated additive.

MATERIALS AND METHODS

Antigens and polymers

Solutions of tetanus toxoid (TT), provided by WHO (Geneva, Switzerland), were from Pasteur Mérieux F-Marcy L'Etoile (conventionally prepared TT-PTC, lot n° PTC 10005: 8500 Lf/ml, 323 Lf/mg protein nitrogen) and from Massachusetts Health Biological Laboratories, Boston, MA (TT-PST, lot PSTxd-20: 1400 Lf/ml, 333 Lf/mg protein nitrogen). Lf and protein N content of the two sources of toxoids were determined by the respective manufacturers. The latter TT preparation was purified by column chromatography. They were used for microencapsulation and enzyme-linked immunosorbent assay (ELISA). TT adsorbed on aluminium hydroxide was also used: TT-PTC-alum (prep. n° 3, 20 Lf/ml, 0.12% aluminium hydroxide, Pasteur Mérieux) and TT-PST-alum. TT-PST-alum was obtained by incubating TT-PST and aluminium hydroxide (0.4 Lf/mg alum) for 30 min at 4°C. After centrifugation (5 min, 3000 rpm), the TT-alum precipitate was diluted to 4 Lf/ml in PBS before use.

Tetanus toxoid containing microspheres (TT-MS) with various additives were prepared as described by Johansen *et al.* (associated paper) by spray-drying and coacervation methods [4,22].

MS characteristics are shown in Table I. The biodegradable polymers used for TT microencapsulation were poly (d,l-lactide-*co*-glycolide) (PLGA 50:50) and poly (d,l-lactide) (PLA) from Boehringer Ingelheim, Germany. The following additives were *co*-encapsulated: calcium carbonate (CaC) and calcium phosphate (CaP), the poloxamers Synperonic L121 and L101 (ICI, Wilton, CT), γ-hydroxypropylcyclodextrin (γ–HPCD) and α-cyclodextrin (α-CD) (Wacker Chemie, CH-Liestal), trehalose (Tre) and BSA. Unless specified otherwise, all substances used were from Fluka, CH-Buchs.

Animals and immunisation

BALB/c female mice 8-10 weeks of age (Harlan Netherlands BV, NL-Zeist) were used in all experiments. Mice (4 per groups) received TT-PTC or TT-PST in various formulations by subcutaneous injection at the base of the tail on day zero. The formulations included various types of PLGA 50:50 or PLA MS, with or without *co*-encapsulated additives, and also alum adsorbed TT. TT-MS were suspended in 5% lecithin solution (Ovothin 170, Lukas Meyer, D-Hamburg) and TT-alum in PBS in a total volume of 100 µl before injection. Lecithin had no effect on antibody production when injected with antigen alone [7]. After immunisation, mouse sera were collected at an interval of 4 weeks by tail bleeding. In some cases, a boost injection of 0.2 Lf of TT in alum was performed 6 months after the first immunisation. Specific anti-TT serum antibodies were measured by ELISA.

ELISA

ELISA was performed as described previously [23]. 50 µl of TT in 0.1 M PBS pH 7.4 (10 µg/ml) were introduced into the individual wells of 96-well flat bottom immunoplates (Maxisorb, NUNC, InterMed, DK-Roskilde). After overnight incubation at 4°C, plates were washed three times with PBS-Tween 20 (0.05%) (PBS-T) and saturated with 200 µl PBS-T 5% (w/v) skimmed powdered milk (PBS-m) for 1 h at 37°C. 50 µl of mouse sera diluted in PBS-T were then added, and plates incubated for 2 h at 37°C. After four washes, 50 µl of a 1/4000 dilution of peroxidase-conjugated goat anti-mouse immunoglobulin (Sigma, St Louis, MO) in PBS-m were added per well. After 1 h incubation at 37°C, plates were washed four times. Then, the substrate o-phenylenediamine dihydrochloride (Sigma) was added in 50 µl of citrate buffer of pH 5.0 containing 1 µl/ml H_2O_2. The enzymatic reaction was stopped with 20 µl of 2 µM H_2SO_4, and plates were read at 492 nm with Microtiter reader (MR 5000, Dynatech Produkte AG, CH-Embrach-Embraport). The antibody titer was expressed as the reciprocal of the lowest negative dilution. International Units (IU) were determined using a mouse standard anti-TT antibody from the National Institute for Biological Standards and Control (UK-Hertfordshire).

Results

The immunisation study was divided into three sets, in which mice received a single subcutaneous injection of various TT preparations (Table I, series a, b and c). In all groups, MS preparations were well tolerated. Neither inflammation nor granuloma were observed at the injection site.

Immunisation with PLGA 50:50 MS containing TT-PTC and one additive

In the first set of experiments, mice (4 per group) received 20 µg of TT-PTC as previously performed corresponding to 6.5 Lf TT. TT-PTC, a tetanus toxoid used in commercial vaccine formulations, was incorporated in MS containing various additives (Table I-a) or adsorbed on alum (TT-PTC-alum). Immune responses were evaluated by measuring anti-TT antibody titers in individual sera for 5 months after immunisation. Specific antibody responses were obtained in each group except for the control group that received MS-empty (data not shown). Responses induced in the TT-MS group were as high as those observed with TT-PTC-alum. In general, additives in MS failed to enhance the antibody response. Lack of any potentiating effect of

additives might be related to the high dose of injected TT-PTC, which already induced nearly maximal response.

Therefore, further experiments were then designed with lower TT doses to induce sub-optimal responses in control TT-alum groups. In addition, chromatography column purified TT was used which represented a more homogenous antigen preparation and was considered to be used for further human and animal studies.

Immunisation with PLGA 50:50 MS containing low-dose TT-PST and one additive

In the second series of immunisation, mice received 0.2 or 1.0 Lf of the HPLC-purified TT-PST incorporated in MS containing various additives (Table I-b). Two groups of mice were immunised with the equivalent dose of TT-PST-alum as positive control.

Table I - Characteristics of TT-MS containing various additives. Particle size was determined by laser light scattering. TT loadings were typically measured by fluorescence spectroscopy. MS_{hyd} were made of a hydrophilic and fast releasing form of low molecular weight PLGA 50:50 (Resomer RG502H), and MS_{sl} were slow release MS made of high molecular weight PLA. n.d.: not determined. a): Theoretical loading, as the *co*-encapsulated BSA interfered with the fluorimetric measurement. b): TT loading was measured by ELISA.

TT-MS preparation	Additive		Mean particle size (μm)	TT loading (Lf/mg)
	Type	Amount % (w/w)		
a : TT-PTC + additives				
TT-MS-Tre	trehalose	15	n.d.	0.84
TT-MS-γ-HPCD	γ-hydroxypropylcyclodextrin	n.d.	n.d.	0.81
TT-MS-αCD	α-cyclodextrin	15	n.d.	0.69
TT-MS-L121	poloxamer L121	20	n.d.	0.62
TT-MS-L101	poloxamer L101	20	n.d.	0.45
TT-MS	-	-	n.d.	0.67
b : TT-PST + additive				
TT-MS-CaC	calcium carbonate	15	9.7	0.18
TT-MS-Tre	trehalose	20	6.8	0.30
TT-MS-γ-HPCD	γ-hydroxypropylcyclodextrin	20	n.d.	0.23
TT-MS-L121	poloxamer L121	22	n.d.	0.25
TT-MS-BSA/2	BSA	5	8.5	0.70 a)
TT-MS-BSA/1	BSA	1	8.2	0.70 a)
TT-MS-CaP	calcium phosphate	15	n.d.	0.11
TT-MS	-	-	7.2	0.20
c : TT-PST + additive mixture				
TT-MS-BSA/Tre	BSA + trehalose	5+15	n.d.	0.42 b)
$TT\text{-}MS_{hyd}$-BSA/Tre	BSA + trehalose	5+15	n.d.	0.84 b))
$TT\text{-}MS_{sl}$-BSA/CaC	BSA + calcium phosphate	5+15	n.d.	0.55 b)

The anti-TT antibody titers in individual sera were determined regularly for six months after immunisation. Specific antibody responses were detected in each group. The geometric mean of individual titers of each group are shown in Fig. 1. Responses peaked at one to two months after immunisation and declined thereafter. Importantly, the level of the response depended on the *co*-encapsulated additive and the dose of antigen injected.

Interestingly, 0.2 and 1 Lf doses of TT-MS-Tre, TT-MS-BSA/2 and TT-MS-γ-HPCD induced early antibody responses (1 and 2 months after immunisation) similar to those obtained with TT-alum. Moreover, antibody titers remained almost stable over time in the groups that had received 1.0 Lf of TT-MS-CaC and TT-MS-CaP. In fact, after the initial peak, the antibody response reached a plateau level 3 months after immunisation. While similar responses were obtained with 0.2 and 1.0 Lf of TT-PST in the groups MS-TT-Tre, MS-TT-BSA/2 or MS-TT-γ-HPCD, the groups of MS-TT-CaC, MS-TT-L121, MS-TT-BSA/1, MS-TT-CaP or MS-TT showed lower responses with the lower dose. On the other hand, antibody titers from TT-alum groups were stable over the entire period studied.

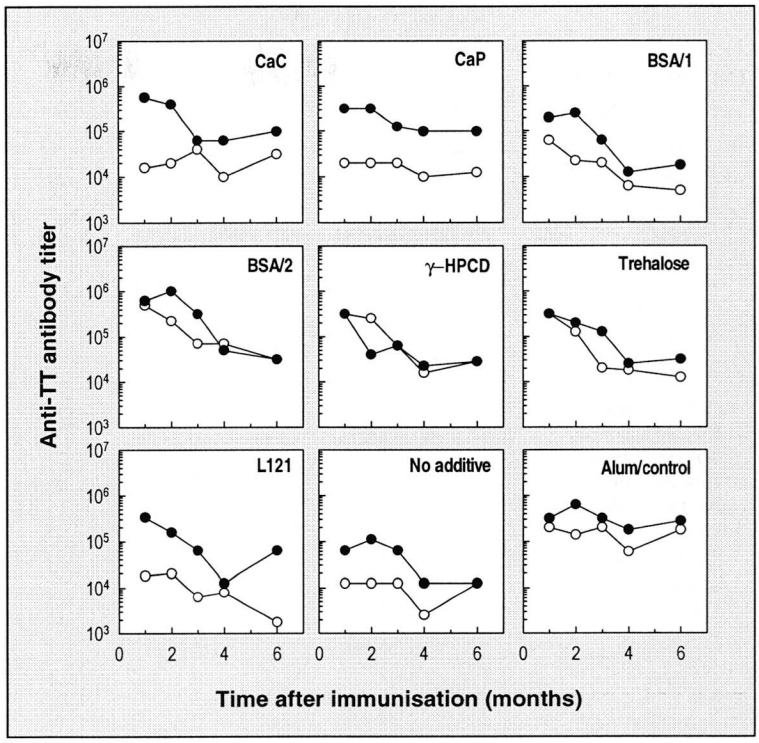

Figure 1 - Time course of antibody responses in BALB/c mice immunised with TT-PST-MS containing various additives. For each formulation, TT-MS or TT-alum, groups of four mice received one dose of 0.2 (○) or 1.0 (●) Lf of TT-PST. Immunising doses were calculated from the values reported in Table I. Specific antibody responses at 1, 2, 3, 4 and 6 months are expressed as the geometric mean of titers obtained in each group.

To evaluate the effect of stabilising agents on the relative TT-PST-MS antigenicity, antibody titers from TT-PST-MS-additive groups were compared to those from the control groups injected with TT-PST-MS without additive. Calculated titer indices are shown in Table II. In general, for the same dose of TT-PST, immunisation with TT-PST-MS-additive induced higher antibody responses than the MS without stabiliser (indices varied between 0.9 for TT-MS-L121 to 15.8 for TT-MS-BSA/2). All groups considered, an averaged 4.8 fold increase in antibody titer was observed, but the effect was more marked with the low dose of TT as compared to the higher dose (average index of 3.4).

The effect of *co*-encapsulated additives was further analysed by comparing the titers of the low dose TT-PST-MS-additive groups to that of the high dose TT-PST-MS (with or without additive) groups (Table II, index b). The determined titer index was clearly greater than unity in the groups having been injected with TT-PST-MS containing Tre, γ-HPCD or BSA/2. Thus, because of the presence of these additives, the 0.2 Lf MS induced a higher response than 1.0 Lf formulations without stabilisers.

Table II - Evaluation of the efficacy of additives in TT-PST-MS on the antibody response in BALB/c mice. The efficacy of the presence of additives in MS was expressed as a), ratio between the mean of the antibody titers (months 1 to 6) of each TT-PST-MS-additive group and the mean of the antibody titers (months 1 to 6) of the TT-MS (without additive) groups, for both doses of TT-PST injected (0.2 or 1.0 Lf) or b), ratio of the titers of the 0.2 Lf TT-PST-MS-additive groups over that of the 1.0 Lf TT-MS (no additive) group. Mean index was calculated for all TT-PST-MS-additive groups together.

TT-PST formulations	Titer index		
	0.2 Lf-additive [a] / 0.2 Lf	1.0 Lf additive [a] / 1.0 Lf	0.2 Lf additive [b] / 1.0 Lf
TT-MS-CaC	1.9	3.9	0.4
TT-MS-Tre	8.7	2.6	1.8
TT-MS-γ-HPCD	11.9	1.8	2.4
TT-MS-L121	0.9	2.3	0.2
TT-MS-BSA/2	15.8	7.9	3.2
TT-MS-BSA/1	2.1	1.8	0.4
TT-MS-CaP	1.6	3.6	0.3
TT-alum	15.8	7.0	15.8
mean index	**6.1**	**3.4**	**1.3**

To examine the boost effect, all groups of mice received 0.2 Lf of TT-PST-alum 6 months after the first injection. Figure 2 shows the antibody responses obtained before and 2 and 7 weeks after the boost injection. In all groups, the booster effect was most pronounced with antibody titers increasing from a range of 0.3-3.1 IU/ml before to a range of 22-224 IU/ml after the boost. The average increase was 103 ± 92 fold in TT-PST-MS and 12 ± 8 fold in TT-alum groups. Moreover, the response persisted for at least 7 weeks after the boost injection. Finally, for the same first immunisation dose, boosting responses reached similar high level in TT-PST-MS and TT-PST-alum groups, suggesting that almost maximal responses were obtained in these experimental conditions.

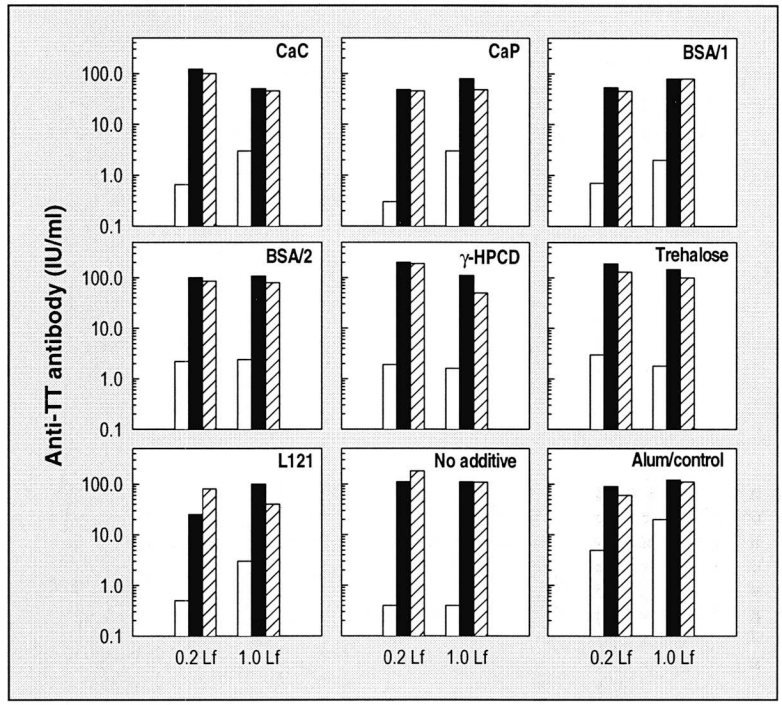

Figure 2 - Booster effects in BALB/c mice immunised with TT-PST-MS containing various additives. Mice were injected first with 0.2 or 1.0 Lf of TT-PST in various formulations and then, all were boosted with 0.2 Lf TT-PST-alum 6 months later. Immunising doses were calculated from the values reported in Table I. Mouse sera were collected before (open bars), and 2 (filled bars) and 7 (hatched bars) weeks after the boost injection. Results are expressed as arithmetic mean IU/ml.

Immunisation with different MS types containing TT-PST and a mixture of additives

Having determined that BSA and trehalose increased the encapsulation efficiency of antigenic TT and presented higher responses at later time points than TT in MS without additives, the next step was to prepare fast- and slow-release MS to obtain high and sustained antibody responses throughout the time course of investigation. For this purpose, TT was encapsulated together into three different polymer types, i.e., the conventional end-group esterified (capped) PLGA 50:50 used previously, a more hydrophilic (uncapped) PLGA 50:50 carrying free hydroxyl and carboxyl end groups, which hydrolyse very fast, and a high molecular weight less hydrophilic PLA, which degrades slowly [4] BSA and trehalose were *co*-encapsulated into the capped and uncapped PLGA 50:50 MS. For the slow-release PLA MS, we speculated that *co*- encapsulation of a pH stabiliser was more appropriate. Therefore, in the last series of immunisation, mice received TT-PST incorporated in three types of MS: fast-releasing capped PLGA 50:50 MS containing BSA and trehalose as additives (TT-MS-BSA/Tre), very fast-releasing hydrophilic uncapped PLGA 50:50 MS (TT-MS_{hyd}-BSA/Tre) and slowly releasing PLA MS containing BSA plus calcium carbonate (TT-MS_{sl}-BSA/CaC) (Table I-c). Four groups received 0.2 or 1.0 Lf TT-PST in mixtures of very fast and slowly releasing MS, or in fast and slowly releasing MS. Two other groups received 0.2 or 1.0 Lf TT in the single PLGA 50:50 and two control groups were injected with 0.2 or 1.0 Lf of TT-alum. Immune responses were analysed over a period of 10 months (Table III). Elevated early antibody responses were detected in all groups except that injected with 0.2 Lf TT-MS-BSA/Tre. In the groups immunised with 0.2 Lf of TT-MS, the mixtures gave better results than TT-MS-BSA/Tre alone. In the groups immunised with 1.0 Lf TT-MS, only minor differences in the antibody

response were detectable in the first 6 months. However, 10 months after immunisation, the group immunised with TT-MS_{hyd}-BSA/Tre + TT-MS_{sl}-BSA/CaC mixture showed a more sustained antibody response than in the group immunised with TT-MS-BSA/Tre + TT-MS_{sl}-BSA/CaC ($p < 0.05$). Nonetheless, the slow-release MS in the two mixtures did not induce a boost antibody response by a delayed antigen release. Finally, the mixture of very fast and slowly releasing MS provided best long term immune responses, similar to those obtained with TT-alum.

Table III - Antibody responses in BALB/c mice immunised with mixtures of fast and/or slowly releasing TT-PST-MS containing additive mixtures. Mice (4 mice per group) received one injection of 0.2 or 1.0 Lf of TT-PST-MS in 3 different formulations (Table I) or the same dose of TT-PST-alum as positive control. Specific antibody responses at months 1, 6 and 10 after immunisation are expressed as the arithmetic mean of individual antibody international units ± s.d. a): TT content in the mixture (Lf TT/mg MS). b): Significant difference with the corresponding values of the (TT-MS_{hyd}-BSA/Tre + TT-MS_{sl}-BSA/CaC) group ($p<0.05$).

TT-PST-MS mixtures		Anti-TT antibody					
Components	weight ratio	0.2 Lf			1.0 Lf		
		month 1	month 6	month 10	month 1	month 6	month 10
TT-MS_{hyd}-BSA/Tre TT-MS_{sl}-BSA/CaC	50% 50% (0.66) [a]	7.42 ±1.54	1.24 ±0.36	0.99 ±0.17	9.18 ±8.18	[b] 2.59 ±1.85	[b] 2.17 ±0.39
TT-MS-BSA/Tre TT-MS_{sl}-BSA/CaC	60% 40% (0.47) [a]	7.79 ±9.41	1.13 ±0.13	0.47 ±0.16	6.13 ±0.61	0.69 ±0.41	0.45 ±0.27
TT-MS-BSA/Tre	100% (0.42) [a]	1.43 ±0.77	0.12 ±0.09	0.04 ±0.01	12.5 ±5.14	1.58 ±0.97	0.61 ±0.21
TT-alum	-	12.0 ±4.65	3.50 ±1.07	1.81 ±1.00	11.5 ±1.57	3.66 ±1.91	4.69 ±2.79

Discussion

When encapsulated proteins are exposed to extracellular fluids at 37°C for a long time period, denaturation or aggregation may occur, possibly by a result of an acidic environment due to polymer hydrolysis. These changes could decrease antigenicity [24]. In this study, we have evaluated the effect of *co*-encapsulating various additives in TT containing MS on the specific antibody response. The additives used are known for their protein or pH stabilising properties. We found that immunisation with TT-PST-MS-additives gave rise to antibody responses higher than those obtained in the absence of stabiliser and that the presence of additives elicited at least equivalent antibody responses with a 5-fold lower dose of TT-PST. Further experiments showed that sustained responses were obtained with mixtures of fast and slowly releasing TT-PST-MS-additives.

