Improving Stability and Release Kinetics of Microencapsulated Tetanus Toxoid by Co-Encapsulation of Additives[1]

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Purpose. Tetanus toxoid (Ttxd) encapsulated in polyester microspheres (MS) for single injection immunization 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. This study examined the effect of co-encapsulated putative stabilizing additives.

Methods. Two different Ttxd were encapsulated in poly(D,L-lactic-co-glycolic acid) (PLGA 50:50) and poly(D,L-lactic acid) (PLA) MS by spray-drying. The influence of co-encapsulated additives on toxoid stability, loading in and release from the MS, was studied by fluorimetry and ELISA.

Results. Co-encapsulated albumin, trehalose and γ-hydroxypropyl cyclodextrin all improved the toxoid encapsulation efficiency in PLGA 50:50 MS. Albumin increased the encapsulation efficiency of antigenic Ttxd by one to two orders of magnitude. Further, with albumin or a mixture of albumin and trehalose ELISA responsive Ttxd was released over 1–2 months following a pulsatile pattern.

Conclusions. Optimized Ttxd containing MS may be valuable for a single-dose vaccine delivery system.

KEY WORDS: antigen delivery; PLGA microspheres; tetanus toxoid; antigenic stability; stabilizing additives.

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 determined 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 stabilizing additives (19,20), although protein stabilization is very common in other pharmaceutical dosage forms (21,22). These studies have shown that additives, including proteins, lipids, polyols, and surfactants, may stabilize biologically active proteins.

Here, we report on attempts to enhance encapsulation efficiency, preserve antigenicity, and optimize the release pattern of Ttxd from PLGA and PLA MS (see also associated paper, Audran et al.[1]). Specifically, the effect of co-encapsulating various agents known for their protein stabilizing properties were investigated: (i) trehalose, serum albumin and cyclodextrins as commonly known stabilizers 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 stabilizers albumin and trehalose may clinically be of 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

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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 Fluka, CH-Buchs.

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, HSA 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, albumin, and calcium carbonate was dispersed in 5% (w/w) PLA in dichloromethane. Coacervation was induced by adding silicone oil (1070 mPas; 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 fluorimetrically, 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 analyzed by fluorimetry or 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 analyzed fluorimetrically using excitation and emission wavelengths of 279 nm 329 nm, respectively (Fluromax™, Spex, Edison, NJ). When albumin 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 effect 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 optimize *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 stabilizing 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 stabilization, 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. The lower loadings were used to screen the effect of the various additives and the higher one to optimize 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 favorably.

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% 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.

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-

Table I. Tetanus Toxoid Loadings of Selected PLGA 50:50 (Resomer® RG502) Microsphere Preparations as Measured by Fluorimetry and by ELISA

Additive		Determined toxoid loading (Lf/mg)[b]		Encapsulation efficiency (%)	
Type	Content (%)[a]	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[c]	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.

Note: n.d.: not determined.
[a] Nominal content relative to microsphere mass (w/w).
[b] Loading per mg microspheres.
[c] HSA was used instead of BSA in view of using most promising preparations for clinical trials.

Table II. Tetanus Toxoid Loadings of Microsphere Preparations Made with a Low MW Hydrophilic PLGA 50:50 Analogue (Resomer® 502H) and with a High MW PLA (Resomer® 206)

Polymer	Additive: Type	Additive: Content (%)[a]	Determined toxoid loading (Lf/mg)[b]	Encapsulation efficiency (%)
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[c,d]	10/5	0.57	27.5

Note: Toxoid content was assayed by ELISA.
[a] Theoretical content relative to microsphere mass (w/w).
[b] Loading per mg microspheres.
[c] PLA MS were prepared by coacervation.
[d] HSA was used instead of BSA in view of using most promising preparations for clinical trials.

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 for assaying. 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.

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 through 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

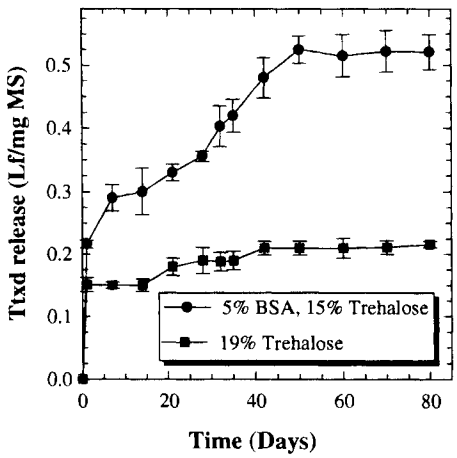

Fig. 1. Release of PSTtdx-20 from BSA and trehalose stabilized PLGA 50:50 MS as measured by ELISA. Nominal loading was 3.5 Lf/mg MS.

