Physico-chemical and antigenic properties of tetanus and diphtheria toxoids and steps towards improved stability

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Abstract

Physico-chemical, antigenic and immunogenic properties may be altered during microencapsulation of antigens and their release from poly(lactic acid) and poly(lactic-co-glycolic acid) microspheres. Here, the physico-chemical, conformational and antigenic stability of tetanus and diphtheria toxoids 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, circular dichroism spectroscopy, turbidimetry, electrophoresis and ELISA. Elevated temperatures altered the physico-chemical and antigenic properties of the toxoids to a greater extent than the acids (50 mM) did. 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 tetanus toxoid 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. This qualifies albumin as potential stabilising additive for toxoid loaded poly(lactic acid) and poly(lactic-co-glycolic acid) microspheres. © 1998 Elsevier Science B.V. All rights reserved.

Keywords: Tetanus toxoid; Diphtheria toxoid; Antigenic stability; Conformational stability; Stabilizing additive

1. 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 (Tttxd, Dttxd) have been studied for use in single injection vaccine deliv-
ery systems [7–11]. Only a 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 disulfide 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 well elucidated [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.

2. Materials and methods

2.1. Materials

Aqueous solutions of tetanus (Ttxd) and diphtheria (Dtxd) toxoid, provided by WHO, Geneva, Switzerland, were from Massachusetts Public Health Biological Laboratories, Boston, MA, USA (Ttxd lot No. PSTtxd-20, 1400 Lf/ml, 5.2 mg/ml protein) and from Pasteur Mérieux, Lyon, France (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 immunoenzyme assay was from Fluka, Buchs, Switzerland. Anti-tetanus antibodies were from Wellcome Biotechnology, Beckenham, UK. Rabbit anti-guinea pig IgG horseradish peroxidase conjugate and 2,2'-azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) were from Sigma Chemical, St. Louis, MO, USA. Unless specified otherwise, all other substances used were of pharmaceutical or analytical grade and were purchased from commercial suppliers.

2.2. 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 mass and aggregation, and finally, enzyme-linked immunosorbent assay (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 LI/ml toxoid) in 67 mM PBS at pH 7.4 were incubated with 0.5 M Gdm-Cl at 37°C for 60 min and analysed fluorimetrically, 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 2³ factorial design (Table 1), with the factors temperature (A; 4 and 60°C) and presence of lactic acid (B) and glycolic acid (C) (0 and 50 mM) in the toxoid solution. Typically, 2 ml of 100 μg/ml toxoid (equivalent to 30 LI/ml) in 67 mM PBS at pH 7.4 for 60 min and analysed fluorimetrically, as specified below. This experiment served as reference for acid and temperature induced conformational changes of the toxoids.

Fluorescence and ELISA responses of 0.1 mg/ml (30 LI/ml) toxoid solutions in PBS contained in borosilicate vials were measured after 7 and 28 days (n = 3).

Table 1
Experimental design for the stability study of tetanus (PTC and PSTtxd-20) and diphtheria toxoids

<table>
<thead>
<tr>
<th>Experimental level</th>
<th>Factor A: temperature (°C)</th>
<th>Factor B: lactic acid (mM)</th>
<th>Factor C: glycolic acid (mM)</th>
<th>pH Ttxd</th>
<th>pH Dtxd</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>4</td>
<td>0</td>
<td>0</td>
<td>7.3</td>
<td>7.4</td>
</tr>
<tr>
<td>a</td>
<td>60</td>
<td>0</td>
<td>0</td>
<td>7.3</td>
<td>7.4</td>
</tr>
<tr>
<td>b</td>
<td>4</td>
<td>50</td>
<td>0</td>
<td>6.0</td>
<td>6.3</td>
</tr>
<tr>
<td>ab</td>
<td>60</td>
<td>50</td>
<td>0</td>
<td>5.2</td>
<td>5.9</td>
</tr>
<tr>
<td>c</td>
<td>4</td>
<td>0</td>
<td>50</td>
<td>5.2</td>
<td>5.8</td>
</tr>
<tr>
<td>ac</td>
<td>60</td>
<td>0</td>
<td>50</td>
<td>5.2</td>
<td>5.8</td>
</tr>
<tr>
<td>bc</td>
<td>4</td>
<td>50</td>
<td>50</td>
<td>3.9</td>
<td>4.1–3.9*</td>
</tr>
<tr>
<td>abc</td>
<td>60</td>
<td>50</td>
<td>50</td>
<td>3.9–3.7*</td>
<td>4.1–3.8*</td>
</tr>
</tbody>
</table>

*The pH range describes a drop over time upon incubation.