The additives calcium carbonate (CaC) and calcium phosphate (CaP) showed similar effects, i.e., increasing the response shortly after immunisation and sustaining the antibody response for the following three months. It has been reported [25] that the intensity of initial antigen burst depends on the antigen loading, because of the large amount of protein located near the MS surface and, consequently, available for initial release. The low antigen loading in these TT-MS-CaP and TT-MS-CaC (0.11 and 0.18 Lf/mg, respectively, Table I), as determined by fluorimetry, may explain the modest immunogenicity observed when the low dose of 0.2 Lf was injected. The sustained action of MS containing the calcium salts may be due to the availability of the intact antigen during MS degradation. We can assume that the stabilisation of the antigen structure results from the buffering action of the calcium salts and the prevention of acid catalysed polymer degradation (see associated paper).

Interestingly, addition of BSA, trehalose and cyclodextrin was associated with higher antibody responses, especially in the early phase, since the resultant antibody titers were at least equal or

even superior to those obtained with TT-alum (Fig. 1). Moreover, these additives were particularly efficient since even the low antigen dose (0.2 Lf) induced high antibody responses (Table II, titer index). TT-MS-BSA released *in vitro* more antigenic TT than MS without BSA. Chang and Gupta previously showed that serum albumin reduced the water content in MS, which might stabilise the antigen in MS during storage [26]. On the other hand, γ–HPCD and trehalose did not preserve encapsulated TT antigenicity, as determined by ELISA after TT extraction from MS (associated paper). However, these two saccharides yielded an increase in TT loading efficiency and mediated the highest *in vitro* burst release, which could therefore explain their *in vivo* efficacy to induce high antibody responses. Thus, BSA and trehalose were subsequently *co*-encapsulated together in PLGA MS. The combination of BSA and trehalose did not lead to a synergistic effect on TT-PST encapsulation efficiency in PLGA-MS, but the immune response appeared to remain more stable with TT-MS-BSA/Tre (Table III) than with TT-MS-BSA/2 or TT-MS-Tre throughout the time of investigation (Fig. 1). In addition, these two additives exerted a synergistic effect on the TT-PST loading efficiency in hydrophilic PLGA MS. This efficient TT loading was accompanied by an increase in antibody response as compared to the response induced with TT in standard PLGA MS-BSA/Tre.

Among the additives used in this study, L121 is an anionic block-*co*-polymer surfactant which stimulates the humoral response through complement activation [27] and by enhancing the ability of macrophages to present antigens to T cells [28], and preferentially induces a Th_1 response [29]. Despite these properties, no enhancement in antibody response could be detected by the *co*-encapsulation of L121 in TT-MS, at least under the experimental conditions used.

Importantly, an efficient and sustained boost effect was achieved after injection of a low dose of TT-alum 6 months after the first immunisation. Despite the differences in antibody levels in the various groups prior to injecting the booster dose, comparable boost responses were obtained in all TT-MS and TT-alum groups. These results suggested the importance of the TT preparation on the quality of the immune responses. Previous TT-MS immunisation schedules [7] did not induce such high concentrations of anti-TT antibodies after boosting injection with TT-alum (10-20 IU/ml with a standard TT versus 22-224 IU/ml with the TT-PST used here). In our previous study, a standard TT of commercial source was used, which contained 85 Lf/mg protein, whereas the TT-PST used here contained 333 Lf/mg protein. So, a lower amount of contaminants in TT preparations may improve the specificity of the response, by avoiding the formation of antibodies against contaminant proteins. In addition, for TT-PST preparation, immune responses were enhanced by the presence of additives (Table II).

These data suggest that a chromatographically purified TT and the mixture of very fast and slowly releasing MS represent key elements for a single injection vaccine. Immunisation with such a preparation ($TT\text{-}MS_{hyd}$-BSA/Tre + $TT\text{-}MS_{sl}$-BSA/CaC, Table III) induced indeed a high and long lasting antibody response even with a low TT-PST dose. Our results also indicate that the additives BSA, trehalose, γ-HPCD and calcium salts, in combination with the use of hydrophilic PLGA 50:50, improved the *in vitro* release of antigenic TT. Combination of hydrophilic PLGA, BSA and trehalose achieved the highest loading of TT-PST and, in combination with slow release MS, the highest level of antibody titer and IU 6 and 10 months after immunisation. As the selected additives may stabilise the antigen in MS during storage and rehydration in body fluids, their use raises the possibility of decreasing the antigen dose in MS-based vaccines. However, the presence of additives in MS was not essential for eliciting a strong secondary response by a booster injection. In conclusion, our data suggest that it is important to concentrate on the design of delayed delivery MS preparations containing additives capable to mimic a booster immunisation 3 to 6 months after the first inoculation. Since a low dose of TT was sufficient to induce a high and sustained boost response, additives may be essential in the manufacturing of delayed delivery particles because of the need to protect the antigen against moisture during a long-lasting latent period prior to antigen release. The combination of some of these stabilising agents and the use of PLGA/PLA MS mixtures with various antigen delivery profiles may allow the optimisation of single-dose vaccine formulations.

ACKNOWLEDGEMENTS

This work was supported by grants from the Swiss Foundation and UNDP/World Bank/WHO.

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5

Immunogenicity of single-dose diphtheria vaccines based on PLA/PLGA microspheres in guinea pigs*

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* Vaccine, *in press*. This chapter was not included in the thesis at the time of the doctoral defence. Associated paper: Johansen *et al.*, Diphtheria and Tetanus Toxoid Microencapsulation into Conventional and End-Group Alkylated PLA/PLGAs. European Journal of Pharmaceutics and Biopharmaceutics, 47: 193-201 (1999).

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INTRODUCTION

A promising approach for single-dose vaccines is by encapsulation of peptide and protein antigens into biodegradable microspheres (MS) of poly(lactide) (PLA) and poly(lactide-*co*-glycolide) (PLGA) [1, 2]. This has been demonstrated both *in vitro* and *in vivo*. *In vitro* experiments showed that antigens are released from MS in a controlled manner as a function of polymer properties [3,4] and that MS are taken up by different types of antigen presenting cells [5,6]. In various animal species, antigen containing MS induced both humoral [4,7-10] and cellular immune responses [11]. However, many protein antigens were found unstable in polyester MS when incubated at body temperature, leading to changes in both physical and chemical structures, weak immune responses, or ambiguous boosting of plasma B cells and neutralising antibodies [4,8,12-15].

Most investigations on single-dose vaccines have been performed with model antigens, birth control drugs [16] and tetanus toxoid [17,18]. The latter two groups have been included in past priority projects of the WHO. Many other antigens such as diphtheria toxoid (Dtxd) [19-21] should be equally good candidates for MS-based vaccines. Circumstantially, the diphtheria incidence has recently increased. Combined single-dose vaccines containing typically diphtheria, tetanus and pertussis toxoids would be a further goal in the development of a new generation of antigen delivery systems.

Here, we studied the microencapsulation of Dtxd into PLA and PLGA of different monomer composition, hydrophobicity, and molecular weight, as well as the effect of co-encapsulated excipients on MS properties. Guinea pigs were immunised with a single dose of selected Dtxd-MS and compared to alum-adsorbed diphtheria vaccine given once or twice.

MATERIALS AND METHODS

Preparation of microspheres

Diphtheria toxoid (Pasteur Mérieux, F-Lyon; lot No. 386; 6500 Lf/ml; 24 mg/ml protein) was microencapsulated into various types of biodegradable polyesters by spray-drying and coacervation, as described previously [21]. The polyesters were a 14 kDa poly(d,l-lactide-*co*-glycolide) 50:50 (PLGA 50:50), a 130 kDa poly(d,l-lactide) (PLA) (Resomer® RG502 and R206 from Boehringer Ingelheim, D-Ingelheim), and non-commercial 10 and 18 kDa poly(l-lactide) with stearyl end-groups (PLAst2 and PLAst1, respectively). The non-commercial polymers were a gift from G. Rafler, Fraunhofer IAP, D-Teltow. Bovine serum albumin (BSA; for immuno enzyme assay, Fluka, CH-Buchs) and d(+)-trehalose dihydrate (Fluka) were co-encapsulated individually or concomitantly in selected MS at nominal levels of 5 and 15% (w/w), respectively.

In vitro evaluation of microspheres

Diptheria toxoid and bovine serum albumin content in MS was determined by reversed phase HPLC on a Vydac® C_4-column (4×250 mm), after hydrolysis of the microspheres in 0.1 N NaOH with 0.05% polysorbate 20 at 37°C for 24 h [21].

Toxoid release from MS (20 mg) was conducted at 37°C in 4 ml of 67 mM PBS of pH 7.4 containing 0.01% polysorbate 20 and 0.02% sodium azid, using rotating borosilicate vials. At regular time intervals, 1 ml of medium was withdrawn, assayed, and replaced by fresh buffer.

Microsphere shape and size were examined by visible light microscopy and laser light diffractometry (Mastersizer X, Malvern Instr., UK-Worcestershire).

Immunisation procedure

Adult guinea pigs (male and female, 250-300 g; strain DH from Harlan, UK) were immunised subcutaneously with 2.5 Lf of Ttxd encapsulated in MS (Ttxd-MS), Ttxd adsorbed on aluminium hydroxide (Alhydrogel, Suerfox, S-Vebaek) or dissolved in saline (Table 1). The Ttxd-MS were dispersed in 5% aqueous lecithin solution (Epikuron® 145, Lucas Meyer, D-Hamburg), whereas the adsorbed and the dissolved toxoid were diluted in saline. A volume of 0.5 ml was injected in

all cases. Animals (10 per group) were bled by cardiac puncture at 4, 8 and 16 weeks post immunisation. Additionally, 2 x 5 animals of each group were bled after 32 and 40 weeks, respectively. Serum was prepared from clotted blood and stored at -20°C until analysis.

Table I - Groups of guinea pigs immunised with non-adsorbed, aluminium adsorbed, and microsphere (MS) based diphtheria toxoid (Dtxd) vaccines (n=10) and negative controls (n=5). SD: spray-dried MS; CO: coacervated MS.

Group code	Vaccine formulation	MS type [a]			Dose (Lf)
Neg. control 1	Saline alone				0.0
Sol	Solution				5.0
Alum-2×0.5	Adsorbate				1.0 (2×0.5)
Alum-1	Adsorbate				1.0
Alum-2.5	Adsorbate				2.5
Neg. control 2	Placebo MS	SD PLGA	+	CO PLAst1	0.0
MS-S1	MS	SD PLGA			2.5
MS-S2	MS	CO PLGA			2.5
MS-S3	MS	CO PLAst1			2.5
MS-S4	MS	CO PLAst2			2.5
MS-Mix1	MS mixture	SD PLGA	+	CO PLAst1	2.5
MS-Mix2	MS mixture	SD PLGA	+	CO PLA	2.5

Determination of serum anti-diphtheria responses

ELISA assay: The antibody responses to diphtheria toxoid in guinea pigs were determined using a method previously described [22]. The coating antigen was Dtxd (0.5 Lf/ml), and results were expressed relative to a reference from the National Institute of Biological Standards and Control (NIBSC, Potters Bar, Great-Britain; guinea pig IgG fraction, 0.04 IU/ml) previously calibrated *in vivo* in IU/ml against the WHO International Standard for diphtheria antitoxin. Detection level was $5\text{-}10\times10^{-4}$ IU/ml of antitoxin.

In vitro neutralisation assay: The *in vitro* diphtheria toxin neutralisation potency of sera was determined using cultured Vero cells as described elsewhere [22]. The results were expressed relative to the 3rd British Standard for equine diphtheria antitoxin (NIBSC 66/153). Detection level was $4\text{-}8\times10^{-4}$ IU/ml.

Statistical analysis: Antibody titres of the immunised groups were expressed as geometric means of IU/ml ± SEM. Six out of 420 values (1.4% of total) deviated more than four times the SD of the mean and were omitted from the data set as outliers. Student's *t*-test was carried out to analyse statistical significance of the results; *P*-values lower than 0.01 were considered to be significant.

Results

In vitro characteristics of the microspheres

The microspheres prepared by spray-drying and coacervation all had regular morphology and smooth surface, independently of the preparation method, polymer type or formulation. The particle size of the spray-dried formulations lay between 1 and 5 µm, and that of the coacervated products between 15 and 60 µm.

The various microsphere types differed greatly in Dtxd-encapsulation efficiency and *in vitro* release of toxoid within the first 48 h, depending on polymer type, *co*-encapsulated excipient and preparation method (Table 2). For the spray-dried 14 kDa PLGA 50:50 MS without additive, the encapsulation efficiency was 75%; the encapsulation efficiency was expressed as actual toxoid loading, as determined by HPLC after extraction, relative to nominal loading. While *co*-encapsulated albumin did not affect the toxoid entrapment (72%), trehalose lowered the encapsulation efficiency to 45-56%. For this polymer type, we also studied the effect of

microencapsulation method on toxoid content. In contrast to the spray-dried PLGA 50:50 MS, coacervated PLGA 50:50 MS showed a lower toxoid content when albumin alone was co-encapsulated (45%), but slightly higher when both albumin and trehalose were used (58%). The high molecular weight (130 kDa) PLA was processed by coacervation only. Here, toxoid entrapment was slightly lower (40-47% efficiency) than in coacervated PLGA 50:50 (45-58% efficiency). Results of the initial release (see below) and by others [23, 24] suggest, however, that the loading values measured with this polymer type are very likely an underestimation of the actual toxoid content. In this polymer, albumin alone or in combination with trehalose appeared to improve the toxoid entrapment (results not shown). Finally, the end-group modified 18 kDa PLAst1 and 10 kDa PLAst2 were both processed by coacervation only. They entrapped the toxoid to a similar extent (32-57%) as did the coacervated 14 kDa PLGA 50:50 and 130 kDa PLA. With the end-group modified PLAs, albumin was a prerequisite for Dtxd-encapsulation to prevent precipitation of the toxoid in the W/O-emulsion prior to coacervation.

An important goal of this investigation was to examine the feasibility of a single-dose delivery system for Dtxd, i.e., to produce polymeric MS providing controlled antigen release thereby mimicking priming and booster doses. Generally, spray-dried PLGA 50:50 MS gave a more pronounced initial release (38-85% of the actual dose) than the corresponding coacervated MS (2-33% of the actual dose) (Table 2). The *co*-encapsulated additives trehalose and albumin increased the initial Dtxd fraction released from the spray-dried PLGA 50:50 MS from 38% to 47-50%, or even to 85% when both additives were *co*-encapsulated concomitantly. In general, concomitant *co*-encapsulation of both additives increased the initial release significantly, except for PLAst2 MS. The initial release values from the high molecular weight PLA MS are not indicated in Table 2, as the determined values cannot be considered as reliable. For this particular polymer type, the available methods for assaying the toxoid content in MS were found inappropriate [23,24], resulting in substantial underestimation. Consequently, the initial fractional release measured amounted to almost the totality or more of the actual loading determined.

Table II - *In vitro* evaluation of PLA and PLGA microspheres (MS) prepared by spray-drying (SD) or coacervation (CO). SD-MS were smaller than 5 µm in diameter, and CO-MS were between 15 and 60 µm. Loading efficiency is the percentage of Dtxd, relative to the nominal loading, recovered from MS after polymer hydrolysis. Initial release is the percentage of Dtxd, relative to the estimated content, released into PBS of pH 7.4 at 37°C. n=3 ± SD. BSA is bovine serum albumin and Tre is trehalose co-encapsulated to the nominal loading of 5 and 15%, respectively. a): See comments in Results.

MS formulation	Group code (see Table I)	Loading efficiency (%)	Initial release within 48 h (%)
SD PLGA	-	75 ± 1	38 ± 6
SD PLGA BSA	MS-S1	72 ± 9	50 ± 2
SD PLGA Tre	-	56 ± 1	47 ± 2
SD PLGA BSA+Tre	in MS-Mix1 & 2	45 ± 5	85 ± 11
CO PLGA BSA	MS-S2	45 ± 4	2 ± 1
CO PLGA BSA+Tre	-	58 ± 2	33 ± 6
CO PLAst1 BSA	MS-S3; in MS-Mix1	32 ± 2	17 ± 1
CO PLAst1 BSA+Tre	-	42 ± 0.4	33 ± 1
CO PLAst2 BSA	MS-S4	57 ± 0.3	49 ± 3
CO PLAst2 BSA+Tre	-	54 ± 2	19 ± 0.1
CO PLA BSA	in MS-Mix2	40 ± 1	120 ± 7 a)
CO PLA BSA+Tre	-	47 ± 1	69 ± 2 a)

Immune responses to Dtxd vaccines

Results of guinea pig serum antibody (IU/ml) titres are summarised in Fig. 1. At four weeks post immunisation, anti-diphtheria antibody levels above protective level (0.01 IU/ml) were detected in animals immunised with 2.5 Lf Dtxd either adsorbed on alum or microencapsulated

into fast-release PLGA50:50 MS (groups *MS-S1, MS-S2, MS-Mix1* and *MS-Mix2*). The PLGA 50:50 MS (*MS-S1* and *MS-S2*) yielded antibody responses comparable in magnitude (0.02 - 0.04 IU/ml) to that induced by the single 2.5 Lf dose of alum-adsorbed Dtxd (*Alum-2.5*). Apparently, the MS size (1 - 5 μm for *MS-S1* and 15 - 60 μm for *MS-S2*) had no noticeable effect on the elicitation of antibodies. The MS mixtures containing besides the fast-release PLGA50:50 MS also slow-release PLAst1 MS or PLA MS (*MS-Mix1* and *MS-Mix2*) gave lower titres (0.01 - 0.015 IU/ml). Dtxd-MS made with the stearyl modified PLAst1 and PLAst2 did not induce responses significantly higher than the negative controls saline and placebo MS. The animals immunised with a single dose of 1 Lf or with the first of two doses of 0.5 Lf of Dtxd adsorbed on alum did not rise significant antibody titres after 4 weeks. Considering the kinetics of antibody response over 40 weeks, the titres of the 2.5 Lf dose groups (all MS-groups and the 2.5 Lf alum-adosrbed group) generally increased slightly between week 4 and 8 and then persisted until week 40. In the low Dtxd dose groups (*Alum-1* and *Alum-2*0.5*), the titres also increased between week 4 and 8 or even up to week 32, but then dropped very noticeably between week 32 and 40.

Diphtheria toxin neutralising antibodies were detectable in guinea pigs 4 weeks post immunisation only in some of the groups. At this early time point, three out of the six Dtxd-MS formulations (rank order: *MS-S2* > *MS-S1* > *MS-Mix2*) and the alum-adsorbed Dtxd at a dose of 2.5 Lf induced measurable antibodies which, however, were below the level considered to be protective (0.01 IU/ml). After 8 weeks, protective antibody levels were induced by *MS-S1, MS-S2* and *MS-Mix2*; these formulations contained small- and large-sized fast-release PLGA 50:50 MS, and mixture of small-sized fast-release PLGA 50:50 MS and large-sized slow-release PLA MS, respectively. The responses obtained with 2.5 Lf DTxd in *MS-S1, MS-S2*, and *MS-Mix2* were statistically not different ($P < 0.01$) from those observed with the same single dose of alum-adsorbed toxoid (*Alum-2.5*). Interestingly, 1 Lf of alum-adsorbed toxoid induced when given in two separate doses (priming and booster; *Alum-2×0.5*) comparable titres to the single 2.5 Lf dose formulations. Significantly lower ($P < 0.01$) titres were observed when 1 Lf of alum-adsorbed Dtxd (*Alum-1*) was given in a single dose. Finally, neutralising antibody titres were maintained over the 40 week study period in the groups immunised with 2.5 Lf Dtxd in *MS-S1, MS-S2, MS-Mix2* and *Alum-2.5*.

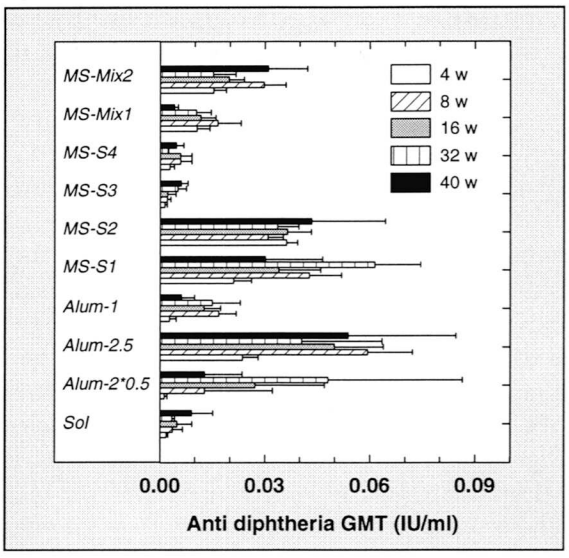

Figure 1 - Total antibody titers in guinea pigs (n=10) immunised with diphtheria toxoid in solution, adsorbed to aluminium hydroxide, or encapsulated in PLA/PLGA microspheres (see Table I for immunisation codes). Serum was sampled 4 (white bars), 8 (grey bars), and 16 weeks (black bars) post immunisation, and measured by ELISA. The results are expressed as IU/ml ± SEM.

