Physicochemical and immunochemical techniques predict the quality of diphtheria toxoid vaccines

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Abstract

The most critical step in the production of diphtheria vaccines is the inactivation of the toxin by formaldehyde. Diphtheria toxoid (DTx) is produced during this inactivation process through partly unknown, chemical modifications of the toxin. Consequently, diphtheria vaccines are difficult to characterise completely and the quality of the toxoids is routinely determined with potency and safety tests. This article describes the possibility of monitoring the quality in diphtheria vaccine production with a selection of physicochemical and immunochemical tests as an alternative to established in vivo tests. To this end, diphtheria toxin was treated with increasing formaldehyde concentrations resulting in toxoid products varying in potency and residual toxicity. Differences in the quality of the experimental toxoids were also assessed with physicochemical and immunochemical techniques. The results obtained with several of these analyses, including SDS-PAGE, primary amino group determination, fluorescence spectroscopy, circular dichroism (CD) and biosensor analysis, showed a clear correlation with the potency and safety tests. A set of criteria is proposed that a diphtheria toxoid must comply with, i.e. an apparent shift of the B-fragment on SDS-PAGE, a reduction of primary amino groups in a diphtheria molecule, an increased resistance to denaturation, an increased circular dichroism signal in the near-UV region and a reduced binding to selected monoclonal antibodies. In principle, a selected set of in vitro analyses can replace the classical in vivo tests to evaluate the quality of diphtheria toxoid vaccines, provided that the validity of these tests is demonstrated in extensive validation studies and regulatory acceptance is obtained.
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1. Introduction

Diphtheria toxoid (DTx) is one of the most successful vaccines and has eliminated diphtheria in many countries. It is prepared from diphtheria toxin, which causes the clinical manifestations of the disease in man [1]. The toxin is converted by formaldehyde into a non-toxic but still immunogenic diphtheria toxoid. This method has already been described by Ramon [2] and, Glenny and Hopkins [3] and in the 1920s. The classical vaccine is produced via a number of steps: cultivation of *Corynebacterium diphtheriae* and clarification of the toxin-containing medium, followed by concentration and inactivation of the toxin, purification of the toxoid through diafiltration, and adsorption to an aluminium salt. The quality of diphtheria toxoid depends mainly on the detoxification process, in which reaction conditions are very important such as formaldehyde concentration, reaction time and temperature, and composition of the matrix. In many cases, the matrix is not chemically defined and is essentially the same as the culture supernatant, which contains non-specified amino acids, peptides and proteins. Some producers use a defined matrix, which consist of a glycine or a lysine solution. During inactivation, formaldehyde reacts first with amino groups; in a second step, cross-links are formed between the reaction product and several other amino acids [4–6]. Thereby, formaldehyde forms intramolecular and intermolecular cross-links. However, the nature of the modifications in the toxoid as well as the location of the modification sites is largely unknown.

The present quality control of diphtheria toxoid is based on an immunogenicity and safety test in animals [7–10]. However, most international regulations allow the use of alternative test methods for the quality control of vaccines [11]. Importantly, the alternative assay should not pass a

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product that fails in the routine quality test. Currently, no functional in vitro tests exist as an alternative for the potency determination of diphtheria toxoids, because it is very difficult to mimic a complex immune response [12]. Another concept for batch release is based on a consistent production process where the vaccine batches predominantly have identical properties [13–15]. This is common practise for well-defined biologicals, e.g. hepatitis B vaccine [16]. In principle, also the potency of a newly produced toxoid can be predicted, if it can be demonstrated that the new product is indistinguishable from a reference toxoid with a proven potency. Immunochemical and physicochemical techniques are instruments to study vaccine properties, such as identity, size, structure, purity, amino acid modifications and antigenicity. The combination of results can verify that vaccines have identical properties and are consistently produced.

The aim of the present study was to investigate the applicability of physicochemical and immunochemical techniques as quality predictors of diphtheria toxoid. In particular, SDS-PAGE, a primary amino group assay, fluorescence spectroscopy, circular dichroism (CD) spectroscopy and biosensor analysis were used to characterise similarities and differences between a set of experimental diphtheria toxoids. Based on the results, we propose to use a selection of these assays to predict the quality of diphtheria toxoid vaccines.