In a second 2³ design (Table 2), the ELISA antigenicity of 30 μg/ml Ttxd was studied at 37°C as a function of lactic acid (A) or glycolic acid (B) (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.

2.3. Methods

2.3.1. Fluorescence spectroscopy

Protein content and structural changes were monitored at 37°C by fluorimetry (Fluoromax, Spex, Edison, NJ, USA) using different excitation and emis-

Table 2
Experimental design for the stability study of tetanus toxoid (PTC and PSTtxd-20)

<table>
<thead>
<tr>
<th>Experimental level</th>
<th>Factor A: lactic acid (mM)</th>
<th>Factor B: glycolic acid (mM)</th>
<th>Factor C: albumin (mg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(1)</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>a</td>
<td>15</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>b</td>
<td>0</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>ab</td>
<td>15</td>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>c</td>
<td>0</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>ac</td>
<td>15</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>bc</td>
<td>0</td>
<td>15</td>
<td>5</td>
</tr>
<tr>
<td>abc</td>
<td>15</td>
<td>15</td>
<td>5</td>
</tr>
</tbody>
</table>

Antigen solutions of 30 μg/ml (10 LI/ml) were incubated at 37°C in borosilicate vials, and ELISA response was measured after 1, 7 and 14 days (n = 3).
sion wavelengths. Toxoid solutions were excited ($\lambda_{ex}$) at 280 nm or 295 nm; with $\lambda_{ex} = 295$ 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 ($\lambda_{max}$) and $I_{350}/I_{329}$ are sensitive measures for conformational changes [20].

2.3.2. ELISA of tetanus toxoid

Flat-bottomed 96-well microtiter plates (Nunc-Immuo Plate Maxisorb, Nunc, Roskilde, Denmark) were filled with 100 $\mu$L of 1 $\mu$g/ml monoclonal anti-tetanus IgG (TT010) in 0.05 M carbonate buffer (pH 9.6), and incubated at 4°C overnight. The plates were washed three times with 300 $\mu$L of 0.05% Tween-20 in PBS (pH 7.4) (PBST) after each incubation step. After 1 h of incubation at 37°C with 150 $\mu$L 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. 25 $\mu$g/ml guinea-pig IgG (25 $\mu$g/ml) was added to each well in 100 $\mu$L of PBSTM, and plates were incubated for another 2 h. Then, rabbit anti-guinea-pig peroxidase conjugate (1/8000 dilution) in 100 $\mu$L PBSTM was added to each well, and plates incubated for further 1 h. Finally, 100 $\mu$L of 0.5 mg/ml peroxidase substrate 2,2'azino-bis(3-ethylbenz-thiazoline-6-sulfonic acid) in 0.05 M citric acid (pH 4.0) was added to the wells, and the end-point optical density measured at 405 nm (Thermomax, Molecular Devices, Menlo Park, CA, USA) after 30 min incubation at room temperature.

2.3.3. 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 $\mu$g/ml toxoid in a 10 mM saline free phosphate buffer (PB) at pH 7.4 were analysed (250–190 nm, Jasco J-720, Japan Spectroscopic, Tokyo, Japan) at regular intervals over 4 weeks. At the end of the study period, the samples were dialysed (10 kDa cutoff) 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°C to 95°C at a rate of 30°C/h and recording the change of specific elipticity at 222 and 288 nm.

2.3.4. Gel electrophoresis

Changes in conformation and molecular mass of Ttxd (200 $\mu$g/ml) upon exposure to lactic or glycolic acid (5–100 mM) were investigated by native gel electrophoresis with 50 mm polyacrylamide (PAGE) of gradient 10–15% (PhastGel, Pharmacia, Uppsala, Sweden). The molecular mass was determined against broad range (6.5–200 kDa) PAGE standards from BioRad (Glattbrugg, Switzerland), and the gels were developed with silver staining.