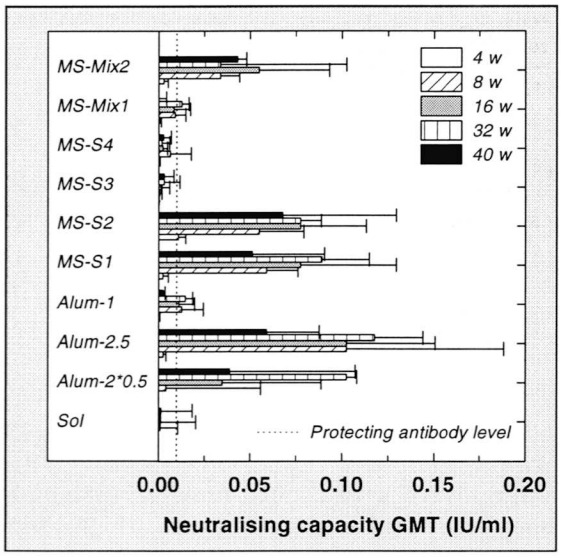

Figure 2 - Neutralising antibody titers in guinea pigs (n=10) immunised with diphtheria toxoid in solution, adsorbed to aluminium hydroxide, or encapsulated in PLA/PLGA microspheres (see Table I for immunisation codes). Titers were determined *in vitro* in cultured Vero cells from blood serum sampled at 4 (white bars), 8 (grey bars), and 16 weeks (black bars) post immunisation. Two values for the *Alum-2×0.5* and the *Alum-2.5* groups and one value from the MS-S4 group were defined as outliers and therefore discarded. All results are expressed as IU/ml ± SEM.

Discussion

Polyester microspheres (MS) have been proposed as immunological adjuvant and controlled antigen release system capable of potentiating both humoral and cellular responses. Adjuvant properties were related to interaction of the MS with both professional APCs [25] or B cells. Furthermore, the possibility for sustained and controlled antigen release from PLA/PLGA MS should allow us to design single-dose vaccines, which may reduce the need for repeated immunisation. The present work focuses on the feasibility of Dtxd containing MS made of conventional PLA and PLGA and of novel end-group stearylated PLA to induce immune responses after a single injection. The results demonstrate high and persisting titers of protective antibodies in guinea pigs, comparable to those induced by Dtxd adsorbed on aluminium hydroxide.

Six groups of guinea pigs were immunised with single doses of 2.5 Lf of Dtxd encapsulated in MS, two groups with respective single doses of 2.5 and 1.0 Lf of Dtxd adsorbed on alum, and one group with two doses of 0.5 Lf of Dtxd adsorbed on alum (Table 1). The MS formulations had the following features, which were potentially relevant to induce an immune response: (i) fast-release MS of low hydrophobicity and of a size (1 - 5 μm) suitable for phagocytosis (*MS-S1*); (ii) fast-release MS of low hydrophobicity and of large size (15 - 60 μm), unsuitable for phagocytosis (*MS-S2*); (iii) very slow-release MS of high hydrophobicity and large size (*MS-S3*); (iv) slow-release MS of high hydrophobicity and large size (*MS-S3*); (v) mixture of *MS-S1* and *MS-S3* (*MS-Mix1*); (vi) mixture of *MS-S1* and slow-release MS of high molecular weight, intermediate hydrophobicity and large size (*MS-Mix2*) (Table 1). The hydrophobic and high molecular weight polymers and the microsphere mixtures, containing both fast and slowly degrading polymers, were intended to provide more prolonged release and, potentially, multiple antigen doses. This strategy was based on the assumption that antigen release from the different MS types is controlled, at least partly, by bulk erosion of the particles.

The data show that the relatively hydrophilic PLGA 50:50 MS (*MS-S1* and *MS-S2*) elicited very strong antibody responses already 4-8 weeks after administration, whereas the end-group stearylated PLA MS (*MS-S3* and *MS-S4*) induced weak responses (Figs 1 and 2). This is consistent with the faster release and erosion properties of the PLGA 50:50 as compared to the hydrophobic PLAst1 and PLAst2. Furthermore, as smaller particles are more readily ingested by APCs [25], the small-sized spray-dried MS (*MS-S1*) were expected to induce a faster and more efficient T cell priming than the larger MS prepared by coacervation (*MS-S2, MS-S3*, and *MS-S4*). Contrary to this expectation, but consistent to some extent with a previous finding [8], the small-sized *MS-S1* and the larger sized *MS-S2* induced comparable antibody responses in guinea pigs; surprisingly, the antibody level at week 4 was even slightly higher upon immunisation with the larger *MS-S2*. As MS larger than 10 -15 μm are generally believed to be excluded from phagocytosis, the above results suggest that full particle uptake by APCs might not be necessary for antigen processing, but a simple surface contact between particles and APCs might be sufficient for transferring the antigen into the APCs, where it can be processed further. On the other hand, in a population of large particles, such as those of *MS-S2*, there will always be a minor number of smaller particles or particle fragments that can be ingested by APCs. At present, it remains unknown which of these two mechanisms facilitates primarily antigen processing. Further, considering that the larger *MS-S2* gave a much lower *in vitro* burst release than *MS-S1* (2% *versus* 50%; Table 2), we may speculate that *MS-S2* possess a higher antigen core loading at the time when they enter into contact with or are taken up by APCs. Consequently, a higher number of T cell epitope-MHC II complexes might be presented on the cell surface. Further, the PLGA 50:50 MS erode substantially within 4 weeks incubation at 37°C [4]. Hence, an earlier bleeding of the guinea pigs might have been more discriminative between *MS-S1* and *MS-S2* for the immediate immune response.

The low early immune response in guinea pigs immunised with the hydrophobic *MS-S3* and *MS-S4* was expected because of the slow erosion rate of these MS. However, as no significant rise in antibody titer was observed over 40 weeks, one may speculate that the toxoid integrity in these

MS was not maintained [13-15, 23]. Hence, the antibody response observed after a single injection of these batches may not reflect their real boosting potential *in vivo*. We therefore studied the immune response in guinea pigs with mixtures of two MS types. The observed antibody responses to *MS-Mix1* and *MS-Mix2* were consistently lower than the response induced by *MS-S1* (PLGA 50:50 MS). This might be due to, at least partly, the presence of trehalose in the PLGA 50:50 MS contained in the MS mixtures. Trehalose, *co*-encapsulated as stabiliser for Dtxd, caused a higher initial release of Dtxd (Table 2). As discussed above, a higher initial release may lower the priming efficacy of MS, because a higher amount of Dtxd is likely released extracellularly and, hence, unavailable for uptake into APCs and priming. Furthermore, *MS-S1* contained 2.5 Lf Dtxd exclusively in fast releasing PLGA 50:50 MS, the MS mixtures contained 1.25 Lf in each of two MS types (PLGA 50:50 + PLA, or PLGA 50:50 + PLAst1). The weaker antibody response observed with the MS mixtures may consequently be a dose-response effect, especially if antigen stability should be less preserved in the PLA- or PLAst1-MS. Indeed, no boosting effect was observed for *MS-Mix1*, indicating that PLAst1 MS did not noticeably contribute to the total immune response; conversely, the *MS-Mix2* seemed to produce a very weak antibody boosting between week 32 and 40.

The stronger immune response produced by *MS-Mix2* as compared to *MS-Mix1* must be attributed to the slow-release MS, as the fast-release MS (spray-dried fast releasing PLGA 50:50 MS, containing trehalose and BSA) were identical in both MS mixtures (Table 2). This clearly shows that the end-group alkylated PLAst1 MS do not contribute to the antibody response, whereas the high molecular weight PLA MS do so. Whether this difference is due to different release characteristics of the two slow-release MS types *in vivo* or to the different hydrophobicity of the MS remains presently unknown. Further interpretation of these results would be premature.

In conclusion, fast-release MS and mixtures of fast-release and slow-release MS generally produced a stronger increase in protective than in total antibodies between weeks 4, 8 and 16 (Figs 1 and 2). This suggests that the MS-formulations favoured maturation of B cells producing high-affinity antibodies. Thus, the presented data clearly show the feasibility of MS-vaccines against diphtheria. The DTxd-MS induced elevated and long-lasting specific humoral immune response after a single immunisation. To what extent long-term protection is achievable with MS preparation should become clearer from future investigations.

ACKNOWLEDGEMENTS

This work was supported by grants from the WHO Global Programme for Vaccines and Immunisation (GPV), Vaccine Research and Development, World Health Organisation, Geneva (Switzerland), and from the Swiss National Foundation for Scientific Work, Berne, Switzerland (grant No. 31-37440.93). We are grateful to Dr. G. Rafler, Fraunhofer IAP, Teltow (Germany) for providing the end-group stearylated PLAs. We also thank Dr. Ying Men for helpful discussion.

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6

Physico-chemical and Antigenic Properties of Tetanus and Diphtheria Toxoids and Steps Towards Improved Stability*

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* Biochemica Biophysica Acta 1425: 425-436 (1998).

INTRODUCTION

The potential of biodegradable microspheres of poly(lactic acid) (PLA) or poly(lactic-*co*-glycolic acid) (PLGA) for single-dose vaccine delivery has been demonstrated in recent years [1-6]. However, it has also become clear from the multiple investigations that the functional stability of microencapsulated antigens is critical. Typically, the preparation of PLA/PLGA microspheres requires materials and processes that might reduce or destroy entirely the antigenicity of encapsulated protein antigens, e.g., toxoids. Moreover, upon release (*in vivo* and *in vitro*) these proteins will be exposed to acidic degradation products from the polymers, i.e., lactic and glycolic acids and their water soluble oligomers, as well as to the surfaces of the polymeric material. Therefore, a better knowledge of antigen stability under stress conditions should help to design more rationally a delivery system with preserved integrity of protein antigens.

Tetanus and diphtheria toxoids (Ttxd, Dtxd) have been studied for use in single injection vaccine delivery systems [7-11]. Only few of these investigations considered the physico-chemical and functional stability of the toxoids upon microencapsulation, or during storage or release from PLA/PLGA microspheres [12]. More basic studies on the native tetanus and diphtheria toxins revealed information on the molecular structure and biological activity of these proteins. The mature tetanus neurotoxin is a 1351 amino acid protein consisting of two chains (N-terminal light chain of 52 kDa and C-terminal heavy chain of 98 kDa) linked by a single disulphide bridge [13,14]. Diphtheria toxin is a 535 residue single chain, which can be proteolytically cleaved into fragments A (N-terminal; 21 kDa) and B (C-terminal; 37 kDa) [15,16]. Conformational studies have demonstrated the importance of conformational domains for the biological activity, i.e., toxicity and antigenicity. Typically, the tertiary structure of diphtheria toxin, which is crucial for its translocation through cell membranes, is strongly influenced by pH, temperature and the hydrophobic environment [17,18]. This has been investigated by spectroscopy and electrophoresis in combination with membrane binding and penetration studies [19-21]. For tetanus toxin, the biological activity and structure have also been examined [14,22-24]. Nonetheless, relatively little is known to date on the mechanisms of loss of activity and related chemical and structural changes. Such knowledge, however, would help to develop more rationally antigen delivery systems such as liposomes or PLA/PLGA microspheres. Indeed, functional antigen stability in these delivery systems is the first requirement for success. Loss of antigen stability during pharmaceutical processing has typically been illustrated for Ttxd and Dtxd which aggregated upon lyophilisation [25]. This result was confirmed by the significant, mostly reversible change observed in the secondary structure of Ttxd during lyophilisation [26]. The addition of stabilising excipients could largely prevent the moisture induced aggregation of Ttxd, but this did not correlate with the extent of structural alterations. In the microencapsulation of Ttxd into PLA/PLGA, lipids, sugars, polyols, surfactants and proteins were *co*-encapsulated to diminish loss of antigenicity [27,28]. In particular, albumin greatly improved both the encapsulation and release of antigenic Ttxd [27,29].

Here, we report on the physico-chemical, conformational and antigenic stability of tetanus and diphtheria toxoids in solution as a function of temperature and the presence of lactic and glycolic acids and albumin. As a consequence, strategies are proposed to preserve toxoid antigenicity in PLA/PLGA microspheres for single-dose immunisation.

MATERIALS AND METHODS

Materials

Aqueous solutions of tetanus (Ttxd) and diphtheria (Dtxd) toxoid, provided by WHO, CH-Geneva, were from Massachusetts Public Health Biological Laboratories, Boston, MA, (Ttxd lot No. PSTtxd-20, 1400 Lf/ml, 5.2 mg/ml protein) and from Pasteur Mérieux, F-Lyon (Ttxd lot No. PTC 10005, 8500 Lf/ml, 26.3 mg/ml protein; Dtxd lot No. 386, 6500 Lf/ml, 24 mg/ml protein). PSTtxd-20 was a column purified and PTC a commercial grade Ttxd. Bovine serum albumin

(BSA) for immuno enzyme assay was from Fluka, CH-Buchs. Monoclonal anti-tetanus antibody (TT010) and guinea pig anti-tetanus IgG were from Wellcome Biotechnology, UK-Beckenham. Rabbit anti-guinea pig IgG horse radish peroxidase conjugate and 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) were from Sigma Chemical, St. Louis, MA. Unless specified otherwise, all other substances used were of pharmaceutical or analytical grade and purchased from commercial suppliers.

Experimental designs

The stability of Ttxd (PTC and PSTtxd-20) and Dtxd in aqueous solution, and the reversibility of acid induced toxoid degradation were studied as a function of temperature and the presence of lactic and/or glycolic acid and of the stabilising agent BSA. Fluorimetry and circular dichroism spectroscopy (CD) was used to study toxoid conformation, turbidimetry was used to study toxoid aggregation, gel-electrophoresis was applied for the study of molecular weight and aggregation, and finally, ELISA was used to determine the antigenicity, i.e., the functional stability of the toxoids (for details, see below).

In a preliminary experiment, conformational changes of Ttxd and Dtxd were investigated by fluorimetry in the presence of the denaturing agent guanidinium hydrochloride (Gdm-Cl). Solutions of each protein (30 µg/ml or approx. 10 Lf/ml toxoid) in 67 mM PBS of pH 7.4 were incubated with 0 - 5 M Gdm-Cl at 37°C for 60 min and analysed fluorometrically, as specified below. This experiment served as reference for acid and temperature induced conformational changes of the toxoids.

Structural and functional changes of the toxoids were examined by fluorimetry and ELISA according to a 2^3 factorial design (Table I), with the factors temperature (A; 4 and 60°C) and presence of lactic (B) and glycolic (C) acid (0 and 50 mM) in the toxoid solution. Typically, 2 ml of 100 µg/ml toxoid (equivalent to 30 Lf/ml) in 67 mM PBS of pH 7.4 and 300 mosmol/kg were incubated in 5 ml air-tight borosilicate vials. The solutions were analysed at regular intervals over 4 weeks. Then, selected samples were neutralised with 10 N NaOH or dialysed (10 kDa cut-off) against 67 mM PBS to study the reversibility of structural changes.

In a second 2^3 design (Table II), the ELISA-antigenicity of 30 µg/ml Ttxd was studied at 37°C as a function of lactic (A) or glycolic (B) acid (0 and 15 mM) and of BSA (C; 0 and 5 mg/ml). Incubation conditions of the solutions were as described above.

The effect of 0 - 10 mg/ml BSA on the ELISA-antigenicity of Ttxd (PTC, 6.3 µg/ml) was studied in aqueous solutions containing 67 mM glycolic acid. For this, 4 ml solutions were incubated in borosilicate vials under moderate horizontal agitation for 6 weeks.

Fluorescence Spectroscopy

Protein content and structural changes were monitored at 37°C by fluorimetry (Fluoromax, Spex, Edison, NJ) using different excitation and emission wavelengths. Toxoid solutions were excited (λ_{ex}) at 280 nm or 295 nm; with λ_{ex}=295 nm, tyrosine (Tyr) emission is eliminated, and tryptophan (Trp) emission can be detected specifically. Further, the ratio of emission intensity at 350 nm over that at 329 nm (I_{350}/I_{329}) was studied. Both changes in emission wavelength maximum (λ_{max}) and I_{350}/I_{329} are sensitive measures for conformational changes [20].

Table I - Experimental design for the stability study of tetanus (PTC and PSTtxd-20) and diphtheria toxoids. Fluorescence and ELISA responses of 0.1 mg/ml (30 Lf/ml) toxoid solutions in PBS contained in borosilicate vials were measured after 7 and 28 days (n = 3). pH ranges describe drop over time upon incubation.

Experimental level	Factor A Temperature (°C)	Factor B Lactic acid (mM)	Factor C Glycolic acid (mM)	pH Ttxd	pH Dtxd
(1)	4	0	0	7.3	7.4
a	60	0	0	7.3	7.4
b	4	50	0	6.0	6.3
ab	60	50	0	5.2	5.9
c	4	0	50	5.2	5.8
ac	60	0	50	5.2	5.8
bc	4	50	50	3.9	4.1-3.9
abc	60	50	50	3.9-3.7 [a)]	4.1-3.8

Table II - Experimental design for the stability study of tetanus toxoid (PTC and PSTtxd-20). Antigen solutions of 30 µg/ml (10 Lf/ml) were incubated at 37°C in borosilicate vials, and ELISA response was measured after 1, 7 and 14 days (n = 3).

Experimental level	Factor A Lactic acid (mM)	Factor B Glycolic acid (mM)	Factor C Albumin (mg/ml)
(1)	0	0	0
a	15	0	0
b	0	15	0
ab	15	15	0
c	0	0	5
ac	15	0	5
bc	0	15	5
abc	15	15	5

ELISA of tetanus toxoid

Flat-bottom 96-wells microtiter plates (Nunc-Immuno Plate Maxisorb, Nunc, DK-Roskilde) were filled with 100 µl of 1 µg/ml monoclonal anti-tetanus IgG (TT010) in 0.05 M carbonate buffer of pH 9.6, and incubated at 4°C overnight. The plates were washed three times with 300 µl of 0.05 % Tween-20 in PBS of pH 7.4 (PBST) after each incubation step. After 1 h incubation at 37°C with 150 µl of 2.5% milk powder in PBST (PBSTM), the plates were incubated at 37°C for 2 h with serial dilutions of standard and test solutions of Ttxd. Guinea pig IgG (25 µg/ml) was added to each well in 100 µl of PBSTM, and plates were incubated for another 2 h. Then, rabbit anti-guinea pig peroxidase conjugate (1/8000 dilution) in 100 µl PBSTM was added to each well, and plates incubated for further 1 h. Finally, 100 µl of 0.5 mg/ml peroxidase substrate 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) in 0.05 M citric acid of pH 4.0 was added to the wells, and the endpoint optical density measured at 405 nm (Thermomax, Molecular Devices, Menlo Park, CA) after 30 min incubation at room temperature.

Circular dichroism

CD measurements were performed to gain more specific insight into conformational changes of Ttxd and Dtxd under stressed conditions. Typically, 0.5 ml of 200 µg/ml toxoid in a 10 mM saline free phosphate buffer (PB) of pH 7.4 were analysed (250-190 nm, Jasco J-720, Japan Spectroscopic CO, J-Tokyo) at regular intervals over 4 weeks. At the end of the study period, the samples were dialysed (10 kDa cut-off) against 10 mM PB and examined for reversibility of conformational changes. In addition, high temperature-induced denaturation of Ttxd and Dtxd was studied by a constant increase of the temperature of the solutions from 20 to 95°C at a rate of 30°C/h and recording the change of specific ellipticity at 222 and 288 nm.

Gel Electrophoresis

Changes in conformation and molecular weight of Ttxd (200 µg/ml) upon exposure to lactic or glycolic acid (5 - 100 mM) were investigated by native gel electrophoresis with 50 mm polyacrylamid (PAGE) of gradient 10 - 15% (PhastGel, Pharmacia, S-Uppsala). The molecular weight was determined against broad range (6.5 - 200 kDa) PAGE standards from BioRad (CH-Glattbrugg), and the gels were developed with silver staining.

Turbidimetry

Aggregation of Ttxd and Dtxd (100 µg/ml) upon exposure to lactic and glycolic acid (0 - 100 mM) and elevated temperatures (37 and 60°C) was studied by incubating solutions of 200 µl per well in a 96-well microtiter plate and monitoring changes in optical density at 450 nm (Thermomax, Molecular Devices, Menlo Park, CA). At regular intervals, the solutions were also examined microscopically for formation of aggregates.

RESULTS

Fluorimetric changes of toxoids upon Gdm-Cl treatment

Solutions of 30 µg/ml of Ttxd and Dtxd were tested with Gdm-Cl to establish a fluorimetric control for conformational unfolding. The solutions were excited (λ_{ex}) at 280 nm or 295 nm. In general, the unfolding of the toxoids were reflected by a red shift of fluorescence (Fig. 1), indicating a change to a more polar environment for the emitting amino acids. Using λ_{ex} = 280 nm, the shift in emission maximum (λ_{max}) began at approx. 1.5 M Gdm-Cl for the column purified PSTtxd-20 and at 2.0 - 2.5 M for commercial grade PTC. Red shifts of both Ttxd increased with increasing Gdm-Cl concentration and occurred at higher Gdm-Cl concentrations (2.5 M for PSTtxd-20 and 4.0 M for PTC) with λ_{ex} = 295. Further, the emission intensity ratio I_{350}/I_{329} increased, and the absolute intensity at 329 nm decreased for all toxoids (results not shown). These results indicated a higher conformational stability of PTC than of PSTtxd-20 and Dtxd and served as reference for the following studies on toxoid stability.