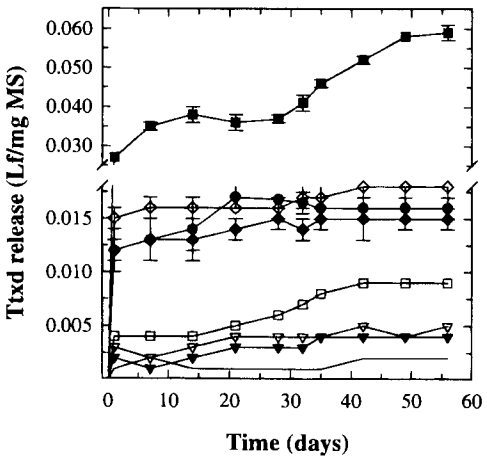

Fig. 2. Release of PSTtdx-20 from PLGA 50:50 MS with various stabilizers as measured with ELISA. Nominal loading of tetanus toxoid was 0.7 Lf/mg MS. (■: BSA 5%; □: BSA 1%; ▼: $CaCO_3$ 15%; ▽: $Ca_3(PO_4)_2$ 15%; ●: γ-HPCD 19%; ◆ Poloxamer L101 19%; ◇: trehalose 19%; continuous line: no additive).

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, as 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

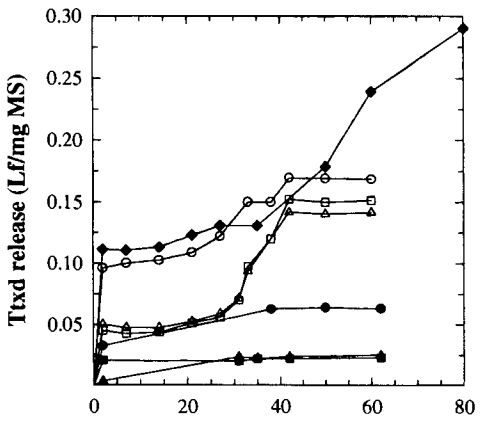

Fig. 3. Release of PTC from PLGA 50:50 MS as assayed by ELISA (filled symbols) and fluorimetry (open symbols). Nominal loadings were approx. 0.4 Lf/mg for the $CaCO_3$ containing MS and 0.9 Lf/mg for the other MS preparations. (▲, △: $CaCO_3$, 15%; ■, □: poloxamer L101 19%; ●, ○: no additive; ◆: BSA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

responsive burst release of 35–50% of the total dose was observed after 24 h. This release behavior 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.

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 behavior 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.

pH Neutralizing Capacity

The pH-stabilizing 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

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 Microspheres (MS) After 80 Days

Additive in MS	Tetanus toxoid release (%)		Protein residues in MS after 80 days (%)	
	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% plus BSA5%	22.5 ± 0.6	53.3 ± 2.9	0.56 ± 0.06	6.7 ± 0.5

Note: 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. The preparations are not identical with those in Table I.

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 stabilizing effect on protein conformation and activity in aqueous solution (21,22) or which we assumed to exert such an effect. The here presented *in vitro* results demonstrate the stabilizing effect of some of the selected additive on the encapsulated antigen. Most

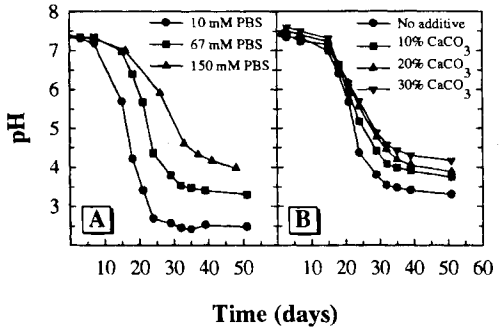

Fig. 4. Effect of buffer capacity of the release medium (A) and amount of $CaCO_3$ in 67 mM PBS (B) on pH as a function of time after incubation at 37°C.

importantly, the data were confirmed in immunization experiments in mice (see associated paper, Audran et al.[1]) and guinea pigs (Sesardic et al., personal communication; independent testing). In the latter study, some stabilized formulations induced protective antibody titers over 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 stabilizer 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, 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 of 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 concommitantly interacts with the PLGA through Van der Waals and H-bonding forces. 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 loadings, although it has been demonstrated that D_2O could exert an important stabilizing 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, Pluronic® 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 stabilize 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 stabilizing effect of 0.4% HSA and 0.2% gelatine on Ttxd in MS have been reported (19). In general, immobilization 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 to 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 stabilize best 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-catalyzed chemical reactions such as proteolysis or Schiff base formation should be reduced for Ttxd.

CONCLUSIONS

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. 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 immunization experiments in mice (see associated paper, Audran et al.[1]) and guinea pigs (Sesardic et al., personal communication; independent testing).

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