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Research paper

Diphtheria and tetanus toxoid microencapsulation into conventional and end-group alkylated PLA/PLGAs

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Abstract

The feasibility of biodegradable polyester microspheres (MS) for single injection vaccines will greatly depend on the toxoid stability within the MS exposed to in vivo conditions. This study examined the effects of polymer type and co-encapsulated additives on diphtheria (Dtxd) and tetanus (Ttxd) toxoid entrapment and stability. The co-encapsulated stabilizers influenced significantly the entrapment of Dtxd and Ttxd in PLA/PLGA MS. Typically, 5% BSA or trehalose decreased the amount of Dtxd entrapped in spray-dried MS, whereas BSA increased the entrapment in coacervated MS. Further, the entrapment of Dtxd decreased as a function of polymer hydrophobicity in spray-dried MS. Without additives, approx. 64, 43 and 16% entrapment efficiency of ELISA-reactive antigen was obtained for 14–17 kDa PLGA 50:50, PLGA 75:25 and PLA, respectively. The novel end-group stearylated l-PLAs were only processed by coacervation. Satisfactory entrapment of 30–60% Dtxd was obtained. Here, albumin was a prerequisite for toxoid encapsulation, as BSA-free formulations produced strong toxoid precipitation. Furthermore, protein burst release increased with the more hydrophobic polymers, with Dtxd, Ttxd and the co-encapsulated BSA following a similar pattern and magnitude. This investigation also revealed that the method of protein extraction from the microspheres (O/W-partition or polymer hydrolysis) as well as the analytical methods (HPLC or ELISA) strongly influenced the determined amount of encapsulated toxoid and BSA. In conclusion, the study revealed the complexity of antigen microencapsulation when using different preparation and analytical techniques, as well as different types of materials.

Keywords: Vaccines; Antigen delivery; Microspheres; Diphtheria toxoid; Tetanus toxoid; Stability; Additives; PLA; PLGA; Stearylated PLA; Loading estimation

1. Introduction

In search of a single-dose vaccine delivery system, one of the most promising approaches 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. However, 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 stabilizing 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 stabilized the toxoid during release. Further, in order to improve the immune response to tetanus toxoid, the antigen has been encapsulated as alum

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adsorbate [7]. However, the peptide growth hormone rhGH was stabilized 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 in the use of polymers with different properties. Block-co-polymerization of polyesters or polyanhydrides with hydrophilic entities such as 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.

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 subunit 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 has 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.

2. Materials and methods

2.1. 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 1. 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.

2.2. Methods

2.2.1. 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

Table 1
Polymers used for the microencapsulation of diphtheria and tetanus toxoids by spray-drying (SD) or coacervation (CO)

Polymer	Code	$\overline{M_w}$[a] (kDa)	Poly-dispersity	Transition temperatures[b] (°C)	Crystallinity[b] (%)	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[c]	PLAst1	18	1.32	52[d], 67, ~160[d]	80	CO
PLA[c]	PLAst2	10	1.27	43[d], 65, ~160[d]	70	CO

[a]Weight averaged molecular weight, $\overline{M_w}$, values were determined by GPC using PS-standards [8].
[b]Transition temperatures and crystallinity were determined by DSC.
[c]PLA end-groups were esterified with stearic chloride and stearyl alcohol.
[d]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.

Table 2
Experimental design to study the effect of co-encapsulated additives (factors A and B) on toxoid containing microspheres[a]

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

[a]The design was used for all polymers tested.

ethyl formate, as described elsewhere [27]. In some cases indicated below, ethyl formate was replaced by dichloromethane. The potential stabilizers for the toxoids, i.e. BSA and trehalose, were co-encapsulated individually or concomitantly (Table 2).

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 2, 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 mPas; Fluka, CH-Buchs), and the hardening of the coacervate droplets took place in octamethylcyclotetrasiloxane (Abil K-4; Goldschmidt, D-Essen).

2.2.2. 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 hydrolyzed 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.

Dtxd and BSA content were assayed by reverse phase HPLC (Merck-Hitachi, D-Darmstadt) on a Vydac C_4 column (4 × 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. Antigenic response of Dtxd and Ttxd was determined by ELISA as described below.

2.2.3. 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 3500 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.

2.2.4. 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 wells 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 0.1 M 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).

2.2.5. Statistical analysis

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

3. Results

3.1. 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 using a factorial design (Table 2).