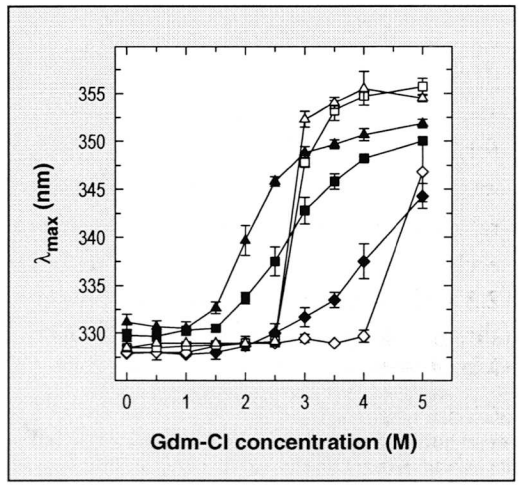

Figure 1 - Conformational changes in terms of wavelength of maximum fluorescence emission (λ_{max}) of Ttxd (diamonds: PTC; squares: PSTtxd-20) and Dtxd (triangles) in solutions of guanidinium hydrochloride (Gdm-Cl). Solutions were measured by fluorimetry after excitation at 280 nm (filled symbols) or 295 nm (open symbols).

Physico-chemical stability of toxoids at elevated temperatures and in the presence of lactic and glycolic acids and BSA as a stabiliser

Fluorimetric properties

Analogous to the Gdm-Cl experiments, fluorimetric changes of toxoid solutions exposed to elevated temperatures or lactic and glycolic acids were interpreted as conformational changes, although chemical changes cannot be excluded. Upon exposure to 4 or 60°C and 0 or 50 mM lactic or glycolic acid (see experimental design in Table I), both Ttxd and Dtxd underwent conformational changes. Temperature proved to be more critical than either of the acids. Incubation at 60°C over 7 days induced a substantial λ_{max}-shift towards longer wavelengths, whereas the effect of either acid was negligible (Fig. 2). The purified PSTtxd-20 underwent a stronger change than the other two toxoids. λ_{max} of PSTtxd-20 increased from 330 to 340 nm when excited at 280 nm (Fig. 2A); a further increase was observed in the presence of lactic and glycolic acid when only Trp-emission was measured (λ_{ex} = 295 nm; Fig. 2B). Interestingly, the acids prevented the temperature induced red shift for PTC and Dtxd with λ_{ex} = 295 nm. As the acids themselves did not generate a blue shift, this indicates an interaction between the factors temperature and acids. Upon longer incubation at 60°C, i.e., 21 days, the shift to higher wavelengths further increased modestly for PTC and Dtxd (0 - 10 nm) and substantially for PSTtxd-20 (85 nm), with λ_{ex} = 295 nm (results not shown). No further shifts were observed for solutions incubated at 4°C.

Incubation of the toxoid solutions at elevated temperature generally increased the I_{350}/I_{329} ratio (Fig. 3A). Here, the results indicated a better conformational stability of Dtxd than of both

Ttxd, with PSTtxd-20 being again the most sensitive to structural perturbations. Conversely, for PTC, I_{350}/I_{329} decreased under the influence of both the acids and high temperature. Further, with λ_{ex} = 280 nm and the combination of high temperature and acidic compounds the I_{350}/I_{329} ratios also decreased between day 7 and 21 for PSTtxd-20 and PTC (results not shown).

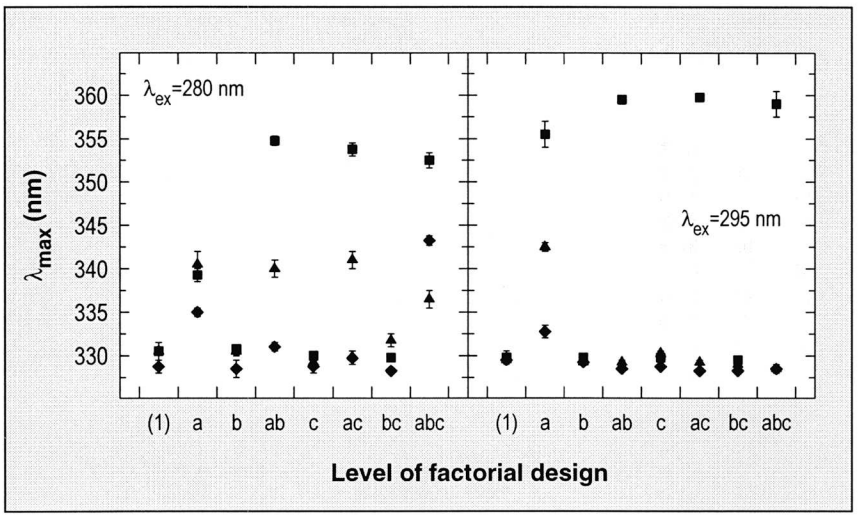

Figure 2 - Effect of temperature (factor A), lactic (factor B) and glycolic (factor C) acid on toxoid conformation (◆: PTC; ■: PSTtxd-20; ▲: Dtxd). Wavelength of maximum fluorescence emission (λ_{max}) was measured after 7 days incubation and at excitation wavelengths of 280 nm (left) and 295 nm (right). Other experimental conditions are described in Table I.

In agreement with the previous parameters, the emission intensity of PSTtxd-20 and PTC stored at 4°C indicated only moderate effect of the acids (λ_{ex} = 295 nm; Fig. 3B). However, incubation at 60°C reduced the emission by approx. 40% within 7 days. Also, an interaction of temperature and acidic compounds was observed as the emission intensity was further lowered to 10 - 20% of the initial value when lactic and glycolic acids were combined with elevated temperature. Contrary to the above described parameters, the emission intensity (at λ_{em} = 329 nm) suggested a slightly better stability of PSTtxd-20 than of PTC. On the other hand, Dtxd emission was reduced by only 20% at 60°C. Furthermore, 50 mM of either acid produced no change in the Dtxd emission at 4°C. When Dtxd was incubated at 4°C with both lactic and glycolic acids, a 20% reduction in emission intensity was found.

The loss of fluorescence intensity at 329 nm and the shift to longer wavelengths may not be attributed to an exposure of initially buried Trp residues upon unfolding of the protein alone, but also to chemical changes of the Trp. Trp can indeed lead to indole formation, emitting light at longer wavelengths, as demonstrated by exposure of Trp and various proteins to 6 M hydrochloric acid [30]. Therefore, we investigated the emission ratio at 450 nm over that of 329 nm, I_{450}/I_{329}, to quantify this chemical reaction. Table III shows the results for Ttxd and Dtxd after 21 days incubation. The emission ratio I_{450}/I_{329} of PTC and Dtxd was not, and that of PSTtxd-20 only slightly influenced by the presence of lactic and glycolic acids. Conversely, temperature increased the I_{450}/I_{329} ratio of all the toxoids substantially, with the PSTtxd-20 showing the highest sensitivity.

Turbidimetric properties and aggregation

All toxoids precipitated visibly when stored at elevated temperatures and in the presence of lactic and glycolic acids. For illustration, the optical density at 450 nm of the toxoid solutions containing 50 mM lactic acid increased from initially 0.04 to 0.10 (PSTtxd-20) and to 0.08 (Dtxd) within 1 day incubation at 60°C. In addition, aggregates were also observed microscopically. At 25 mM lactic acid, the optical density reached similar levels after 3 days. For PTC solutions, the optical density did not change over three days and no aggregation was observed. When incubated at 37°C, 100 mM lactic acid was necessary to induce aggregation within 3 - 7 days, depending on the toxoid. Again, PTC showed less aggregation than PSTtxd-20 and Dtxd.

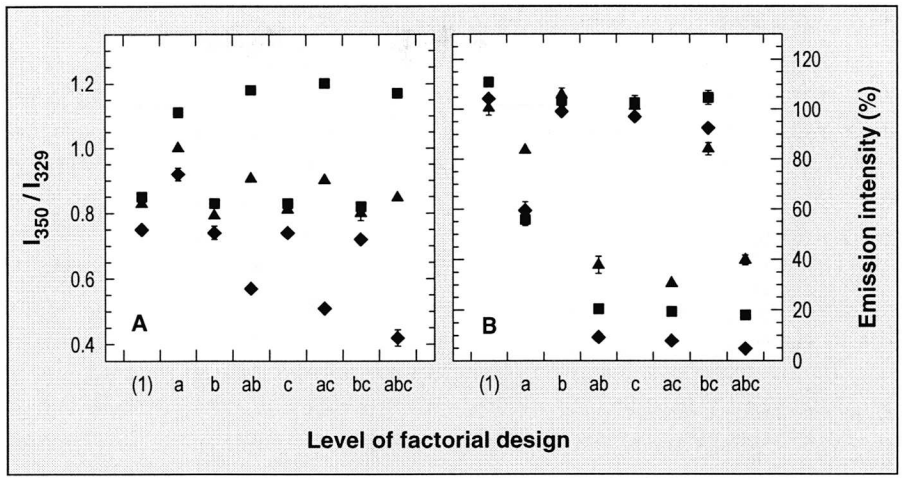

Figure 3 - Effect of temperature (factor A), lactic (factor B) and glycolic (factor C) acid on toxoid conformation and fluorescence intensity (◆: PTC; ■: PSTtxd-20; ▲: Dtxd). All solutions were excited at 295 nm; other experimental conditions are described in Table 1. (A) Ratio of fluorescence emission at 350 nm over that at 329 nm (I_{350}/I_{329}). (B) Emission intensity at 329 nm.

CD properties

Conformational stability of Ttxd solution was further studied by far UV-CD (results not shown). In PB, the spectra indicated significant α-helical conformation. After 7 days incubation at 60°C, the reduction of the ellipticity at 208 nm and 222 nm was not significant. In the presence of glycolic acid, the ellipticity minimum shifted from 208 nm to 213 nm indicating a slight increase in β-sheet structure. However, considering the aggregation of Ttxd at low pH and high temperature, the CD-result may have been affected by scattering and absorbance flattening. Nonetheless, the data showed that the toxoids did not become random coil under the actual experimental conditions.

Electrophoretic properties

The influence of lactic and glycolic acids on Ttxd stability was investigated by native PAGE (results not shown). PTC or PSTtxd-20 samples of 50 and 250 μg/ml were incubated with either acid at 37°C. The intensity of the toxoid bands in the gel were reduced as a function of acid concentration (0 - 100 mM). The bands disappeared completely when the toxoids were incubated with 100 mM of both lactic and glycolic acids (pH 3.1). This was probably caused by aggregation of the toxoids in solution, as shown above. Interestingly, when 0.2% BSA was added to the toxoid solutions, the deleterious effect of the acids was lowered. In the presence of BSA, the toxoid band at 130 kDa showed a much higher intensity and occasionally a weak band at 200 kDa appeared, suggesting a dimerisation between Ttxd and BSA. When the higher concentration of PTC (250 μg/ml) was incubated with 100 mM lactic or glycolic acid at 37°C over 7 days, band retention in the PAGE stacking gel zone indicated aggregation. This immobility was not observed when the toxoid was incubated in PBS without acids.

Table III - Effect of temperature (factor A), lactic acid (factor B) and glycolic acid (factor C) on the fluorescence ratio at 450 nm over that at 329 nm (I_{450}/I_{329}) of tetanus and diphtheria toxoids after 21 days incubation. I450/I329 was used as a measure of chemical stability of Trp. Experimental conditions are given in Table I.

Experimental	Fluorescence ratio I_{450}/I_{329}					
	λ_{ex}=280 nm			λ_{ex}=295 nm		
level	PTC	PSTtxd-20	Dtxd	PTC	PSTtxd-20	Dtxd
(1)	1.7	13.0	4.7	2.0	21.6	5.7
a	15.0	80.1	17.9	15.2	159.0	20.8
b	1.7	20.6	3.3	1.9	35.3	4.7
ab	12.9	354.0	25.5	14.7	1374.0	40.4
c	1.7	25.5	2.9	2.1	45.3	4.5
ac	16.8	354.0	26.2	19.1	1425.0	42.1
bc	1.8	34.3	11.5	2.2	58.7	6.3
abc	9.4	174.0	8.3	16.1	639.0	43.4

Antigenic stability of tetanus toxoid

The effect of temperature and of lactic and glycolic acids on Ttxd antigenicity was studied according to the experimental design in Table I. Fig. 4 shows the ELISA-responsive antigenicity after incubation of 100 µg/ml toxoid solutions for 7 days. Temperature proved to be more harmful than lactic or glycolic acid. At 4°C, lactic and glycolic acids (50 mM) caused only minor loss of Ttxd ELISA-antigenicity, i.e., 75 - 85% of the antigenicity was preserved; PTC was tendentiously more stable than PSTtxd-20. Incubation at 60°C (level a_2) led to almost complete loss of antigenicity for both Ttxd grades. At 37°C (level a_1), the antigenicity of PTC and PSTtxd-20 was reduced by 40 and 50%, respectively. Additionally, the presence of acids further decreased the antigenicity.

The influence of albumin on the antigenicity of Ttxd at 37°C was also investigated (Fig. 5). In the absence of BSA, the antigenicity was reduced by 34 - 42% after 1 day incubation with 15 mM of either acid and by 42 - 50% when both acids were present. Interestingly, the prolonged incubation period of 14 days had only a minor additional effect of approx. 10%. Thus, the major loss in Ttxd antigenicity occurred immediately after contact with the acidic aqueous solutions. Most promising, the addition of 5 mg/ml BSA improved tremendously the Ttxd stability. The deleterious effect of both acids on Ttxd antigenicity at 37°C was almost overcome by BSA. However, BSA did not prevent loss of Ttxd antigenicity at 60°C. Only 5 to 7% of the antigenicity of a 100 µg/ml PTC solutions in PBS or 50 mM glycolic acid was preserved (results not shown); no antigenicity remained in solutions without BSA.

Finally, the antigenicity of PTC in glycolic acid (67 mM; pH = 5; 37°C) was studied as a function of time and BSA content (Fig. 6). In the absence of BSA, the decrease in ELISA response was 85% within 1 day, and the remaining antigenicity declined over 42 days. Increasing amounts of BSA *co*-incubated with the Ttxd solutions improved significantly the Ttxd antigenicity. With 4.8 mg/ml BSA, the antigenicity was preserved almost completely over 42 days. In the insufficiently stabilised solutions, the apparent decomposition rate of Ttxd followed a first or higher order kinetics.

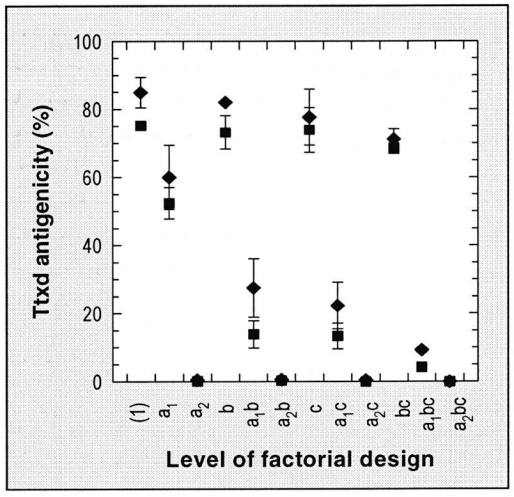

Figure 4 - Effect of temperature (factor A), lactic (factor B) and glycolic acid (factor C) on the antigenicity of Ttxd after 7 days incubation (◆: PTC; ■: PSTtxd-20). Incubation temperature was 4°C at level (1), 37°C at level a_1 and 60°C at level a_2. Other experimental conditions are described in Table I.

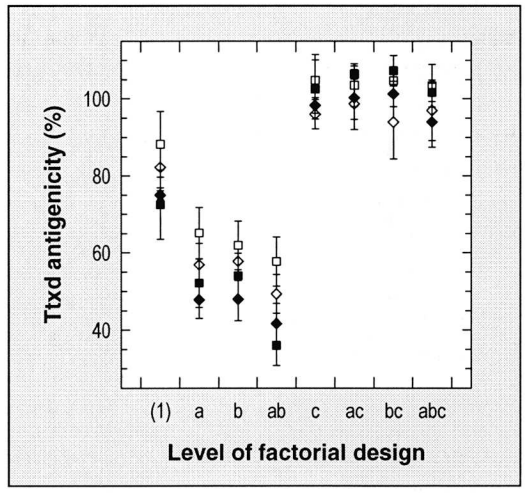

Figure 5 - Effect of lactic acid (factor A), glycolic acid (factor B) and albumin (factor C) on the antigenicity of PTC (diamonds) and PSTtxd-20 (squares) after 1 day (open symbols) and 14 days (filled symbols) incubation at 37°C. Other experimental conditions are described in Table II.

Reversibility of changes in physico-chemical properties and antigenicity

When microencapsulated antigens are released from polyester microspheres, they probably diffuse from a more or less acidic micro-environment into a isohydric physiologic fluid. Thus,

the reversibility of acidity induced changes in the physico-chemical properties and of antigenicity is an important issue. In the present study, the shifts in λ_{max} fluorescence and I_{350}/I_{329} ratio were partially reversed upon pH neutralisation and subsequent equilibration for 7 days at 37°C (results not shown). For the PSTtxd-20 samples incubated with 50 mM of either acid at 60°C, λ_{max} was reverted from approx. 445 nm to 367 nm. Similarly, the I_{350}/I_{329} ratio was reverted from 1.1 to 1.0. For the other toxoids (PTC and Dtxd), the neutralisation and incubation caused further denaturation. Finally, the loss of antigenicity after 21 days incubation at 60°C was not reversible by pH neutralisation or dialysis of acidic residues.

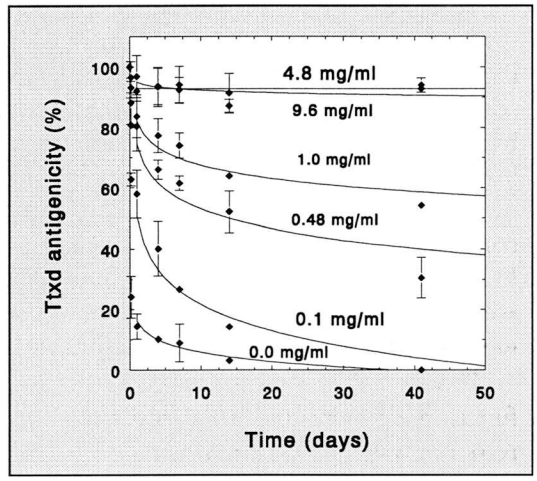

Figure 6 - Influence of albumin on the ELISA-antigenicity of 6.5 µg/ml (2.1 Lf/ml) PTC in 67 mM glycolic acid of pH 5 at 37°C. The fitted curves visualise the trend and do not reflect a kinetic evaluation. (n = 3)

Discussion

Alterations of physico-chemical and antigenic or immunogenic properties may be induced during the encapsulation of antigens and during their release from microspheres, both *in vitro* and *in vivo*. During microencapsulation, antigens are typically exposed to mechanical forces, elevated temperature, organic solvents and stringent drying. During release from the microspheres, antigens encounter a temperature of 37°C and possibly also an acidic environment caused by polymer hydrolysis. Indeed, the present study revealed that elevated temperatures and the presence of lactic or glycolic acid are detrimental factors for the *in vitro* stability of Ttxd and Dtxd. The commercial type Ttxd (PTC) and Dtxd exhibited similar physico-chemical stability, which was significantly superior to that of the column purified Ttxd (PSTtxd-20) (e.g., Fig. 2B). Further, elevated temperature altered to a greater extent the physico-chemical and antigenic properties of the toxoids than did the presence of the acidic compounds (e.g., Figs. 2 and 4). Finally, conformational changes were partly reversible, but the loss of antigenicity was irreversible.

Fluorimetric analysis was used here as a major tool to study conformational alterations of the toxoids in solution. With the denaturant Gdm-Cl, spectra of conformationally altered toxoids were first established as reference. The conformational changes were reflected by a red shift and a reduction in fluorescence intensity (Fig. 1). PTC showed a higher stability than PSTtxd-20 and Dtxd, i.e., the fluorescence changes of PSTtxd-20 and Dtxd occurred at lower denaturant concentrations. However, it should be noted that the accompanying unknown proteins of the toxoid solutions (from *Cl. tetani* and *C. diphtheriae*) may have substantially influenced this results. The higher emission signal of PTC as compared to PSTtxd-20 and Dtxd, may be related to (i) the higher number of Trp and Tyr in Ttxd (13 and 79 residues) than in Dtxd (5 and 18 residues), (ii) a higher number of total Trp and Tyr in the proteinaceous impurities, (iii) a higher fraction of Trp-moieties in the hydrophobic core, or (iv) differences in formalinisation. The effect of formalinisation on thermal unfolding has recently been reported [17].

The parameter λ_{max} is generally considered to be a sensitive indicator for Trp exposure to the aqueous environment [19]. In diphtheria toxin, Trp exposure in PBS of pH 7.0 was restricted as shown in ref. [19]. Upon gradual addition of hydrochloric acid, the toxin underwent a conformational transition at pH 5 and 37°C and unfolded partly at a pH of less than 5, accompanied by an increase of λ_{max}. Conversely, λ_{max} of Ttxd exposed to pH 2.5 at 37°C remained unchanged over one week, but decreased slightly after four and seven weeks [31]. These results are in good agreement with our findings, although the acidifying component (hydrochloric versus lactic and glycolic acid) in the buffer solutions of the toxins and toxoids differed. In 50 mM lactic or glycolic acid (pH 4 - 6), the Trp environment did not change greatly (Figs. 2 and 3). This suggests that toxoid encapsulated in PLA/PLGA might preserve its functionality during polymer hydrolysis. This assumption is supported by the observation that encapsulated albumin and carbonic anhydrase remained intact above pH 4.2, but degraded partly at pH 2.9 [32]. The apparent pH-sensitivity within the pH-range of 2.9 to 4.2 may be related to hydrolysis of Asp-X peptide bonds after protonation of Asp (pK_a = 3.9). Especially, Asp-Pro bonds are labile [35], and the Ttxd contains seven Asp-Pro bonds [24]. Furthermore, deamidation of Asn is another common destructive process in proteins that must be considered [35], and the particular unstable sequence Asn-Gly is located at six sites in Ttxd [24]. Although, the fluorimetrically and CD spectroscopically observed toxoid conformation (Figs. 2 and 3) was not sensitive to pH > 4, the antigenicity was significantly lowered (Figs. 4 and 5) and depended on the lactic or glycolic acid concentration.