2. Materials and methods

2.1. Biochemical and immunochemicals

Diphtheria toxin-containing culture fluid (clarified and concentrated) was obtained from the production department of The Netherlands Vaccine Institute (NVI). Monoclonal anti-diphtheria toxin antibodies Dim 5, Dim 25, Dim 27 and Dim 33 were obtained from the Laboratory for Clinical Vaccines of the NVI. Horse anti-diphtheria toxoid serum, horse anti-diphtheria peroxidase conjugate, diphtheria toxin DTa 79/1 and diphtheria toxoid DTa 93/1 were obtained from the Laboratory for the Control of Biological Products of the NVI.

2.2. Preparation of diphtheria toxoids

Before the inactivation of diphtheria toxin, the toxin-containing culture fluid was extensively dialysed (MWCO: 10–12 kDa) against phosphate buffered saline (PBS); 0.15 M NaCl, 7.7 mM Na_2HPO_4 and 2.3 mM NaH_2PO_4, pH 7.2) to remove medium components of low-molecular weight, such as amino acids and peptides. After dialysis, the toxin was filter sterilised (0.22 μm) and the protein concentration was determined to be 3.0 mg/ml by the BCA protein assay (Pierce) [17]. The antigenicity was 900 Lf/ml as measured by the Ramon flocculation test [18]. For the production of a series of experimental diphtheria toxoids (Table 1), a glycine

Table 1
List of experimental toxoids[a] prepared by adding various concentrations formaldehyde and glycine to diphtheria toxin.

Name	Formaldehyde and glycine (mM)
DTx	–
DTd1	1
DTd2	2
DTd3	4
DTd4	8
DTd5	16
DTd6	32
DTd7	48
DTd8	64
DTd9	80
DTd10	128

[a] Toxoids were prepared from diphtheria toxin DTx with a final concentration of 2.6 mg/ml (ca. 785 Lf/ml). See Section 2 for further details.

solution of 2.0 M was added to dialysed toxin to a final concentration of 1, 2, 4, 8, 16, 32, 48, 64, 80 or 128 mM. To start the inactivation reaction, a diluted formaldehyde solution (Merck) of 2.0 M was added to a final concentration equimolar to that of glycine (Merck). A certain amount of PBS was added to the toxoids to obtain a protein concentration of 2.6 mg/ml. The mixtures were incubated for 6 weeks at 35 °C. Diphtheria toxin DTx (2.6 mg/ml) was also incubated for 6 weeks at 35 °C and used as a control. The flocculation titre of the toxin and toxoids was measured and ranged between 750 and 825 Lf/ml. The toxoids were stored at 4 °C prior to analysis.

2.3. SDS-PAGE and immunoblotting

SDS-PAGE under reducing conditions and subsequent immunoblot analyses were performed as described [19]. For reduction of the disulphide bridges, 2 μg protein was diluted in the sample buffer (60 mM Tris, 70 mM SDS, 0.1 M dithiothreitol, 0.1 mM tetrabromophenol blue and 35% glycerol diluted in water) to a volume of 20 μl and boiled for 10 min. The samples of 20 μl were loaded onto 10% SDS-PAGE gels and electrophoretically separated. SDS-PAGE molecular weight (broad range; Bio-Rad) were used for calibration. Protein bands were visualised by using Coomassie brilliant blue or via immunoblotting. The gels were scanned and the intensity of protein bands was quantified by using the program Phoretix 1D quantifier (Phoretix International, UK).

For immunoblotting, proteins were transferred to a 0.45 μm nitrocellulose membrane (Bio-Rad) by using a semi-dry electroblot system (Ancos). The protein blots were incubated by shaking for 1 h with anti-diphtheria toxin antibodies Dim 5 or Dim 25 (1:1000) dissolved in buffer (0.15 M NaCl and 10 mM tris(hydroxymethyl) aminomethane), blocked by incubation for 5 min with 0.5% Protifar (Nutricia) diluted in buffer, and treated for 1 h with horse anti-mouse peroxidase conjugate (1:2000; Organon) dissolved in buffer with 0.5% Protifar. After each incubation step, the blot was thoroughly washed with buffer. Antibody

reactive proteins were visualised on the blot by using a substrate solution (10 mM 3,3′,5,5′-tetramethylbenzidine, 18 mM dioctyl sulphosuccinate, 82.5 mM Na acetate-buffer, pH 5.5, 25% ethanol and 0.0625% (v/v) hydrogen peroxide (30%)). After the peroxidase-catalysed colour reaction, the blots were washed with water.