3.1.1. 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 ($\alpha = 0.01$) Dtxd content in 10 out of 11 MS batches than method B (up to 40% higher, results not shown). This difference increased with increasing polymer hydrophobicity. For the coacervated MS, how-

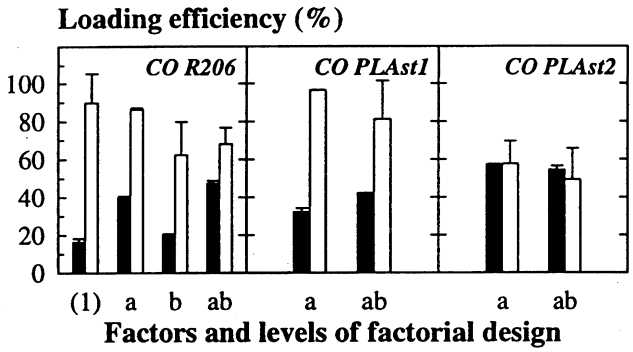

Fig. 1. Effect of extraction method on diphtheria toxoid content in three different PLA MS prepared by coacervation. Method A (extraction of protein in dichloromethane and PBS): filled bars; method B (hydrolysis of polymer in NaOH): open bars. See Table 1 for polymer specification and Table 2 for experimental design.

ever, a partly opposite behaviour was found (Fig. 1). For PLA R206 and for PLAst1 (18 kDa) MS, method B indicated significantly higher ($\alpha = 0.05$) 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.

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

Table 3 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 ($\alpha = 0.001$) with increasing lactide-to-glycolide ratio of the polymer, and a significant ($\alpha = 0.05$) 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 analyzed by HPLC were generally 5–15% higher than those determined by ELISA. Similarly, the coacervated RG502-MS contained higher ($\alpha = 0.001$) 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 ($\alpha = 0.001$) 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 with the standard PLGA 50:50 (esterified end-groups).

Table 3 further demonstrates that the additives BSA (levels a and ab) and trehalose (levels b and ab) had a significant ($\alpha = 0.001$) 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. On the opposite, in coacervation, BSA and trehalose improved slightly the Dtxd encapsulation 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

Table 3

Diphtheria toxoid loadings of selected spray-dried and coacervated PLA/PLGA microspheres according to the experimental design in Table 2[a]

Experiment[b]	Loading efficiency (%)							
Spray-dried	RG502		RG752		R202			
microspheres	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	RG502[c]		R206		PLAst1		PLAst2	
microspheres	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

[a]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.
[b]Level a: 5% BSA; level b: 15% trehalose.
[c]Microspheres prepared to examine the effect of preparation method (spray-drying vs. coacervation).

Table 4
Tetanus toxoid loading and burst release (2 days) of spray-dried PLA/PLGA microspheres according to the experimental design in Table 2[a]

Experiment	ELISA-responsive loading efficiency (%)			ELISA-responsive burst release (%)		
	RG502	RG752	R202	RG502	RG752	R202
a	33.5 ± 5.9	23.4 ± 7.1	11.5 ± 7.4	17.7 ± 2.5	53.0 ± 4.4	73.1 ± 1.6
ab	27.9 ± 5.0	14.9 ± 1.3	21.3 ± 1.3	39.8 ± 4.1	67.8 ± 3.4	70.3 ± 13.1

[a]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.

only relatively minor changes in loading efficiency as compared with BSA alone.

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 4). 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 ($\alpha = 0.001$). 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).

3.1.3. Effect of polymer solvent on Dtxd encapsulation

Microspheres made from RG752 and R202 gave 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 ($\alpha = 0.001$) 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 approximately 20 and 15% to 56 and 84%, respectively, whereas for RG752 MS, it increased from approximately 8 to 35–40% for both proteins (R202 and RG752 were studied at level a and ab, respectively).

3.1.4. Quality of BSA co-encapsulation

The additive BSA can exert a stabilizing 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).

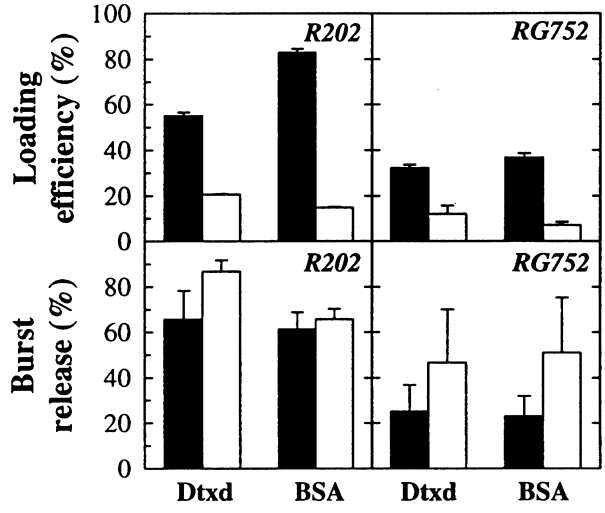

Fig. 2. Effect of polymer solvent on Dtxd and BSA content (top) and burst release (bottom) from spray-dried PLA R202 (level a) and PLGA RG752 (level ab) MS. Ethyl formate: open bars; dichloromethane: filled bars. The proteins were extracted from the MS by method A and assayed by HPLC. See Table 1 for polymer specification.