In contrast to pH, temperature was very critical for toxoid conformation. At 60°C, Trp and Tyr in PTC were only little (λ_{max} = 332 nm), in Dtxd partly (λ_{max} = 342 nm), and in PSTtxd-20 completely (λ_{max} = 355 nm) exposed to the aqueous solvent; these λ_{max} values possibly represent different stages of unfolding (Fig. 2). The general increase in the I_{350}/I_{329} ratio observed with all toxoids incubated in 50 mM lactic and/or glycolic acid at 60°C can be interpreted as unfolding (Fig. 3A). Once more, PTC showed only minor changes. Surprisingly and in contrast to all other experiments, I_{350}/I_{329} decreased slightly for Dtxd and substantially for PTC exposed to 60°C (with λ_{ex} = 295 nm). This suggests a reduced Trp exposure to the solvent under acidic and elevated temperature conditions. This may be partly explained by the hydrophobic character of the toxoid at a pH close to its isoelectric point (pI of 5.1 for Ttxd). Moreover, as the reduction of I_{350}/I_{329} was only observed at 60°C, but not at 4°C, Trp exposure might also depend on energy input and time. Further, a combined influence of the effect of temperature and lactic and glycolic acids should be considered. Surprisingly though, CD spectroscopy indicated only minor changes in conformation of Ttxd after seven days incubation at 60°C, with slight loss of α-helical and slight increase of β-sheet conformation. This is in agreement with similar investigations on Ttxd stability by FTIR [26]. It has been observed that α-helices may undergo structural transitions in acidic solutions and at elevated temperatures, whereas β-sheets are more stable [33]. Further, we investigated the thermal stability of Ttxd by both far and near UV CD spectroscopy, but no significant conformational change was detected. This was unexpected as the change in Ttxd ELISA-antigenicity occurred rapidly upon contact with the acids.

The appearance of a fluorescence maximum at 450 nm of the toxoids stored at 60°C must be ascribed to the formation of new chemical structures. Both Trp and Tyr are quite reactive [34], with Trp being readily oxidised below pH 4 [35]. Further, hydrolytic degradation products of proteins in hydrochloric acid generate Trp-related fluorescence with a λ_{max} of above 400 nm, and indole derivates such as kynurenine emit light near 450 nm when excited at 300 nm [30,31]. Finally, formaldehyde may be released during acidic hydrolysis of formalinised proteins [36]. Candidate functional groups for reversible reaction with formaldehyde are hydroxymethylamine, aminal [37], and Lys [38]. The reactive intermediate imine group was assumed to form intermolecular cross-linking and possibly aggregation of Ttxd, as the imine group of one protein molecule may covalently bind to Lys (unstable product) or Tyr (stable product) of an adjacent molecule [12]. Hence, one can speculate that this binding to Tyr alters its electrical dipole

making it less accessible for excitation. However, the present investigation cannot reveal more precisely the molecular mechanisms of fluorimetric changes.

The profound drop in fluorescence intensity of the toxoids stored at 60°C and low pH must be attributed to the concomitant aggregation observed with all toxoids. Aggregation is also the most plausible explanation for the total loss of Ttxd antigenicity at 60°C and, to a lesser extent, at 37°C, and for the partial or complete loss of the intensity of toxoid bands on the electrophoresis gel. As the CD spectra did not show any conformational change of Ttxd after 7 days incubation at 60°C and low pH, we assume that the toxoids unfolded via an intermediate soluble conformation. This intermediate conformation should have a short life time, be strongly reactive and quickly lead to insoluble aggregates. Therefore, aggregation of Ttxd well explains the incomplete release of toxoids from PLGA microspheres and the lack of distinct booster responses in animals after single injection of encapsulated toxoid.

By co-encapsulating albumin together with Ttxd in PLGA microspheres, we have improved the antigenic stability of the toxoid [27]. Both the total amount of antigenic material encapsulated and the antigenic fraction released were enhanced. The present results confirm the previous observations as the antigenic lability of Ttxd in acidic solutions or at elevated temperatures was counterbalanced by the presence of albumin (Figs. 5 and 6). We might speculate that hydrophobic and electrostatic interactions between Ttxd and albumin provide a better stability. Such interactions were indeed suggested by native PAGE. On the other hand, Ttxd stability was also improved at higher toxoid concentrations, in the absence of albumin (results not shown). This suggests that albumin is not specific for stabilisation at low pH, but that increased protein concentration is important; as shown also for several enzymes, albumin can substitute for naturally occurring protein stabilisers in cells [39,40]. This is in agreement with the common notion that proteins are generally more unstable in dilute solutions due to higher exposure to denaturants (solvent, salts, surfaces). Hence, encapsulated antigens may be also stabilised with proteins other than albumin. However, high protein concentration may sometimes lead to aggregation, especially at elevated temperature and low pH [41], which indeed was confirmed here with Ttxd and albumin in acidic solutions at 60°C.

The present investigation demonstrates the superiority of the commercial grade PTC as compared to the column purified PSTtxd-20, regarding conformational and antigenic stability at elevated temperatures, low pH and in the presence of the denaturant Gdm-Cl. Hence, from a stability point of view, purified toxoid might not be preferable over commercial grades. Nonetheless, despite the poor antigenic stability of Ttxd in acidic solution at 37°C, the presence of albumin appeared to protect Ttxd not only in aqueous solutions, but also during release from PLGA microspheres [27]. Most importantly, immune responses in mice [29] and guinea pigs (Sesardic *et al.*, personal communication) were clearly improved after single injection of Ttxd microspheres with *co*-encapsulated albumin.

ACKNOWLEDGEMENTS

This work was partly supported by the WHO global programme for vaccines and immunisation (GPV), Vaccine Research and Development, World Health Organisation, CH-Geneva.

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7

Release of Tetanus Toxoid from Adjuvants and PLGA Microspheres:

How Experimental Set-up and Surface Adsorption Fool the Pattern*

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INTRODUCTION

Biodegradable poly(lactic acid) (PLA) or poly(lactic-*co*-glycolic acid) (PLGA) microspheres are considered attractive for controlled antigen delivery [1]. It is currently assumed that the frequently observed pulsatile release pattern extending over several months may mimic booster doses of conventional vaccines and obviate the need for repeated injections in young children. Surprisingly, the antigen release kinetics of PLA/PLGA microspheres is probably better established than that of conventional adjuvants, such as alum and Freund's Incomplete Adjuvant (IFA). Thus, it appears rather presumptuous to propose an optimised antigen release pattern for microspheres potentially suitable for boosting, considering that the release kinetics of standard vaccine formulations is not even known. A further difficulty for the rational design of pulsatile release microspheres is the frequently observed incomplete protein release from microspheres, as typical for albumin [2], carboxyanhydrase [3], interferon [4], and toxoids [5-7]. For tetanus toxoid (Ttxd), the incomplete release was found to partly due to stability problems [7].

Release experiments with PLA/PLGA microspheres are commonly performed in glass or plastic tubes by dispersing microspheres in a buffer and subsequent incubation at 37°C under agitation [8]. However, it has been demonstrated that the experimental set-up exerts a great effect on the release of indomethacin from PLA microspheres [9]. In case of protein drugs, a major problem may also arise from the acidic polymer degradation products, which can compromise the stability of entrapped and released protein [5,10]. Typically, aggregation and loss of activity may be an important cause of incomplete release of intact protein.[11-13]. Thus, to circumvent these effects, it was suggested to eliminate continuously the mono- and oligomeric acids formed from the release medium by dialysis [14]. Finally, protein adsorption on surfaces (container for release test and polymeric microspheres) may influence the release observed in a given release test. Protein adsorption mechanisms and conformational changes that occur on hydrophobic surfaces were described by Norde and Favier [15]. More specifically, protein adsorption on PLGA microspheres was examined by Calis *et al.* [16] and Crotts *et al.* [17]. The latter study concluded that non-specific protein adsorption is a critical factor in controlling protein release kinetics from PLGA microspheres and that it can be suppressed by adding sodium dodecyl sulphate to the release medium.

The goal of this study was to characterise first the *in vitro* release kinetics of tetanus toxoid adsorbed on alum or dispersed in IFA. We believe that this information may be useful for designing pulsatile delivery systems mimicking priming and booster doses. Secondly, we examined whether the incomplete *in vitro* release of tetanus toxoid is caused by adsorption to polymeric particles or borosilicate glass surfaces. For this, we studied the release from fast degrading PLGA microspheres under different experimental conditions as well as the adsorption of the toxoid on placebo PLGA particles.

MATERIALS AND METHODS

Materials

Tetanus toxoid (Ttxd) in aqueous solution (lot No. PTC 10005: 8500 Lf/ml or 26.3 mg/ml, Pasteur Mérieux, F-Lyon) and adsorbed on aluminium oxide (lot No. 10005: 19.7 Lf/ml) were provided by WHO. Poly(d,l-lactic-*co*-glycolic acid) (PLGA 50:50) with a M_W of approx. 12 kDa was purchased from Boehringer Ingelheim, D-Ingelheim (Resomer RG502H). Freund's Incomplete Adjuvant (IFA) was from Sigma Chemical, St. Louis, MO. Monoclonal anti-tetanus antibodies (TT010) and guinea pig anti-tetanus IgG were from Wellcome Biotechnology, UK-Beckenham. Rabbit anti-guinea pig IgG horse radish peroxidase conjugate and 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) were from Sigma Chemical, St. Louis, MO. Unless specified otherwise, all other substances used were of pharmaceutical or analytical grade and purchased from commercial suppliers.

In vitro release of tetanus toxoid from adjuvants

The release of Ttxd from alum and IFA was studied according to Model D in Table I. Briefly, 50 µl of Ttxd (20 or 200 Lf/ml) formulated in alum or IFA were embedded in 250 µl of 2%

agarose at 60°C. A second layer of 250 µl agarose was added on the top of the first one. Finally, 3.4 ml of 67 mM (isotonic) PBS of pH 7.4, containing 0.2% BSA to prevent toxoid adsorption on the glass, were added as receiver medium. Samples were taken at regular intervals and replaced with fresh buffer. As a control, the same experiment was carried out with an aqueous solution of Ttxd.

Preparation of microspheres

PLGA microspheres (MS) were prepared by spray-drying (Büchi 190, CH-Flawil) a dispersion of aqueous toxoid solution in a 5% (w/w) solution of PLGA in ethyl formate as described elsewhere [8]. For improving the antigenicity of Ttxd, BSA (5%) and trehalose (15%) were *co*-encapsulated [7].

In vitro release of tetanus toxoid from microspheres

Toxoid release experiments from 10 mg MS were conducted at 37°C in 5 ml borosilicate vials (Chromacol, GB-London) sealed with a Teflon cap, according to the experimental conditions given in Table I; a buffer was chosen to account for a pH drop caused by degrading MS. At regular intervals (time points in Fig. 2), samples were assayed by ELISA. For this, the release medium with the MS was centrifuged at 3,500 rpm (2000 G) for 10 min, and the particle-free supernatant withdrawn for analysis and replaced with fresh buffer. All experiments were repeated five times.

Adsorption of toxoid

Adsorption of Ttxd on the borosilicate vials used for the release study and on placebo MS (15 mg), loaded or not loaded with BSA, was carried out either in PBS or in PBS with 0.2% BSA at pH 7.4. Varying concentrations of Ttxd in 3.4 ml solution were added to the vials and incubated at 37°C for 24 h under horizontal rotation. The equilibrium concentration was assayed by ELISA after separating the particles from the supernatant by centrifugation at 3,500 rpm for 10 min at room temperature.

ELISA of tetanus toxoid

Tetanus toxoid antigenicity was measured by ELISA. Briefly, flat-bottom 96-well microtiter plates (Nunc-Immuno Plate Maxisorb, Nunc, DK-Roskilde) were filled with 100 µl of 1 µg/ml monoclonal anti-tetanus IgG (TT010) in 0.05 M carbonate buffer of pH 9.6, and incubated overnight at 4°C. The plates were washed three times with 300 µl of 0.05 % Tween 20 in PBS of pH 7.4 (PBST) after each incubation step. After 1 h incubation at 37°C with 150 µl 2.5% milk powder in PBST (PBSTM), the plates were incubated at 37°C for 2 h with serial dilution of standard and test solutions of tetanus toxoid. Guinea pig IgG (25 µg/ml) was added to each well in 100 µl of PBSTM, and plates incubated for another 2 h. Then, rabbit anti-guinea pig peroxidase conjugate (1/8000 dilution) in 100 µl PBSTM was added to each well, and plates incubated for further 1 h. Finally, 100 µl of 0.5 mg/ml peroxidase substrate 2,2′-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) in 0.05 M citric acid of pH 4.0 was added to the wells, and the endpoint optical density measured at 405 nm (Thermomax, Molecular Devices, Menlo Park, CA) after 30 min incubation at room temperature.

Table I - Experimental conditions for the release of tetanus toxoid from PLGA 50:50 microspheres. The volume of the release medium was 3.4 ml, the pH 7.4 and the osmolarity 300 mosmol/kg. In A, B and C, the MS were suspended directly in 3.4 ml buffer and the vials rotated vertically. In D, MS were suspended in 40 µl 5% soya lecithin and immobilised in 250 µl of 2% agarose in the vials. Another 250 µl of 2% agarose was added to separate the gel-embedded MS from the recipient release medium. The vials were horizontally shaken.

Release system and medium	Sampling volume
A: MS suspended in 67 mM PBS	1 ml
B: MS suspended in 67 mM PBS + 0.2% BSA	1 ml
C: MS suspended in 67 mM PBS + 0.2% BSA	2 ml
D: MS immobilised in agarose and 67 mM PBS + 0.2% BSA	1 ml

RESULTS AND DISCUSSION

Release of tetanus toxoid from adjuvants

Classical adjuvants have not been considered so far as delivery systems with controlled release properties. Hence, *in vitro* release or dissociation of antigens from adjuvants (alum or IFA) has attracted only minor experimental interest. As the combination of antigen and adjuvant is crucial to elicit an efficient immune response, the release kinetics are likely to play a role in this process. One might speculate that, if the antigen dissociates too rapidly, it might be degraded enzymatically and escape before its recognition and uptake by antigen presenting cells.

Release of Ttxd from alum and from IFA was studied by immobilising the formulations in agarose to mimic a subcutaneous or intramuscular injection site [18]. At a dose of 1 Lf Ttxd, the amounts released from alum and IFA after one day were 26 and 47%, respectively, and 79 and 89% after nine days (Fig. 1). At a dose of 10 Lf, the release profiles were similar to 1 Lf, except for the Ttxd fraction released during the first day, which was lower with the 10 Lf dose. In a control experiment, 1 and 10 Lf Ttxd solution were directly embedded in the agarose gel. Approx. 75% (1 Lf) or 51% (10 Lf) of the dose were released within one day and 90 to 100% after three days.

The initially faster release of Ttxd from IFA as compared to alum might be due to an instability of the emulsion in the agarose gel. During the experiment, mineral oil appeared in the receiver compartment. If the W/O-emulsion coalesced inside the gel, Ttxd partly became available for diffusion controlled release which may contribute to an initial fast release, suggesting a limitation of the agarose release model for such emulsions. On the other hand, the release of Ttxd from alum was more linear in shape as compared with Ttxd released from IFA. Although adsorption of proteins at interfaces generally occurs through weak interaction forces of the van der Waals type, hydrophobic interactions and hydrogen bonds may in some cases result in irreversible adsorption. The slow and incomplete release of Ttxd from alum suggests the involvement of such strong interactions between Ttxd and alum. In contrast to the Ttxd release profiles observed here, the release of ovalbumin (10%, w/w) from alum in PBS was reported to be of nearly zero order over 30 days with a total of 45% albumin released [19], although this release experiment was performed by dispersing the formulation directly in a buffer (e.g., system A, Tab. I). Apparently, ovalbumin adsorbs stronger on alum than does Ttxd. Further, it was reported that polyvalent cations such as Al^{3+} were able to neutralise negatively charged proteins [20] and thereby, making them more hydrophobic at neutral pH. A subsequent change in the tertiary structure of a protein can influence the kinetics and the reversibility of the adsorption and desorption processes [21]. By the same token, the release of Ttxd from alum may be partly controlled by such changes in interaction.

The analysis of the release kinetics revealed an approximate linear square root-of-time dependency with both alum ($r^2 = 0.990$) and the IFA ($r^2 = 0.934$ for 10 Lf, and $r^2 = 0.968$ for 1 Lf). However, as the release occurs by a two step process, i.e., dissociation of Ttxd from the adjuvant and diffusion through the 3 - 6 mm 2% agarose gel, it would be rather speculative to interpret a diffusion controlled release of Ttxd. By extrapolating the square root-of-time plot to time zero, a pronounced burst release appeared with IFA, but not with alum, which clearly demonstrates the higher affinity of Ttxd for alum than for IFA.

Release of tetanus toxoid from microspheres using different test systems

The release kinetics of Ttxd from PLGA microspheres (MS), containing the additives BSA and trehalose, depended on the test system used (Fig. 2). In general, when the MS were directly suspended in the buffer solution (test systems A, B and C), a triphasic release pattern was seen, consisting of an initial burst release phase, a dormant period, and a second release pulse. This is in contrast with the more regular release pattern of MS immobilised in 2% agarose where 27 - 35% of the total Ttxd were released (relative to Ttxd loading determined) within the first 24 h. This burst phase was followed by a dormant period of 14 - 20 days, before a second pulse of Ttxd was released over a period of 15 - 30 days. The total fraction released from these samples lay in the range of 64 - 77% of the total dose. The effect of the release test system was most marked during the second and third release phases. Typically, the second phase lasted slightly shorter when the MS were dispersed in PBS containing 0.2% BSA (PBS-BSA) as compared to PBS without BSA. Further, the rate of release in the third phase and the maximum fraction released were higher in

PBS-BSA samples (72 - 77%) than in PBS alone (64%). The effect of aliquot volumes withdrawn and replaced with fresh buffer at each sampling time (test systems B, 1 ml, and C, 2 ml) was most important in the third phase. The second release pulse began slightly earlier, and the total amount of Ttxd released was slightly higher when 2 ml of medium were replaced (system C) instead of 1 ml.

In previous experiments, we observed that the total fraction of Ttxd released from PLGA 50:50 MS was lower than the percentage measured here (previously, 5 - 40% depending on the *co*-encapsulated additives used; here, 64 - 77% depending on the experimental conditions). Since previous investigations indicated a major effect of *co*-encapsulated BSA on the total Ttxd amount released, one MS batch with, and a second without the additive BSA as stabiliser were studied here (Fig. 2, Inset). The two batches showed a comparable burst release, but there was no further Ttxd release from MS without BSA. At present, it remains unknown whether this is due to Ttxd adsorption on the release vials and the polymeric material itself (see below), or to the deterioration of Ttxd inside the MS prior to release.

Interestingly, although various studies have demonstrated the importance of the *in vitro* test systems and conditions for prolonged drug release from biodegradable MS, no official recommendations are, to our knowledge, available to date. Conti *et al.* [9] evaluated different dissolution methods and test conditions for the release of indomethacin from PLA MS. It appeared that the drug release kinetics depended greatly on the test parameters. For peptides and proteins, release from PLA/PLGA MS is generally not solely diffusion controlled, but follows a pulsatile pattern. It is assumed that this is due to strong interactive forces between peptide or protein compounds and polyesters. Thus, this very complex release behaviour is particularly sensitive to the actual *in vitro* test conditions.

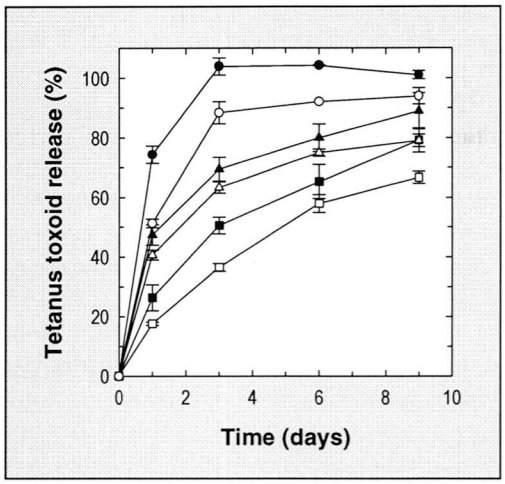

Figure 1 - Release of 1 Lf (filled symbols) or 10 Lf (open symbols) tetanus toxoid from the adjuvants IFA (triangles) and alum (squares). Vaccine formulations were immobilised in agarose, and toxoid determined in a recipient compartment of BSA-containing PBS buffer. As controls toxoid solutions (circles) were embedded in the agarose without any adjuvant (n = 3).