2.3.5. Turbidimetry

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

3. Results

3.1. Fluorimetric changes of toxoids upon Gdm-Cl treatment

Solutions of 30 $\mu$g/ml of Ttxd and Dtxd were tested with Gdm-Cl to establish a fluorimetric control for conformational unfolding. The solutions were excited ($\lambda_{ex}$) 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 $\lambda_{ex} = 280$ nm, the shift in emission maximum ($\lambda_{max}$) 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 $\lambda_{ex} = 295$. 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.

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

3.2.1. Fluorimetric properties

In analogy 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°C or 60°C and 0 or 50 mM lactic or glycolic acid (see experimental design in Table 1), 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 change of toxoid solutions exposed to elevated temperatures or lactic and glycolic acids.
tional 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 \( \lambda_{ex} = 280 \text{ nm} \) 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).

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 \( (\lambda_{ex} = 295 \text{ nm}; \text{Fig. 3B}) \). 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 \( \lambda_{em} = 329 \text{ nm} \)) 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

**Table 3**

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 of incubation

<table>
<thead>
<tr>
<th>Experimental level</th>
<th>( \lambda_{ex} = 280 \text{ nm} )</th>
<th>( \lambda_{ex} = 295 \text{ nm} )</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PTC</td>
<td>PSTtxd-20</td>
</tr>
<tr>
<td>(1)</td>
<td>1.7</td>
<td>13.0</td>
</tr>
<tr>
<td>a</td>
<td>15.0</td>
<td>80.1</td>
</tr>
<tr>
<td>b</td>
<td>1.7</td>
<td>20.6</td>
</tr>
<tr>
<td>ab</td>
<td>12.9</td>
<td>354.0</td>
</tr>
<tr>
<td>c</td>
<td>1.7</td>
<td>25.5</td>
</tr>
<tr>
<td>ac</td>
<td>16.8</td>
<td>354.0</td>
</tr>
<tr>
<td>bc</td>
<td>1.8</td>
<td>34.3</td>
</tr>
<tr>
<td>abc</td>
<td>9.4</td>
<td>174.0</td>
</tr>
</tbody>
</table>

\( I_{450}/I_{329} \) was used as a measure of chemical stability of Trp. Experimental conditions are given in Table 1.
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 3 shows the results for Ttxd and Dtxd after 21 days of 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.

3.2.2. 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 of 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 3 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.

3.2.3. CD properties

Conformational stability of Ttxd solution was further studied by far UV CD (results not shown). In PB, the spectra indicated significant $\alpha$-helical conformation. After 7 days of 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 $\beta$-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.

3.2.4. 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 $\mu$g/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 a weak band at 200 kDa appeared, suggesting a dimerisation between Ttxd and BSA. When the higher concentration of PTC (250 $\mu$g/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.

3.3. 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 1. 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 a2) led to almost complete loss of antigenicity for both Ttxd grades. At 37°C (level a1), 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 encouragingly, 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.

3.4. 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 $\lambda_{max}$ 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, $\lambda_{\text{max}}$ 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 of incubation at 60°C was not reversible by pH neutralisation or dialysis of acidic residues.

4. 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 physicochemical 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 these 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 $\lambda_{\text{max}}$ 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 $\lambda_{\text{max}}$. Conversely, $\lambda_{\text{max}}$ of Ttxd exposed to pH 2.5 at 37°C remained unchanged over 1 week, but decreased slightly after 4 and 7 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]. This 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 ($pK_a = 3.9$). Asp–Pro bonds especially 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
ELISA-antigenicity occurred rapidly upon contact

of proteins in hydrochloric acid at 60°C can be interpreted as 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 $\lambda_{ex} = 295$ 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 7 days of incubation at 60°C, with slight loss of $\alpha$-helical and slight increase of $\beta$-sheet conformation. This is in agreement with similar FTIR investigations on Ttxd stability [26]. It has been observed that $\alpha$-helices may undergo structural transitions in acidic solutions and at elevated temperatures, whereas $\beta$-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 prevents unfolding 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 $\lambda_{max}$ of above 400 nm, and indole derivatives such as kynurenine emit light near 450 nm when excited at 300 nm [30,31]. Finally, formaldehyde may be released during acidic hydrolysis of formalised proteins [36]. Candidate functional groups for reversible reaction with formaldehyde are hydroxymethylamine, aminal [37], and Lys [38]. The reactive intermediate imine group (R1$^-$NH$^+\text{-CH}_2$) 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, and it becomes less accessible for excitation. However, the present investigations 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 of 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 im-
portant; 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.

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References