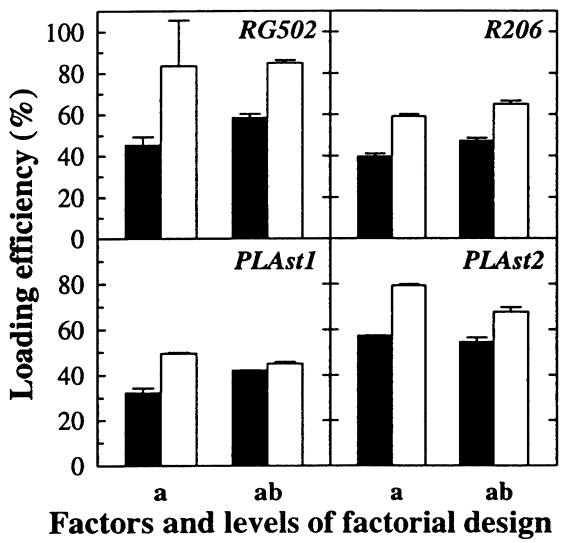

Fig. 3. Loading efficiency of encapsulated diphtheria toxoid (filled bars) and BSA (open bars) in selected coacervated MS (experimental levels a and ab according to Table 2). The proteins were extracted from the MS by method A and assayed by HPLC.

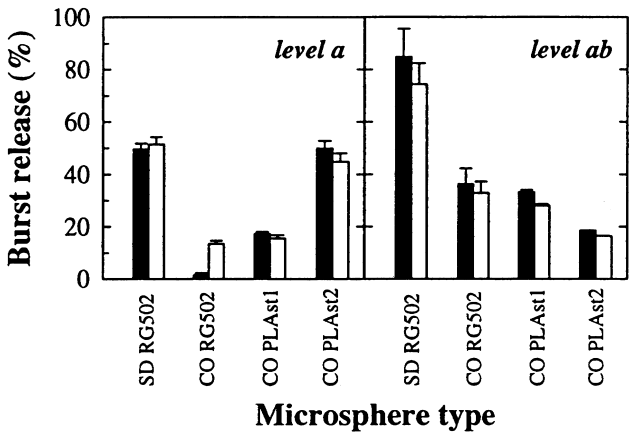

Fig. 4. Release of diphtheria toxoid from spray-dried (SD) and coacervated (CO) PLGA RG502 PLAst1 and PLAst2 MS after 2 days (filled bars) and 30 days (open bars). (Left) Experiments at level a (co-encapsulated BSA). (Right) Experiments at level ab (co-encapsulated BSA and trehalose). Release was determined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

For spray-dried MS, there were only minor differences in BSA and Dtxd loading efficiencies (results not shown).

3.2. 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 < 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 ($\alpha = 0.001$). 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.

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 ($\alpha = 0.001$). Furthermore, the release of Dtxd from R202 MS (65–85%) was significantly higher ($\alpha = 0.001$) 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 ($\alpha = 0.01$) (Table 4). In addition, the burst release was higher ($\alpha = 0.005$) when both trehalose and BSA were co-encapsulated (level ab) than when only BSA was co-encapsulated (level a).

4. 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 < 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 (Table 3 and Table 4). However, the present investigation only allows us to speculate on the mechanisms behind this. Thanks to 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 with 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. However, 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, approximately 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 approximately 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 stabilizing 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]. However, 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. Firstly, 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 solubilize 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 coencapsulated 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 (D. Sesardic and A Sasiak, personal communication) with Ttxd MS containing stabilizing 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.