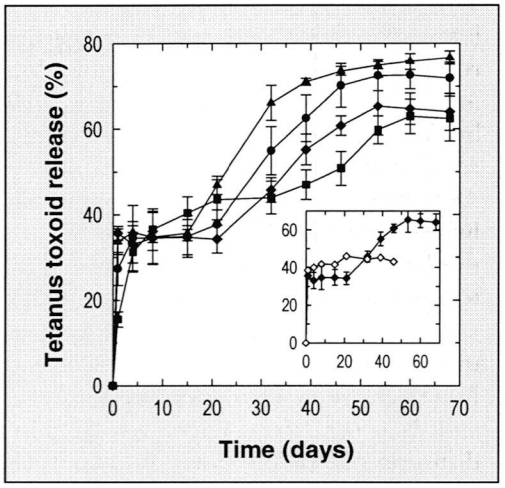

Figure 2 - Release profiles of Ttxd from PLGA MS obtained in the four release test systems described in Table I. System A: PBS (◆); System B: PBS-BSA-1 ml (●); System C: PBS-BSA-2 ml (▲); System D: agarose gel (■). The inset compares the Ttxd release in system A obtained from MS containing 5% BSA and 15% trehalose (◆) with MS containing only 15% trehalose (◊) as additives (n = 3).

Adsorption characteristics of tetanus toxoid

A better understanding of *in vitro* protein release from PLA/PLGA microspheres may require knowledge about protein adsorption on the glassware used for the release test and on the surface of the MS themselves. The adsorption isotherms of Ttxd indicate a Langmuir-type monomolecular adsorption on both the glass vials used here and on placebo MS (Fig. 3). Adsorption on both

materials was rather important when PBS was used as incubation medium, but became negligible when the medium contained BSA.

The adsorption data were also plotted along the Langmuir equation

$$\frac{x}{m} = \frac{abc}{1+bc}$$

where c is the Ttxd concentration (Lf/ml) at equilibrium and x the amount (Lf) of toxoid adsorbed to the surface area m (m^2). The value of the Langmuir constant a (Lf/m^2) is a measure of the adsorptive capacity of a particular surface (glass or MS) for the toxoid, and b (ml/Lf) is related to the enthalpy of adsorption. The adsorption parameters for the experiments in PBS are summarised in Table II. The adsorption in PBS containing 0.2% BSA did not follow the non-linear Langmuir model ($r^2 < 0.68$) and, therefore, the Langmuir parameters were not calculated. It should also be noticed that the values do not exclusively reflect protein adsorption, but adsorption of ELISA-responsive toxoid. Considering that conventional Ttxd solutions contain, besides the antigen itself, substantial amounts of accompanying proteins, i.e., up to 60%, the values have more immunochemical rather than pure physicochemical significance. The calculated Langmuir parameters clearly demonstrate the influence of BSA on the adsorption of Ttxd onto the materials studied. First, the addition of 0.2% BSA to the buffer solution produced a significant reduction of the adsorption capacity, a, of both borosilicate glass and PLGA MS. Second, the surface area-related adsorption capacity of glass for Ttxd in PBS was approx. eight-fold higher than that of PLGA. However, this difference diminished upon adding 0.2% BSA to PBS. Importantly, the Ttxd adsorption capacity of MS containing 5% encapsulated BSA was reduced to less than half of that of the PLGA MS without *co*-encapsulated BSA.

Borosilicate vials showed an adsorption capacity for Ttxd in PBS of 280 Lf/m^2 at an equilibrium toxoid concentration of approx. 0.2 Lf/ml (Fig. 3, Inset A). Assuming monomolecular adsorption as well as a quantitative correlation between ELISA-responsive toxoid and protein content (323.2 Lf/mg protein), this should correspond to a molecular dimension of approx. 268 nm^2 (surface occupied by one adsorbed Ttxd molecule). This is larger than the expected dimension of dissolved Ttxd in its native state (for comparison, albumin has a dimension of 31 nm^2 in water [16]). The difference might be caused by a partial toxoid defolding upon adsorption or by a preferential adsorption of accompanying proteins. Upon adsorption, hydrophobic interactions between the protein and the adsorbent and also between neighbouring protein molecules can cause conformational rearrangements which may lead to dimensional changes. Therefore, the number of adsorbed molecules in a monolayer will be inversely proportional to the protein's ability to unfold, i.e., its flexibility. Such unfolding properties of Ttxd have been observed previously (unpublished results). When 0.2% BSA was added to the incubation solution (Fig. 3, Inset B), an approx. 100-fold decrease in adsorption was observed and the plateau was reached at a Ttxd concentration of less than 0.1 Lf/ml. However, at this very low adsorption level, scattered values rather than distinctive isotherm were observed. The reduced toxoid adsorption most likely resulted from competitive adsorption of albumin to the glass and MS surfaces.

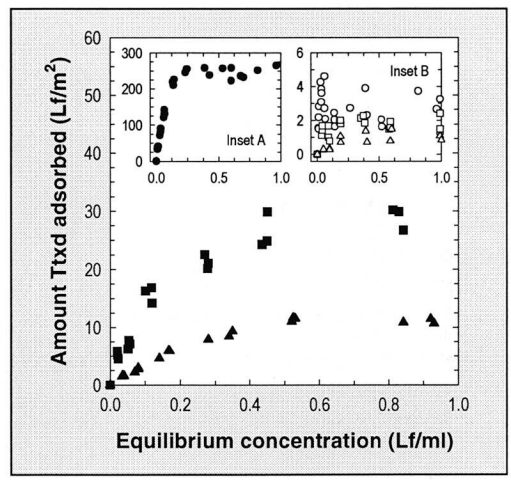

Figure 3 - Adsorption isotherms of Ttxd on PLGA placebo MS containing trehalose (■) and on PLGA MS containing BSA and trehalose (▲) in PBS at 37°C. Inset A: adsorption of Ttxd in PBS on borosilicate glass vials (●). Inset B: adsorption of Ttxd in PBS containing 0.2% BSA on borosilicate glass vials (○), PLGA placebo MS containing trehalose (□), and on PLGA MS containing BSA and trehalose (△). (n = 3)

Table II - Langmuir parameters a and b and correlation coefficient r^2 of the non-linear Langmuir transformation of the adsorption of tetanus toxoid on borosilicate glass and PLGA (50:50) microspheres (MS) in PBS. The determined surface area of glass and of MS samples (15 mg) were approx. 0.0027 and 0.0250 m^2, respectively. All adsorption data were plotted along the Langmuir equation. Adsorption in PBS containing 0.2% BSA did not follow the Langmuir model ($r^2 < 0.68$).

Surface Type	a (Lf/m²)	b (ml/Lf)	r²
Borosilicate glass	280.1	16.1	0.961
PLGA MS	33.4	6.9	0.974
PLGA MS with *co*-encapsulated BSA	15.6	3.7	0.964

Similar to borosilicate glass, placebo PLGA MS adsorbed well Ttxd (Fig. 3). In PBS, a plateau adsorption capacity of 33 Lf/m^2 (Tab. II) was reached at an equilibrium concentration of 0.6 Lf/ml Ttxd. Further, the adsorption capacity was much higher (approx. 20-fold) in PBS than in PBS containing BSA (Fig. 3, Inset B), which probably reflects again competitive adsorption between the two proteins. Competitive adsorption was also observed for both calcitonin and BSA adsorption on PLGA microparticles in the presence of ionic and non-ionic surfactants [17,22]. It has indeed been shown that surfactants displace protein molecules from polymer surfaces by competitive hydrophobic interactions [23]. In the present study, BSA (also surface active) appeared to interact more strongly than the toxoid with glass or MS surfaces.

Finally, when Ttxd adsorption was measured on PLGA MS containing *co*-encapsulated BSA, it was clearly lower than the adsorption on placebo MS (free of protein) (Fig. 3 and Tab. II). An apparent plateau of approx. 16 Lf/m^2 was reached at an equilibrium concentration of 0.6 Lf/ml in PBS, but the low gradient slope of the isotherm may indicate a non-Langmuir type process. The low adsorption of Ttxd from PBS may be explained by the concomitant release of BSA from the MS and subsequent competition for adsorption within the period of time of the adsorption experiment. Hence, the more complete release of Ttxd from the MS containing *co*-encapsulated BSA, as compared to those without BSA, can be explained by the concomitant release of BSA which competes with the toxoid for adsorption. In this competition, BSA might adsorb preferentially, because it is expected to be released faster from the MS than Ttxd, thereby getting prior access to the particle surface.

For *in vitro* release experiments, protein adsorption on glassware and polymers is of high relevance as the measured amounts of released protein may be greatly falsified by concomitant surface adsorption. In light of this, significant adsorption on PLA/PLGA microspheres has previously been reported for proteins such as BSA, IgG, calcitonin and Ttxd [16,20,24,25]. Particular attention should be given to this process when release experiments are performed over a prolonged period of time during which only minute amounts will be released. We can speculate that the incomplete Ttxd release from PLGA MS shown in Figure 2 is a result of such competitive processes. Figure 2 also reveals that the differences in the measured Ttxd release was minor after one day (burst release), but became increasingly important during the later stages. As the release medium was replaced by fresh medium at regular intervals, low toxoid concentrations were maintained. Therefore, we expect adsorption to become more important than release. When freely available Ttxd in solution (equilibrium concentration) was plotted as a function of initial Ttxd concentration, a hyperbolic relationship was observed (Fig. 4). This illustration emphasises the practical importance of the *in vitro* release medium. In PBS, the maximum fraction of available Ttxd varied between 40 and 100% depending on the initial toxoid concentration. It appears from Fig. 3 and 4 that adsorption of Ttxd on glass and on MS in PBS is very important at low toxoid concentrations and that the presence of MS exerts only a minor additional effect. When MS with *co*-encapsulated BSA were *co*-incubated, 80 to 90% of the toxoid remained available, i.e., non-adsorbed, almost independently of the initial toxoid concentration.

Considering the continuous release pattern observed in the *in vitro* release test system D (MS immobilised in agarose) (Fig. 2), one might speculate that the pulsatile release obtained in the test systems A, B, and C (MS freely suspended in the buffer) can be ascribed, at least partly, to adsorption of the released Ttxd. Furthermore, the earlier onset of the second release pulse and the higher total amount of Ttxd released, when twice the sampling volume of the release medium was

replaced at each time point, also suggest that adsorption and desorption phenomena may interfere. On the other hand, adsorption can only be partly responsible for the pulsatile release pattern because the pulsatile behaviour appears in both PBS and PBS containing 0.2% BSA. Hence, the presence of BSA in the medium did not fundamentally alter the Ttxd release pattern, although it prevents adsorption to a great extent.

A parameter not considered here that should be of great importance for protein adsorption on glass and microspheres surfaces is the pH of the solution [15,26]. Affinity normally increases with decreasing repulsion between the protein and the sorbent, and maximum adsorption is generally observed at the isoelectric point of the protein. When studying release from PLA and PLGA matrices, the pH in the release medium will drop, depending on the buffer capacity, as the polymer degrades. If the release medium is not replaced regularly, this pH-change may enhance protein adsorption as pH will approach the pI of the protein, which is in the range of 4 - 6 for most toxoids.

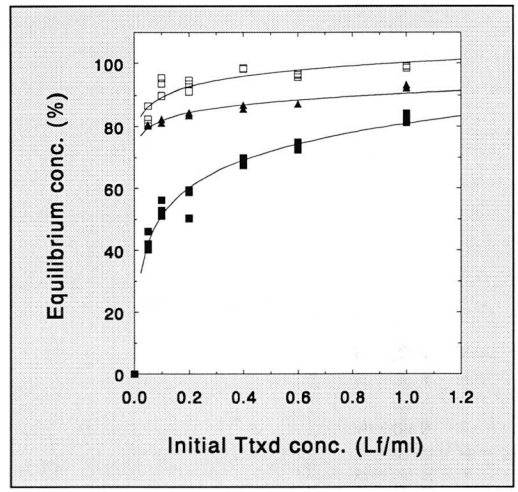

Figure 4 - Free Ttxd in solution after 24 h incubation at 37°C with PLGA placebo MS containing trehalose in PBS (■), PLGA placebo MS containing BSA and trehalose in PBS (▲), or with PLGA placebo MS in PBS containing 0.2% BSA (□) (n = 3). The lines represent a logarithmic fit to visualise the trend and do not reflect a physical evaluation. The equilibrium Ttxd concentration is expressed relative to the initial Ttxd concentration.

Conclusions

This study showed that the observed *in vitro* release of tetanus toxoid from PLGA microspheres (MS) depends on the model chosen for this purpose. As the toxoid strongly adsorbed to hydrophobic surfaces such as glass and MS, attention should be paid to the experimental conditions for the purpose of investigating such release. The adsorption capacity of borosilicate glass for Ttxd was significantly higher than that of PLGA MS and should particularly be taken into consideration when designing a release experiment. However, the use of BSA in the release medium or *co*-encapsulated in MS competed with the adsorption of Ttxd on borosilicate glass and MS and improved the release characteristics of the toxoid. Further, MS immobilised in an agarose gel (model for subcutaneous injection site) produced a quite continuous release pattern, whereas particles freely dispersed in the buffer gave a pulsatile release. This evidences the limitations of release data, which depend not only on the formulation, but also on the test system. Finally, the release of tetanus toxoid from the adjuvants alum and IFA showed a stronger association to alum than to IFA. Interestingly, the release was relatively slow and lasted between three and nine days. Therefore, a single dose vaccine formulation mimicking the priming and one or two booster injections, might require similar kinetics to alum vaccine at the conventional vaccination time points.

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Thermodynamic Approach to Protein Microencapsulation into Poly(d,l-Lactide) by Spray Drying*

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* International Journal of Pharmaceutics 129: 51-61(1996).
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INTRODUCTION

Biodegradable microspheres based on poly(d,l-lactic acid) (PLA) as well as poly(d,l-lactic-*co*-glycolic acid) (PLGA) have become very attractive parenteral delivery systems for peptides and proteins [1-3], hormones [4-6], vaccines [7-12] and other active agents. Great effort has been devoted on improving microencapsulation technologies, and the scientific as well as the patent literature covers numerous aspects of process parameters. However, all of these investigations had adopted a rather empirical strategy to examine the consequences of changing operational parameters [13-17]. The few studies considering a more fundamental approach are limited to purely qualitative physico-chemical aspects. In a solvent evaporation process, different polymer solvents were used showing that drug entrapment and other PLA microspheres properties tend to depend on the Hansen partial solubility parameters [18]. On the other hand, the characteristics of spray dried PLA microspheres loaded with bovine serum albumin (BSA) were shown to correlate only to some extent to physico-chemical parameters such as solubility parameters, surface and interfacial tension, and vapour pressure of the solvent [19]. For coacervation, the effect of solvent/non-solvent pairs on properties of ethyl cellulose microcapsules using a critical interaction parameter was described [20]. The results suggest that microspheres properties must depend on the interaction capacity of all components forming the system, i.e., polymer, solvent, drug and water. Indeed, the components of a microencapsulation mixture represent a thermodynamic system with different interactions occurring simultaneously. Therefore, more generally valid concepts of microencapsulation should be based on the thermodynamic interaction energy balance.

The present investigation focuses on the influence of various solvents on different properties of PLA microspheres loaded with BSA used as a model protein. As our approach should be of practical relevance, all solvents taken into consideration were environmentally and toxicologically acceptable. Therefore, the commonly used chlorinated solvent dichloromethane was only used as reference and should be exchanged for less harmful organic solvents. The main object was to design solvent systems on the basis of interaction energies between solvent-polymer-aqueous BSA-phase, which produce microspheres with optimum properties, i.e., high loading efficiency and low burst release. In a first step, single polymer solvents and solvent mixtures were selected on the basis of the Hansen solubility parameters [21]. Second, a refined selection of single polymer solvents was made according to calculated interaction energies of solvent-polymer, solvent-water, and polymer-water by using four interaction capacity parameters, i.e., two partial solubility parameters of Hansen (δ_d, δ_p), and the *E* and *C* parameters of Drago [22,23].

THEORY

Microencapsulation by spray-drying generally involves the three components polymer, drug and solvent (or solvent mixture). It can be easily recognised that product characteristics such as drug encapsulation efficiency, burst release of drug and residual solvent greatly depend on the molecular interactions between the components. These interactions can be illustrated graphically by a schematic triangular model, as shown in Fig. 1.

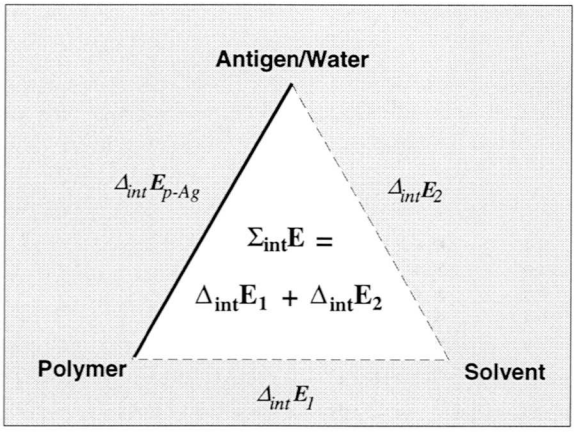

Figure 1 - Schematic model of intermolecular interactions taking place during BSA-microencapsulation into PLA by spray drying. Good drug encapsulation is expected if the interaction protein-polymer *dominates* over the sum ($\Sigma_{int}E$) of the interactions polymer-solvent ($\Delta_{int}E_1$) and protein-solvent ($\Delta_{int}E_2$).

The interaction capacity of a compound can be estimated by a set of three partial solubility parameters: δ_d (dispersive), δ_p (polar), and δ_h (hydrogen bonding) [21]. The first two parameters are good descriptors for the dispersive and polar interactions, while δ_h is considered to be a very poor one for the hydrogen bonding interaction. Hence, the parameters E (electrostatic) and C (covalent) to quantify hydrogen bonding capacity of a Lewis acid or a Lewis base were introduced [24]. Although these parameters are semi-empirical, they are, for the time being, the best descriptors of the interaction enthalpy of hydrogen bonding [25].

The interaction energy, $\Delta_{int}E_1$, between a solvent A and a polymer B, wherein the polymer is dissolved, can be expressed by the following equation [25]:

$$\Delta_{int}E = -2^{A}V^{A}\delta_d{}^{B}\delta_d - 2^{A}V^{A}\delta_p{}^{B}\delta_p - ({}^{A}E^{B}E + {}^{A}C^{B}C) \quad (1)$$

where ${}^{A}V$ is the molar volume of the solvent (cm^3/mol) (with ${}^{A}V \approx {}^{A}\overline{V}$), δ_d, δ_p are the Hansen partial solubility parameters ([MPa]$^{1/2}$) for dispersive and polar forces, respectively, and E, C are the Drago parameters (kJ$^{1/2}$/mol$^{1/2}$) for electrostatic and covalent contributions, respectively.

In the case of poor mixing between two components, such as occurs between water and water-insoluble polymers or between water and organic solvents which are only partly miscible with water, adsorption type interaction must be considered. The corresponding interaction energy, $\Delta_{int}E_2$, is calculated by:

$$\Delta_{int}E = -{}^{A}V^{A}\delta_d{}^{B}\delta_d - {}^{A}V^{A}\delta_p{}^{B}\delta_p - ({}^{A}E^{B}E + {}^{A}C^{B}C) \quad (2)$$

For simplicity, parameters for pure water were used in this work although the presence of BSA should modify the properties of the aqueous component. When the solvent is miscible with water, equation 1 must be used.

For this work, we have made the hypothesis that for good drug encapsulation into the polymer, drug-polymer interaction must dominate over the interactions of drug-solvent and polymer-solvent. For given drug-polymer components, this interaction should become dominant through weakening the interactions with the solvent, illustrated by the dotted lines in Fig. 1. In other terms, if the sum of interaction energies $\Delta_{int}E_1$ and $\Delta_{int}E_2$ is low, the encapsulation efficiency should be high.

When a single polymer solvent is replaced by a solvent mixture, the interaction energies cannot be calculated with the above equations. At present, no appropriate models exist for solvent mixtures. In these cases, Hildebrand's total solubility parameter for the mixture, δ_{mix}, has to be considered:

$$\delta_{mix} = \phi_A\delta_A + \phi_B\delta_B \quad (3)$$

where ϕ_A and ϕ_B are the mole fractions for component A and B, respectively, and δ_A and δ_B are the corresponding Hildebrand parameters. The mole fractions can be calculated by

$$\phi_A = \frac{n_A V_A}{n_A V_A + n_B V_B} \quad (4)$$

and

$$\phi_B = 1 - \phi_A \quad (5)$$

where n_A and n_B are the number of moles, and V_A and V_B the molar volumes (cm^3/mol) for A and B, respectively.

MATERIALS AND METHODS

Material

Poly(d,l-lactic acid) (PLA), Resomer R202 (M_n = 7,700; M_w = 15,600, as determined in our laboratory) was purchased from Boehringer Ingelheim (D-Ingelheim) and bovine serum albumin

(BSA) was obtained from Fluka (CH-Buchs). The solvents of analytical grade used for PLA were from Fluka, and their relevant physico-chemical characteristics are summarised in Table I.

Microencapsulation

Microspheres were prepared by typically dispersing 2 g of a 2.0% (w/w) aqueous BSA solution in 40 g of 5.0% (w/w) solutions of PLA, using the organic solvents or solvent mixtures listed in Table I and III, respectively. The W/O-mixture was homogenised by ultrasonication (Model Vibra Cell, Sonic & Materials, US-Danbury) during two times 30 seconds at output control 7 under ice-cooling. Subsequently, the obtained emulsion was spray dried in a Büchi 190 laboratory spray dryer (Büchi, CH-Flawil) at a rate of 2 - 5 ml/min. The process parameters were the following: flow of pressurised air: 400 - 500 Nl/h, aspiration: - 40 mbar; inlet temperature: 37 - 80°C (depending on the solvent), outlet temperature: 33 - 60°C (depending on the solvent).

To break up any agglomerates or remove any BSA adsorbed on the microspheres surface, the product was washed in 0.1% aqueous poloxamer F68 solution and distilled water, and sieved through a 100 µm sieve. After drying under vacuum at room temperature for 24 hours, the spheres were washed in hexane, thereby removing any adsorbed water, and dried again under vacuum for 12 hours.

Particle morphology and size

Microspheres shape and size were examined by visible light microscopy, scanning electron microscopy (SEM) and laser light scattering (Mastersizer X, Malvern Instruments, GB-Worcestershire). For SEM, the microspheres were mounted on a double faced adhesive tape, sputtered with platinum and viewed in a Hitachi S-700 scanning electron microscope.

Protein loading

BSA was extracted from the accurately weighed (50 mg) microspheres by first dissolving the polymer in dichloromethane. After filtration on a 0.2 µm membrane filter (RC 58, Schleicher & Schuell, D-Dassel), the protein was eluted from the filter with 67 mM phosphate buffer of pH 7.4. The filter was washed three times with 3 ml buffer, and the combined solution assayed fluorometrically (Fluoromax, Spex, Edison, NJ).

In vitro release

Release studies were conducted in rotating 4 ml glass vials at 37°C. Microspheres were accurately weighed (50 mg) into the vials and 4 ml of 67 mM phosphate buffer of pH 7.4 was added. Prior to incubation the suspensions were sonicated for 15 - 30 s in a sonication bath, at 25 kHz. After 24 hours, the samples were collected and centrifuged for 15 min at 3500 rpm. The concentration of BSA in the supernatant was subsequently measured fluorometrically at 37°C.

Table I - Selected physico-chemical characteristics of the polymer solvents. δ is the Hildebrand solubility parameter, and δ_p,δ_d and δ_h are the Hansen partial solubility parameters $(MPa)^{1/2}$ representing the polar, dispersive and H-bonding cohesive forces [26]. p,m and s represent poor, moderate and strong H-bonding forces.
a): Solvents used only in mixtures with EtAc.

Solvent	Kp (°C)	δ	δ_d	δ_p	δ_h	H-bond
Dichloromethane (DCM)	40	20.3	18.2	6.3	6.1	p
Methylal	42	17.5	15.1	1.8	8.6	m
Acetone	56	20.0	15.5	10.4	7.0	m
Tetrahydrofuran (THF)	66	19.4	16.8	5.7	8.0	m
1,3-Dioxolane	76	23.2	14.8	11.3	13.9	m
Ethyl acetate (EtAc)	77	18.1	15.8	5.3	7.2	m
Cyclohexane [a)]	80	16.8	16.8	0.0	0.2	p
Isopropanol [a)]	82	23.5	15.8	6.1	16.4	s
Dimethyl carbonate	90	20.2	15.9	6.6	10.6	m
Isobutanol [a)]	99	22.2	15.8	5.7	14.5	s
1,4-Dioxane	100	20.5	19.0	1.8	7.4	m
Toluene	111	18.2	18.0	1.4	2.0	p
Nitroethane	115	22.7	16.0	15.5	4.5	p

RESULTS AND DISCUSSION

Solvent selection

Single organic solvents for PLA were selected to cover a wide range of boiling point, i.e., 40 to 115°C, and solubility parameters, i.e., 16.8 to 23.5 $(MPa)^{1/2}$ (Table I). More interestingly, the solvents also show varying polar and hydrogen bonding cohesive energies, two parameters which should greatly influence the behaviour of PLA in solution, as PLA is a polar molecule and a Lewis base. Dichloromethane (DCM) was used as reference for studying new individual solvent systems. Some of the solvents were selected for their low calculated interaction capacity with the polymer and with water (see below).

With the solvent mixtures, three different strategies were followed. The first group of solvent mixtures was characterised by a total Hildebrand solubility parameter comparable to DCM, i.e., EtAc/isopropanol (58.8:41.2) and EtAc/isobutanol. In the second group, the volume fraction of solvent with polar cohesive energy was reduced by mixing with a non-polar solvent, i.e., EtAc/cyclohexane. In the third group, proton donors and proton acceptors were mixed providing mutual *neutralisation* of the hydrogen bonding capability, i.e., EtAc/DCM and DCM/nitroethane. On a qualitative basis, these mixtures were expected to provide insight into the interaction between BSA and PLA.

Microencapsulation process

The solvent systems used for PLA substantially influenced both the manufacturing process and the quality of the microspheres. In the production process, the stability of the W/O-emulsions varied. Methylal, 1,3-dioxolane, and the mixtures EtAc/alcohols and EtAc/cyclohexane (61:39) all required additional sonication at some point before or during the spray drying process to maintain the mixtures homogeneous. The spheres produced with the higher boiling solvents nitroethane and toluene showed a similar behaviour. With all other solvent systems, good W/O-emulsion stability and satisfactory powder flow in the cyclone was achieved. Generally, the total yield was in the order of 50 - 70% after sieving and drying; for the higher boiling solvents, i.e., nitroethane and toluene, the yield was only 8 - 40%.

Particle morphology and size

Microencapsulation of BSA by spray drying generally resulted in spherical particles of about 1 - 10 µm in size. This size range was found by SEM and confirmed by laser light scattering. The particle size distribution was uniform, with a mean diameter of about 3.4 µm. The SEM-micrographs showed regularly shaped microspheres with a non-porous surface. With DCM, marked surface indentations were visible. Ethyl acetate, dimethyl carbonate, toluene and nitroethane all gave very satisfactory particle morphology. By contrast, the water miscible solvents acetone, methylal, THF, and 1,4-dioxane produced a substantial part of coalesced microspheres with irregular morphology. These results with the single solvents confirm our previous findings [11]. With the solvent mixtures, particles with regular, spherical morphology and a smooth surface were obtained.

Protein loading

Drug incorporation efficiency is one of the key criteria for optimising microencapsulation techniques. As presented in Table II, BSA incorporation efficiency depends greatly on the polymer solvent used. Ethyl acetate and dichloromethane proved to be appropriate solvents, providing efficiencies of 65 - 75% (w/w). For ethyl acetate, this efficiency appears to depend greatly on the loading level.

Microencapsulation with toluene, nitroethane and dimethyl carbonate resulted in intermediate loading efficiencies, i.e., 41 - 58%. The first two, however, were expected to give higher incorporation rates due to low interactions solvent-PLA and solvent-water (BSA), as discussed below. This discrepancy might be due to the higher boiling points of these solvents leading to a less dense surface structure of the microspheres. From the SEM-micrographs, however, no surface pores were visible.

Finally, methylal, acetone, THF and 1,3-dioxolane gave low encapsulation efficiencies. This was anticipated since these compounds are highly or even entirely miscible with water, thereby having

the potential to precipitate the protein and/or compete for adsorption on the polymer. In the light of this, the surprisingly high loading efficiency of nearly 70% attained with 1,4-dioxane cannot be explained.

Table II - BSA-loading, loading efficiency and burst release (after 24 h) from PLA microspheres prepared with a single polymer solvent. a): Microspheres were prepared to examine the effect of BSA-loading.

Solvent	Loading (%)	Loading efficiency (%)	Burst release (%)
DCM [a)]	1.28	64.9 ± 5.3	9.2 ± 0.1
DCM [a)]	3.77	66.8 ± 2.9	36.4 ± 1.1
Methylal	0.21	10.8 ± 0.5	45.0 ± 1.0
Acetone	0.49	25.0 ± 1.0	41.8 ± 1.3
THF	0.69	35.0 ± 1.0	16.6 ± 0.3
1,3-Dioxolane	0.20	10.2 ± 1.5	50.3 ± 1.3
EtAc [a)]	1.44	72.3 ± 1.8	8.6 ± 1.0
EtAc [a)]	3.22	56.6 ± 1.3	33.6 ± 0.3
Dimethyl carbonate	0.81	41.2 ± 4.3	60.9 ± 1.0
1,4-Dioxane	1.30	67.8 ± 3.3	12.3 ± 0.4
Toluene	1.16	58.8 ± 1.9	27.2 ± 1.2
Nitroethane	0.88	44.8 ± 2.6	16.5 ± 0.3

The experiments with the solvent mixtures provided highly relevant results (Table III). The mixtures of ethyl acetate and alcohol having the same total solubility parameter as DCM resulted in very low loadings. This might be attributed to a partial precipitation of BSA in the presence of alcohol. Isobutanol, however, gave even lower efficiencies, although it is less water-miscible than isopropanol and therefore was expected to cause less pronounced precipitation of BSA. With the 80:20 mixture of ethyl acetate and isopropanol, a rather inconsistent result was obtained. The incorporation rate for this mixture was expected intermediate between pure ethyl acetate and the 58.8:41.2 mixture, but only about 5% was found. At present, no rational explanation can be provided, but the result provides clear evidence that an optimum ratio of individual components in a mixture must exist. The effect of protein loading with the two EtAc/isopropanol mixtures was negligible (Table III), as found for the single solvent DCM.

Table III - BSA-loading, loading efficiency and burst release (after 24 h) from PLA microspheres prepared with polymer solvent mixtures. Solvent ratios are expressed in weight percentage. δ_{mix} was calculated by equation 3. EtAc/isopropanol batches were prepared also to examine the effect of different BSA-loadings (high, mediate, low). a): δ_{mix} was calculated according to Eq. 3.

Solvent mixture	δ_{mix} [a)]	Loading (%)	Loading efficiency (%)	Burst release (%)
EtAc/isopropanol (58.8:41.2)	20.3	0.14	28.8 ± 5.2	11.6 ± 0.6
EtAc/isopropanol (58.8:41.2)	20.3	0.55	27.9 ± 3.4	40.3 ± 1.2
EtAc/isopropanol (58.8:41.2)	20.3	0.64	32.2 ± 1.0	23.1 ± 1.4
EtAc/isopropanol (58.8:41.2)	20.3	1.43	30.0 ± 2.5	62.3 ± 1.9
EtAc/isopropanol (80:20)	19.3	0.03	5.2 ± 3.0	54.3 ± 1.8
EtAc/isopropanol (80:20)	19.3	0.09	4.7 ± 0.3	69.3 ± 3.5
EtAc/isopropanol (80:20)	19.3	0.24	5.0 ± 1.0	65.9 ± 5.4
EtAc/isobutanol (50.1:49.9)	20.3	0.08	4.1 ± 0.1	not determined
EtAc/cyclohexane (81:19)	17.9	1.56	79.4 ± 5.8	31.6 ± 0.7
EtAc/cyclohexane (61:39)	17.6	0.68	34.8 ± 0.9	47.1 ± 0.6
DCM/nitroethane (71.6:28.4)	21.2	1.49	75.9 ± 3.8	4.6 ± 0.1
DCM/nitroethane (38.6:61.4)	21.7	0.29	14.8 ± 0.8	30.8 ± 0.5
DCM/EtAc (49:51)	19.0	1.24	76.2 ± 4.4	21.1 ± 0.5
DCM/EtAc (65.8:34.2)	19.3	1.21	62.3 ± 1.8	19.5 ± 1.3

Two different mixtures of ethyl acetate and cyclohexane, a polymer non-solvent, were used to examine the influence of reduced volume fraction of the solvent with polar cohesive energy contribution. The findings partly support our hypothesis that a reduced volume fraction of the polar solvent in a mixture may weaken the solvent-polymer interaction, whereby the drug-polymer interaction becomes more dominant, as illustrated in Fig. 1. With the EtAc/cyclohexane 81:19 mixture, the highest loading of all solvent systems studied was obtained (Table III). However, addition of cyclohexane seems to have limitations since spray drying of the 61:39 mixture resulted in more than 50% reduction of the loading efficiency. This confirms the observation made above for the ethyl acetate/isopropanol mixture that the ratio of individual components in a solvent mixture is crucial for obtaining good quality microspheres.

The third approach was based on a possible neutralisation mechanism of hydrogen-bonding forces by mixing organic solvents with proton donor and proton acceptor properties. DCM, a proton donor, was mixed with the acceptors nitroethane and ethyl acetate, respectively. The DCM/nitroethane mixture 71.6:28.4 (molar ratio 2.2:1) seems to confirm a synergistic effect as loading efficiency is higher as compared to the individual solvents. The DCM/EtAc mixtures 49:51 (molar ratio 1:1) and 65.8:34.2 (molar ratio 2:1) also indicate favourable properties, even though the effect is less prominent. With the DCM/nitroethane mixture 38.6:61.4 (molar ratio 1:1.8), an inconsistent finding was observed as only 15% of the theoretical amount of protein was incorporated. This strongly emphasises the importance of a finely adjusted volume ratio in solvent mixtures.

In vitro release

Protein release from a biodegradable polymer matrix such as PLA microspheres is a complex process, composed of (i) the release of material located near the particle surface, (ii) diffusion in macropores or in the polymer phase itself, and (iii) the more delayed release taking place due to degradation of the polymer matrix. In this study, we were mainly interested to examine the first stage of protein release, the so-called burst release. If the protein concentration near the surface of the microspheres is high, a more pronounced burst release will be observed. High protein concentration near the microsphere surface may be expected when strong interactions between the polymer solvent and the protein occur during manufacturing of the spheres, whereby the solvent carries along the protein towards the surface during evaporation. As biodegradable microspheres are commonly used for controlled drug release over several weeks or months, the burst release should be moderate (< 10%) and controlled.

The data in Table II and III show that for most of the solvent systems, burst release is inversely proportional to the loading efficiency. Clearly, the solvent systems giving the highest incorporation rate tend to give the lowest burst release; DCM, EtAc and 1,4 dioxane prove to be the superior solvents. These three solvents provide acceptable loading efficiency combined with a moderate burst release in the order of 10%. The increased burst release from the formulations with higher actual BSA-loading (EtAc and DCM; Table II) is in agreement with results in the literature [2,10].

Dimethyl carbonate and the two acetals produced microspheres with a very pronounced burst. As the SEM-micrographs did not reveal any surface pores, this high burst might be attributed to BSA transported by the organic solvent towards the microsphere surface during the evaporation process. These particular solvents are characterised by a relatively high hydrogen bonding capacity (Table I). A quite important burst release was also observed with toluene, which, in contrast to the afore mentioned acetals and carbonate, interacts only very weakly through hydrogen bonding and polar forces. For toluene, the SEM-micrographs reveal indeed that the particles are relatively porous.

Among the solvent mixtures, EtAc/alcohol produced microspheres with very high burst release. The results suggest that strong hydrogen-bonding solvents interact intensely with the protein, thereby transporting the protein towards the microsphere surface during the solvent evaporation in the manufacturing process. With the EtAc/isopropanol mixture (58.8:41.2), an increasingly important burst release is observed from the microspheres with increasing actual loadings.

The intended reduction of the polar and hydrogen bonding forces through lowering the volume fraction of EtAc in the mixture with cyclohexane did obviously not lead to improved release

characteristics. Compared to pure EtAc, the mixtures with cyclohexane (81:19 and 61:39) gave a threefold increase of the burst release. Here, however, a higher final loading seems to give a lower burst. We can speculate that, in the final step of particle drying, cyclohexane as a non-solvent for the polymer would possibly not diffuse readily through the polymer matrix but remain as residual in the microspheres. Consequently, this might create a more porous matrix morphology.

The microspheres prepared from the solvent mixtures consisting of a Lewis acid (DCM) and a Lewis base (nitroethane, ethyl acetate) gave, with one exception, a burst release which lies above that observed when single solvents were used. The DCM/nitroethane mixture (71.6:28.4) produces spheres, however, with excellent release properties and, as mentioned previously, high loading efficiency. This particular mixture consisting of 2 moles of DCM and 1 mole of nitroethane appears to fulfil best our requirements for good quality microspheres, i.e., high loading efficiency and low burst release. Although the assumed mutual neutralisation between Lewis acid and Lewis base had generally a favourable effect on the loading efficiency, this was not generally the case for the burst release.

Evaluation of thermodynamic parameters

The results presented in Tables II and III show the wide range of solvents suitable for successful spray drying of PLA. Table II illustrates this in terms of increasing boiling point. Solvents with boiling points ranging from 40 to 110°C produce good quality microspheres. There is, however, clear evidence that poor quality particles are obtained from solvents which are highly water miscible such as, the acetals, acetone, and THF. In this respect, 1,4-dioxane represents a striking exception which cannot be explained for the time being.

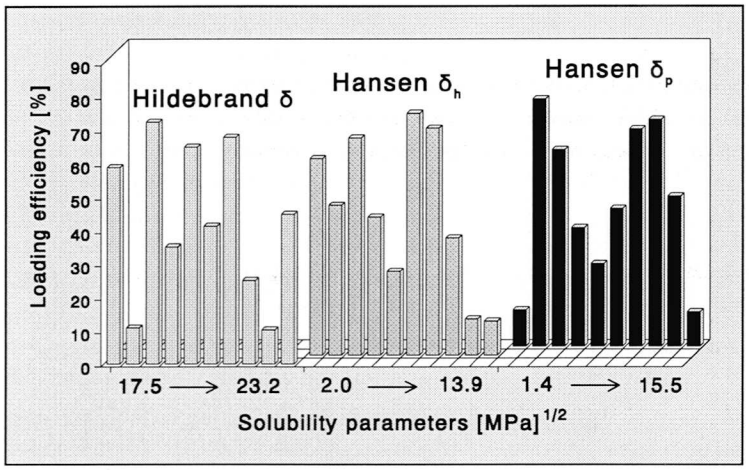

Figure 2 - Microspheres loading efficiencies versus the Hildebrand solubility parameter (δ), the Hansen hydrogen-bonding (δ_h) and polar (δ_p) cohesive parameters.

Figure 2 illustrates the pattern of BSA encapsulation efficiency plotted versus the Hildebrand solubility parameter δ (18 - 23 $[MPa]^{1/2}$), and the Hansen hydrogen bonding δ_h (2.0 - 13.9 $[MPa]^{1/2}$) and polar δ_p (1.4 - 15.5 $[MPa]^{1/2}$) cohesive parameters. For the Hildebrand solubility parameter, the borderline solvents methylal and 1,3-dioxolane do not reflect the real boundary of the suitable solubility parameter range. These two solvents are particular compounds as they exhibit relatively strong H-bonding interactions (Table I). Overall, the irregularity of the profile demonstrates that the Hildebrand parameter does not represent an adequate tool to optimise solvent systems for protein microencapsulation.

The weakness of the Hildebrand parameter lies in the fact that it does not discriminate between dispersive, polar and hydrogen-bonding forces involved in the drug-polymer-solvent interactions. For aliphatic polyesters, which obviously interact greatly through polar and hydrogen bonding forces, the Hildebrand parameter is clearly not appropriate.

A more discriminative picture on the type and energy of interactions should be expected when the Hansen partial solubility parameters are considered, which distinguish between dispersive, permanent dipole-dipole, and H-bonding contributions. The plot representing the H-bondings (Fig. 2) tendentiously shows the superiority of low or moderate H-bonding solvents. This

observation is further supported by the negative results obtained with the EtAc/alcohol mixtures; the hydrogen bonding parameters of isopropanol and isobutanol are 16.3 and 14.5 $(MPa)^{1/2}$, respectively. By contrast, the importance of the polar cohesion parameter in a single solvent system is ambiguous (Fig. 2). Clearly, solvents with polar cohesion parameters ranging from 1.4 to 15.5 $(MPa)^{1/2}$ gave suitable product quality. Therefore, the microencapsulation process appears to be much less influenced by the polarity of the solvent than by the hydrogen-bonding capacity.

Table IV - Hansen and Drago interaction parameters used for calculating interaction energies. E- and C-values taken from Drago *et al.* [23]. a): Value of ethyl acetate (used as a molecular model for PLA). b): Value of benzene. c): Value of nitrobenzene.

Substance	δ_d $([MPa]^{1/2})$	δ_p $([MPa]^{1/2})$	E $(kJ^{1/2}/mol)$	C $(kJ^{1/2}/mol)$	V (cm^3/mol)
PLA	15.5	1.6	3.31 a)	2.00 a)	55.7
water (acid)	15.6	16.0	2.68	1.60	18.0
water (base)	15.6	16.0	4.66	0.20	18.0
DCM	18.2	6.3	1.76	0.22	63.9
Acetone	15.5	10.4	3.56	2.58	74.0
THF	16.8	5.7	3.35	4.46	81.7
EtAc	15.8	5.3	3.31	2.00	98.5
1,4-Dioxane	19.0	1.8	3.80	2.64	85.7
Toluene	18.0	1.4	1.43 b)	0.92 b)	106.8
Nitroethane	16.0	15.5	2.64 c)	0.98 c)	71.5

Although the Hansen parameters do differentiate between dispersion, polar and hydrogen bonding forces, they still appear to lack specificity as far as H-bonding interactions are concerned. These interactions can be considered as the mutual reactions between H-donors and H-acceptors. As PLA is a Lewis base, hence H-acceptor, we can reasonably assume that this polyester interacts very differently with H-donors (DCM, alcohols) and H-acceptors (esters, ketoses, acetals). Moreover, the presence of water (and also of the protein) affects this interaction. All these factors have not been taken into account so far.