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References

[1] M.T. Aguado, P.-H. Lambert, Controlled-release vaccines – Biodegradable polylactide/polyglycolide (PL/PG) microspheres as antigen vehicles, Immunobiology 184 (1992) 113–115.
[2] C.R. Howard, A.J. Beale, M.T. Aguado, The development of controlled release vaccines – the role of the world health organization, in: B. Gander, H.P. Merkle, G. Corradin (Eds.), Antigen delivery systems, Harwood Academic, Amsterdam, 1998, pp. 1–14.
[3] M.J. Alonso, R.K. Gupta, C. Min, G.R. Siber, R. Langer, Biodegradable microspheres as controlled-release tetanus toxoid delivery systems, Vaccine 12 (1994) 299–306.
[4] J.L. Cleland, A.L. Lim, L. Barron, E.T. Duenas, M.F. Powell, Development of a single-shot subunit vaccine for HIV-I: part 4. Optimizing microencapsulation and pulsatile release of MN rgp120 from biodegradable microspheres, J. Control. Rel. 47 (1997) 135–150.
[5] M. Singh, X.M. Li, H. Wang, J.P. McGee, T. Zamb, W. Koff, W. Chang Yi, D.T. O'Hagan, Immunogenicity and protection in small-animal models with controlled release tetanus toxoid microparticles as a single-dose vaccine, Infect. Immun. 65 (1997) 1716–1721.
[6] W. Lu, T.G. Park, Protein release from poly(lactic-co-glycolic acid) microspheres: protein stability problems, J. Pharm. Sci. Tech. 49 (1995) 13–19.
[7] I. Esparza, T. Kissel, Parameters affecting the immunogenity of microencapsulated tetanus toxoid, Vaccine 10 (1992) 714–720.
[8] C. Thomasin, G. Corradin, Y. Men, H.P. Merkle, B. Gander, Tetanus toxoid and synthetic malaria antigen containing poly(lactide)/poly(-lactide-co-glycolide) microspheres: importance of polymer degradation, J. Control. Rel. 41 (1996) 131–145.
[9] L. Youxin, C. Volland, T. Kissel, In-vitro degradation and bovine serum albumin release of the ABA triblock copolymers consisting of poly(L(+)lactic acid), or poly(L(+)lactic acid-co-glycolic acid) A-blocks attached to central polyoxyethylene B-blocks, J. Control. Rel. 32 (1994) 121–128.
[10] S.P. Schwendeman, H.R. Costantino, R.K. Gupta, Stabilization of tetanus and diphtheria toxoids against moisture-induced aggregation, Proc. Natl. Acad. Sci. USA. 92 (1995) 11234–11238.
[11] A.-C. Chang, R.K. Gupta, Stabilization of tetanus toxoid in poly(dl-lactic-co-glycolic acid) microspheres for the controlled release of antigen, J. Pharm. Sci. 85 (1996) 129–132.
[12] M. Morlock, H. Koll, G. Winter, T. Kissel, Microencapsulation of rh-erytropoietin, using biodegradable poly(D,L- lactide-co-glycolide): protein stability and the effects of stabilizing excipients, Eur. J. Pharm. Biopharm. 43 (1997) 29–36.
[13] P. Johansen, Y. Men, R. Audran, G. Corradin, H.P. Merkle, B. Gander, Improving stability and release kinetics of microencapsulated tetanus toxoid by co-encapsulation of additives, Pharm. Res. 15 (1998) 1103–1110.
[14] O.L. Johnson, J.L. Cleland, H.J. Lee, M. Charnis, E. Duenas, W. Jaworowicz, D. Shepard, A. Shahzamani, A.J. Jones, S.D. Putney, A month-long effect from a single injection of microencapsulated human growth hormone, Nature Med. 2 (1996) 795–799.
[15] O.L. Johnson, W. Jaworowicz, J.L. Cleland, L. Bailey, M. Charnis, E. Duenas, C. Wu, D. Shepard, S. Magil, T. Last, A.J. Jones, S.D. Putney, The stabilization and encapsulation of human growth hormone into biodegradable microspheres, Pharm. Res. 14 (1997) 730–735.
[16] B. Jeong, Y.H. Bae, D.S. Lee, S.W. Kim, Biodegradable block copolymers as injectable drug delivery systems, Nature 388 (1997) 860–862.
[17] M. Chiba, J. Hanes, R. Langer, Controlled protein delivery from biodegradable tyrosine-containing poly(anhydride-co-imide) microspheres, Biomaterials 18 (1997) 893–901.
[18] B.R. Conway, J.E. Eyles, H.O. Alpar, Immune response to antigen in microspheres of different polymers, Proc Int. Symp. Control. Rel. Bioactive Mater. 23 (1996) 335–336.
[19] Y. Tabata, Y. Ikada, Phagocytosis of polymer microspheres by macrophages. Adv. Polym. Sci., 94 (1994) 107–141.
[20] C. Yan, J.H. Resau, J. Hewetson, M. West, W.L. Rill, M. Kende, Characterization and morphological analysis of protein-loaded poly(lactide-co-glycolide) microparticles prepared by water-in-oil-in-water emulsion technique, J. Control. Rel. 32 (1994) 231–241.
[21] T. Uchida, K. Yoshida, A. Ninomiya, S. Goto, Optimization of prepeparative conditions for polylactide microspheres containing ovalbumin, Chem. Pharm. Bull. 43 (1995) 1569–1573.
[22] Y. Men, B. Gander, H.P. Merkle, G. Corradin, Induction of sustained and elevated immune responses to weakly immunogenic synthetic malarial peptides by encapsulation in biodegradable polymer microspheres, Vaccine 14 (1996) 1442–1450.
[23] Y. Men, H. Tamber, R. Audran, B. Gander, G. Corradin, Induction of a cytotoxic T lymphocyte response by immunization with a malaria specific CTL peptide entrapped in biodegradable polymer microspheres, Vaccine 15 (1997) 1405–1412.
[24] M. Singh, A. Singh, G.P. Talwar, Controlled delivery of diphteria toxoid using biodegradable poly(d,l-lactide) microcapsules, Pharm Res. 8 (1991) 958–961.
[25] M. Singh, O. Singh, A. Singh, G.P. Talwar, Immunogenicity studies on diphtheria toxoid loaded biodegradable microspheres, Int. J. Pharm. 85 (1992) R5.
[26] P.H. Lambert, Research priorities for vaccines to be used at a global level, Behring Inst. Mitteilungen 95 (1994) 1–6.
[27] B. Gander, P. Johansen, HoNam-Trâî, , H.P. Merkle, Thermodynamic approach to protein microencapsulation into poly(d,l-lactide) by spray drying, Int. J. Pharm. 129 (1996) 51–61.
[28] C. Thomasin, P. Johansen, R. Alder, R. Bemsel, G. Hottinger, H. Altorfer, A.D. Wright, E. Wehrli, H.P. Merkle, B. Gander, A contribution to overcoming the problem of residual solvents in biodegradable microspheres prepared by coacervation, Eur. J. Pharm. Biopharm. 42 (1996) 16–24.
[29] B.R. Bloom, Vaccines for the third world, Nature 342 (1989) 115–120.
[30] Y. Men, C. Thomasin, H.P. Merkle, B. Gander, G. Corradin, A single administration of tetanus toxoid in biodegradable microspheres eli-