A more powerful tool for estimating polymer-solvent-drug interactions is the calculation of energies of interaction using, in addition to δ_d and δ_p, the E (electrostatic) and C (covalent) parameters which account for acid-base interactions. The parameters are reported to give improved agreement with experimental data [22,25]. The values of the E and C parameters used, taken from Drago's work [23], are summarised in Table IV. One should notice, however, that the values for PLA, toluene and nitroethane are assumptions from ethyl acetate, benzene and nitrobenzene, respectively.

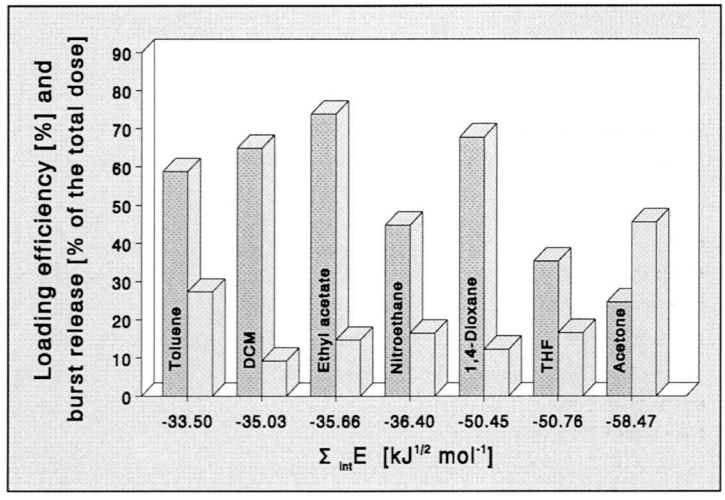

Figure 3 - Microspheres loading efficiency (filled columns) and burst release (open columns) versus the sum of change in interaction energy (equations 1 and 2).

Figure 3 shows a plot of loading efficiency and burst release versus the sum of interaction energies, $\Sigma_{int}E$, from solvent-polymer and from solvent-water interactions (water was used instead

of aqueous BSA-solution for simplicity). By reducing the solvent-polymer and the solvent-water interactions, the water-polymer interaction is assumed to become dominant, resulting in higher protein incorporation efficiency and lower burst release. The tendency of the results demonstrates the validity of our thermodynamic model, although 1,4-dioxane does not fit into this model. One also should keep in mind, that the calculated interactions energies for toluene and nitroethane are approximations, as the E and C values for these solvents are not known. The values used here are those reported for benzene and nitrobenzene, considered as valid assumptions for toluene and nitroethane, respectively. The proposed interaction model for drug microencapsulation require confirmation by additional experiments. Clearly, taking into consideration the heat of dissolution of the polymer in the different solvents, and the knowledge of the parameters δ_d, δ_p, E, C for the polymer and the solvents would definitely improve the validity of the calculated interaction energies. In this respect, a new and more developed thermodynamic model to predict protein encapsulation efficiency in PLA microspheres has recently been proposed [12].

Acknowledgements

We thank Dr. E. Wehrli, Laboratory of Electron Microscopy I, ETH, Zurich, for providing the SEM-micrographs and C. Thomasin, Department of Pharmacy, ETH, Zurich, for helpful discussions.

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Appendicies

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Summary

Single-Dose Diphtheria and Tetanus Vaccines

Tetanus (Ttxd) and diphtheria (Dtxd) toxoids encapsulated in polyester microspheres (MS) for single injection immunisation have so far given pulsatile *in vitro* release and strong immune response in animals, but no boosting effect. This has been ascribed to insufficient toxoid stability within the MS exposed to *in vivo* conditions over a prolonged time period. The effect of *co*-encapsulated putative stabilising additives on encapsulation, release and stability of two different Ttxd and one Dtxd preparations was therefore examined in microspheres based on poly(d,l-lactic-*co*-glycolic acid) (PLGA) and poly(d,l-lactic acid) (PLA). Further, we investigated the influence of polymer hydrophobicity of commercially available and novel more hydrophobic polymers on encapsulation and burst release of the toxoids from MS prepared by spray-drying and coacervation. For this purpose fluorimetry, ELISA and HPLC were used. Finally, candidate tetanus vaccines were tested in mice for immune response.

The *co*-encapsulated stabilisers influenced significantly the entrapment of Dtxd and Ttxd in PLA/PLGA MS. *Co*-encapsulation of albumin, trehalose and γ-hydroxypropyl-cyclodextrin all improved the Ttxd encapsulation efficiency in spray-dried PLGA MS. Albumin increased the encapsulation efficiency of antigenic Ttxd by one to two orders of magnitude. Furthermore, with albumin or a mixture of albumin and trehalose ELISA responsive Ttxd was released over 1-2 months following a pulsatile pattern. For Dtxd, albumin or trehalose decreased the amount of toxoid entrapped in spray-dried MS, whereas albumin increased the entrapment in coacervated MS. Moreover, the entrapment of Dtxd decreased as a function of polymer hydrophobicity in spray-dried MS. Without additives, approx. 64, 43 and 16% entrapment efficiency was obtained for PLGA 50:50, PLGA 75:25 and PLA (approx. 15 kDa), respectively. Modified PLAs, carrying stearyl end-groups were only processed by coacervation. Satisfactory entrapment of Dtxd of between 30 and 60% was obtained. Here, albumin was a prerequisite for toxoid encapsulation, as albumin-free formulations produced strong toxoid precipitation. Furthermore, protein burst release increased with the more hydrophobic polymers, with Dtxd, Ttxd and the *co*-encapsulated albumin following a similar release pattern and magnitude. These investigations also revealed that entrapment estimation methods (extraction or polymer hydrolysis) as well as analytical methods (HPLC or ELISA) strongly influenced the determined amount of encapsulated toxoid and albumin.

Antibody responses were analysed after immunisation of mice with Ttxd microencapsulated in the presence of additives. Immunisation gave rise to higher responses than those obtained in the absence of additive. Albumin, trehalose, γ-hydroxypropylcyclodextrin and calcium salts preserved the immunogenicity of the incorporated antigen with the highest efficacy. Sustained responses were obtained with mixtures of fast and slowly releasing MS containing albumin plus trehalose or calcium salts. The selected additives may stabilise the antigen in MS during storage and rehydration in body fluids. Regulated antigen release from MS-based vaccines permits a reduction of the antigen dose and optimisation of single-dose vaccine formulations.

Further, we investigated the influence of polymer type and *co*-encapsulated excipients on Dtxd immunogenicity in guinea pigs. Guinea pigs immunised with Dtxd encapsulated into hydrophilic polymers exhibited specific antibody responses over 40 weeks that were comparable to the responses to aluminium adsorbed toxoid. In contrast, none or very low antibody responses were determined after immunisation with MS of hydrophobic polymers. All MS formulations designed

to provide both priming plus boosting doses elicited protective antibody responses 40 weeks post immunisation.

PHYSICO-CHEMICAL AND ANTIGENIC STABILITY

Physico-chemical, antigenic and immunogenic properties may be altered during microencapsulation of antigens and their release from PLA/PLGA MS. Here, the physico-chemical, conformational and antigenic stability of Ttxd and Dtxd was studied in aqueous solutions stressed by elevated temperature and the presence of lactic and glycolic acids. Further, the stabilising effect of albumin was investigated. The analytical tools used were fluorimetry, CD-spectroscopy, turbidimetry, electrophoresis and ELISA. Elevated temperatures altered the physico-chemical and antigenic properties of the toxoids to a greater extent than did the acids (50 mM). Substantial unfolding and chemical changes of tryptophan were observed upon 1 - 4 weeks of incubation at 60°C. At 4°C, only minor conformational changes were observed, even in the presence of the acids. Furthermore, 40% of the Ttxd antigenicity was lost after 7 days at 37°C. This loss increased in the presence of the acids. At 60°C, the antigenicity had completely vanished. Very importantly, 0.5% albumin preserved the tetanus antigenicity over 6 weeks incubation at 37°C, regardless of the presence of glycolic acid.

ANTIGEN RELEASE SET-UP ASSESSMENT AND SURFACE ADSORPTION

The classical adjuvants alum and Freund's Incomplete Adjuvant (IFA) are frequently used as references for the design of new adjuvants and antigen delivery systems, e.g., microspheres. PLGA MS have been proposed for delivering antigen booster doses *in vivo* after a single injection. However, as antigen release kinetics from conventional adjuvants are generally unknown, it appears presumptuous to propose a desired antigen release pattern from PLGA MS. Therefore, we have studied the Ttxd *in vitro* release from alum, IFA formulations and MS in four different test systems. The results showed a stronger Ttxd association to alum than to IFA, and the release from both formulations lasted between 3 - 9 days. The total of ELISA-responsive antigen released was 60 - 85% of the actual dose. Both the total amount and the prolongation of release depended on the Ttxd dose. Furthermore, the incomplete *in vitro* release of Ttxd from the adjuvants and also from PLGA 50:50 MS was shown to be partly due to experimental conditions. Typically, Ttxd adsorbed on the glass vials used for the release test and also on the surface of the PLGA MS wherefrom it was released. In conclusion, the test system depending rate and quantity of release observed evidence the limitations of *in vitro* release data. Finally, for mimicking conventional vaccination schedules, i.e., 0, 4, and 8 weeks, PLGA MS should release antigen doses at the corresponding time points, and the release pulses should last for a few days.

THERMODYNAMIC MICROENCAPSULATION APPROACH

Microencapsulation was studied by a thermodynamic approach taking into account quantitatively the molecular interactions between polymer, solvent and the aqueous protein phase. Bovine serum albumin was microencapsulated into PLA by spray-drying using single solvents and binary solvent mixtures. The use of binary solvent mixtures allowed the design of systems with adjustable solubility parameters. MS characteristics like entrapment efficiency, burst release after 24 h and surface morphology were investigated and proved to be highly dependent on the polymer solvent system. Hildebrand or partial Hansen solubility parameters (δ, δ_d, δ_p, δ_h) proved to be insufficient for predicting MS properties, although low or moderate hydrogen-bonding solvents and solvent mixtures were found to be generally appropriate, whereas strongly H-bonding solvents gave poor quality MS. Similarly, water-miscible solvents were shown to give often unsatisfactory products. A more powerful tool for optimising a microencapsulation process is estimating polymer-solvent-drug interactions by using δ_d, δ_p, and the Drago parameters E (electrostatic) and C (covalent) of the components involved. Entrapment efficiency is increased and burst release reduced if polymer-drug interaction is dominant and if polymer-solvent or drug-solvent interactions are reduced. This thermodynamic approach provides a rational basis for optimising microencapsulation processes.

Zusammenfassung

Einfachimpfungen gegen Diphtherie und Tetanus

Der Einbau von Tetanustoxoid (Ttxd) und Diphtherietoxoid (Dtxd) in Polyester-Mikropartikel aus Poly(d,l-laktid-*co*-glykolid) (PLGA) und Poly(d,l-laktid) (PLA) ergibt ein Antigenfreigabesystem, das nach parenteraler Verabreichung am Tier starke Immunantworten bewirkt. Dieses Antigenfreigabesystem zeigt *in vitro* ein pulsatiles Freisetzungsverhalten, welches *in vivo* bisher keinen signifikanten *Boost-effect* bewirken konnte. Dies wird einer ungenügenden Stabilität der Toxoide gegenüber den *in vivo* herrschenden Bedingungen zugeschrieben. Deshalb wurde in dieser Arbeit der Einfluss von ausgewählten Hilfsstoffen auf Verkapselungsgüte, *in vitro* Freigabe und Stabilität von zwei verschiedenen Tetanus- und einem Diphtherietoxoid in PLA/PLGA Partikeln untersucht. Zudem wurde der Einfluss der Hydrophobizität von kommerziell erhältlichen und neuen, hydrophoberen Polyestern auf die initiale *in vitro* Freigabe der Toxoide aus Mikrosphären (MS) untersucht. Die verwendeten Mikroverkapselungstechniken waren die Sprühtrocknung und die Koazervation. Analytisch wurden die Toxoide mittels Fluorimetrie, ELISA und HPLC erfasst. Ausgewählte Impfstofformulierungen wurden schliesslich an Mäuse und Meerschweinchen verabreicht und die spezifischen Antikörperreaktionen bestimmt.

Es zeigte sich, dass die verkapselten Hilfstoffe den Einbau von Dtxd und Ttxd in PLA/PLGA MS signifikant beeinflussten. Sowohl Albumin als auch Trehalose und γ-Hydroxypropyl-Cyclodextrin steigerten die Verkapselungseffizienz von Ttxd in sprühgetrockneten PLGA MS. Albumin verbesserte die Verkapselungseffizienz von ELISA-reaktivem Ttxd um ein bis zwei Grössenordnungen. Mit Albumin und Mischungen von Albumin und Trehalose folgte die Freigabe des ELISA-reaktiven Ttxd während 1-2 Monaten einer pulsatilen Kinetik. Während Albumin und Trehalose die Verkapselung von Dtxd in sprühgetrocknete MS verschlechterte, verbesserte Albumin den Einbau dieses Toxoids in koazervierte MS. Auch sank die Verkapselungseffizienz von Dtxd in sprühgetrockneten MS mit der Hydrophobizität des Polymers. Ohne Hilfsstoffe betrug die Verkapselungseffizienz in PLGA 50:50, PLGA 75:25 und PLA (ca. 15 kDa) rund 64%, 43% und 16%. Die hydrophoberen, Stearyl-Endgruppen tragenden PLAs wurden ausschliesslich mittels Koazervation verarbeitet. Mit diesen Polymeren erreichte die Verkapselungseffizienz 30% bis 60%. Die Anwesenheit von Albumin war eine Voraussetzung für die Koazervation dieser Polymere, da ohne Zusatz von Albumin eine starke Toxoidausfällung auftrat. Die initiale *in vitro* Freigabe nahm mit zunehmender Hydrophobizität der Polymere zu, wobei Dtxd, Ttxd und mitverkapseltes Albumin in vergleichbaren Ausmassen und nach einer ähnlichen Kinetik freigegeben wurden. Überdies stellte sich in diesen Untersuchungen heraus, dass sowohl die Methode der Proteinextraktion aus den MS wie auch die Methode der Proteinbestimmung (HPLC, Fluorimetrie oder ELISA) einen grossen Einfluss auf die gefundene Menge der verkapselten Proteine hatten.

Die Immunogenität von mikroverkapseltem Ttxd wurde anhand der Antikörperreaktion nach subkutaner Injektion in Mäusen bestimmt. Die MS, die auch Albumin, Trehalose, γ-Hydroxypropyl-Cyclodextrin und Calziumsalze enthielten, ergaben höhere Antikörpertiter als die MS ohne Hilfsstoffe. Eine anhaltende Immunantwort konnte mit einer Mischung von MS erreicht werden, die unterschiedlich schnell freisetzende MS und mitverkapseltes Albumin, Trehalose und Calziumsalz enthielt. Dieser Effekt lässt sich möglicherweise auf eine durch die Hilfsstoffe stabilisierte Antigenität und die kontrollierte Antigenfreigabe zurückführen. Diese MS mit

kontrollierter Antigenfreigabe könnten die Grundlage bilden zur Herstellung von niedriger dosierbaren und von nur einmal zu verabreichenden Impfstoffen.

Physikalisch-chemische und antigene Stabilität

Der Prozess der Mikroverkapselung, aber auch die Freisetzung von Antigenen aus MS kann die physikalisch-chemischen und antigenen Eigenschaften von Antigenen beeinflussen. In dieser Studie wurden deshalb die physikalisch-chemische und antigene Stabilität Ttxd und Dtxd in wässrigen Lösungen untersucht, die erhöhten Temperaturen und dem Einfluss von Milch- und Glykolsäure ausgesetzt wurden. Zusätzlich wurde die stabilisierende Wirkung von Albumin untersucht. Für den Nachweis allfälliger Veränderungen wurden Fluorimetrie, CD-Spektroskopie, Turbidimetrie, Gel-Elektrophorese und ELISA angewandt. Es zeigte sich, dass die Toxoide vor allem durch die erhöhte Temperatur und weniger durch die Anwesenheit der Säuren verändert wurden. Substanzielle Proteinentfaltung und chemische Änderungen am Tryptophan konnten nach einer bis vier Wochen Lagerung bei 60°C beobachtet werden. Lagerung bei 4°C hingegen führte, selbst bei zusätzlicher Anwesenheit der Säuren, nur zu minimalen Konformations–änderungen. Weiter zeigte sich, dass bereits nach 7 Tagen bei 37°C rund 40% der Ttxd-Antigenität zerstört war, wobei dieser Effekt durch die Anwesenheit der Säuren noch verstärkt wurde. Bei 60°C war im gleichen Zeitraum sogar gar keine Antigenität mehr messbar. Als sehr vorteilhaft erwies sich der Zusatz von 0.5% Albumin, welches die Tetanusantigenität in bei 37°C gelagerten Lösungen über 6 Wochen stabilisierte, und zwar unabhängig vom Glykolsäurezusatz.

Freisetzungsanforderung und Oberflächenadsorption des Antigens

Bei der Entwicklung neuer Adjuvantien und Antigen-Verabreichungssysteme, wie zum Beispiel Mikrosphären, werden häufig die klassischen Adjuvantien Alum oder Freund's Inkomplettes Adjuvans (IFA) als Referenzformulierungen mituntersucht. Es wird angenommen, dass PLGA MS nach einfacher Injektion Wiederholungsimpfungen hinfällig machen könnten. Bis heute sind jedoch die Freisetzungskinetiken mit herkömmlichen Adjuvantien weitgehend unbekannt. Es erschien uns deshalb naheliegend, ein erwünschtes Antigen-Freigabe-Muster zu definieren. Dazu wurde das Freigabeverhalten von Ttxd aus Alum, IFA Formulierungen und MS in vier verschiedenen Testsystemen *in vitro* untersucht. Die Resultate zeigten, dass das Antigen stärker mit Alum als mit IFA assoziiert war und, dass die Freisetzung aus beiden Formulierungen innerhalb von drei bis neun Tagen beendet war. Insgesamt wurde 60% - 85% der eingesetzten Dosis an ELISA-reaktivem Antigen freigesetzt. Sowohl die wiedergefundene Menge wie auch die Dauer der Freisetzung hing stark von der Ttxd-Dosis ab. Zudem konnte die unvollständige Freisetzung von Ttxd aus den Adjuvansformulierungen und auch aus den PLGA 50:50 MS zum Teil auf die experimentellen Bedingungen zurückgeführt werden. Es zeigte sich nämlich, dass Ttxd an den Glassfläschchen, welche für die Freisetzungsuntersuchungen verwendet wurden, und an den MS selber adsorbiert wurde. Dies lässt den Schluss zu, dass *in vitro* erhaltene Daten nur beschränkte Aussagekraft besitzen, da sie stark vom verwendeten Testsystem abhängig sind. Ausserdem sollten PLGA MS zu denselben Zeitpunkten, wie dies herkömmliche Impfpläne vorsehen, also nach zum Beispiel 0, 4 und 8 Wochen, Antigene freisetzen, wobei die Freisetzung einige Tage dauern sollte.

Thermodynamischer Ansatz der Mikroverkapselung

Durch den Einbezug molekularer Interaktionen zwischen Polymer, Lösungsmittel und wässeriger Proteinphase wurde versucht, den Verkapselungsprozess nach thermodynamischen Parametern zu beschreiben. Dazu wurde Rinderserumalbumin (BSA) mittels Sprühtrocknung in PLA mikroverkapselt, wobei einzelne Lösungsmittel und binäre Lösungsmittelgemische eingesetzt wurden. Der Einsatz von Lösungsmittelgemischen ermöglichte die Untersuchung von Systemen mit einstellbaren Löslichkeitsparametern. Der Einfluss der Kohäsionsenergien wurde anhand von verschiedenen MS-Eigenschaften wie Beladung, Initialfreigabe von BSA innerhalb 24 Stunden und Oberflächeneigenschaften untersucht. Es stellte sich heraus, dass die Messparameter sehr stark von den Eigenschaften des Lösungsmittelsystems abhängig waren. Die Löslichkeitsparameter nach Hildebrand oder Hansen (δ, δ_d, δ_p, δ_h) konnten jedoch keine befriedigende Voraussagen über die

entstehenden MS-Eigenschaften liefern. Immerhin, ergaben Lösungsmittel und Lösungsmittelgemische mit eher geringer Wasserstoffbrückenbildungskapazität qualitativ bessere MS als solche mit hoher Kapazität. Ebenso führten mit Wasser mischbare Lösungsmittel zu unbefriedigenden Resultaten. Der Prozess der Mikroverkapselung liess sich schliesslich besser optimieren, wenn die Polymer-Lösungsmittel-Protein-Interaktionen mit Hilfe von δ_d, δ_p und den Drago Parametern E (elektrostatisch) und C (kovalent) berechnet wurden. Durch Minimierung der Polymer-Lösungsmittel-Interaktion konnte die Verkapselungseffizienz gesteigert und die Initialfreigabe gesenkt werden, was auf die Dominanz der Polymer-Protein-Interaktion zurückgeführt wurde.

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Curriculum vitae

Pål Johansen was born on March 28th, 1967 at Vega in Norway as the youngest child of Irene Johanne and Gunnar Erik Johansen. After courses in Philosophy, Mathematics, and Chemistry at the University in Oslo (UiO) 1998-1990, a graduation at in Pharmacy in 1994 followed. The student period included 6 months practical training in a hospital pharmacy in Bærum, Norway, as well as 9 months diploma work at ETH Zurich, Switzerland. During the doctoral study, Johansen was also working as teaching assistant in Galenical Pharmacy at ETH Zurich. He is presently post doctoral fellow at ETH Zurich preparing for a clinical trial with single-dose vaccines.