cits T cell and antibody responses similar or superior to those obtained with aluminium hydroxide, Vaccine 13 (1995) 683–689.
[31] J.H. Eldridge, J.K. Staas, J.A. Meulbroek, J. R McGhee, T.R. Tice, R.M. Gilley, Biodegradable microspheres as a vaccine delivery system, Mol. Immunol 28 (1991) 287–294.
[32] P. Johansen, H.P. Merkle, B. Gander, Physico-chemical and antigenic properties of tetanus and diphtheria toxoids and steps towards improved stability, Biochim. Biophys. Acta 1425 (1998) 425–436.
[33] N. Nihant, C. Schugens, C. Grandfils, R. Jerome, P. Teyssie, Polylactide microparticles prepared by double emulsion/evaporation technique. I. Effect of primary emulsion stability, Pharm. Res. 11 (1994) 1479–1484.
[34] R.K. Gupta, A.C. Chang, P. Griffin, R. Rivera, Y.Y. Guo, G.R. Siber, Determination of protein loading in biodegradable polymer microspheres containing tetanus toxoid, Vaccine 15 (1997) 672–678.
[35] S. Sharif, D.T. O'Hagan, A comparison of alternative methods for the determination of the levels of proteins entrapped in poly(lactide-co-glycolide) microspheres, Int. J. Pharm. 115 (1995) 259–263.
[36] D.K. Xing, K. McLellan, M.J. Corbel, D. Sesardic, Estimation of antigenic tetanus toxoid extracted from biodegradable microspheres, Biologicals 24 (1996) 57–65.
[37] R. Audran, Y. Men, P. Johansen, B. Gander, G. Corradin, Enhanced immunogenicity of microencapsulated tetanus toxoid with stabilizing agents, Pharm. Res. 15 (1998) 1111–1116.
[38] P. Johansen, G. Corradin, H.P. Merkle, B. Gander, Release of tetanus toxoid from adjuvants and PLGA microspheres: how experimental set-up and surface adsorption fool the pattern, J. Control. Rel. 56 (1998) 209–217.