2.4. TNBS assay

The reaction of formaldehyde with diphtheria toxin results in a reduction of the number of primary amino groups in the molecule. The toxoid samples were dialysed against PBS to remove unreacted glycine. After the dialysis, the protein concentrations and the primary amino group concentration of the toxoids were determined by the BCA protein assay and by a colourimetric assay using 2,4,6-trinitrobenzenesulphonic acid (TNBS) [20], respectively.

2.5. Denaturation experiment and fluorescence spectroscopy

The sensitivity of the experimental toxoids to denaturation by guanidine–HCl was studied by fluorescence spectroscopy [21,22]. Toxoid samples of 2.5 µg/ml were incubated for 2 h with various guanidine–HCl concentrations from 0 to 4 M in steps of 0.2 M. The spectra of the toxoids and toxin were recorded at 25 °C with a Perkin-Elmer LS50B fluorescence spectrometer. The excitation wavelength was 295 nm (band width 2.5 nm) and the emission wavelength was measured between 330 and 360 nm (band width 5 nm). For each sample, the emission maximum was determined from five averaged scans (corrected for background).

2.6. Circular dichroism

Circular dichroism spectra were recorded at 25 °C with a dual-beam DSM 1000 CD spectrophotometer (On-Line Instrument Systems, Bogart, GA). The subtractive double-grating monochromator was equipped with a fixed disk, holographic gratings (2400 lines per mm, blaze wavelength 230 nm) and 1.24 mm slits. Far-UV and near-UV spectra were taken from 260 to 190 nm (path length 0.2 mm) and from 320 to 250 nm (path length 10 mm), respectively. The protein concentration was 0.5 mg/ml for far-UV measurements and 1 mg/ml for near-UV measurements. For each measurement, six repeated scans (step resolution 1 nm) were averaged and the corresponding buffer spectrum (also six averaged scans) was subtracted. The near-UV CD spectra were smoothed by using a smoothing factor 13. The measured CD signals were converted to molar CD ($\Delta\varepsilon$), based on a mean residual weight of 109.

2.7. Biosensor analysis

Biosensor analyses were performed on a Biacore 1000 to measure the affinity of monoclonal antibodies for diphthe-

ria toxin and experimental toxoids. Monoclonal antibodies Dim 5 and Dim 33 are directed against A-fragment of diphtheria toxin, while Dim 25 and Dim 27 can bind the B-fragment. The binding of the experimental toxoids to each antibody was measured. Fc-specific antibodies (Rabbit anti-mouse; Biacore AB) were coupled to a CM5 sensor chip by using an amine coupling kit (Biacore AB) and gave a response of about 5000 resonance units (RU). Subsequently, 500–650 RU of anti-diphtheria toxin monoclonal (Dim 5, Dim 25, Dim 27 or Dim 33) diluted in HBS-P buffer (Biacore AB) was bound by the Fc-specific antibody. The experimental toxoids were diluted in HBS-P buffer to a concentration of 300 nM. Finally, the real-time binding and release of the toxoids to the individual monoclonal antibodies were analysed at flow rates of 20 µl/min and for 2.5 and 5 min, respectively. For kinetic analysis, the Fc-specific antibodies bound 200–400 RU of an individual monoclonal. The binding and release of the toxoid samples were measured for 3 and 5 min, respectively. The flow rate was 30 µl/min and the toxin DTx and toxoids DTd4 and DTd5 were diluted to concentrations of 20, 40, 60, 80 and 100 nM. The kinetic data were calculated with BIA evaluation software.

2.8. Specific toxicity

The sensitivity of Vero cells to diphtheria toxin provides an opportunity to determine residual toxicity of toxoids [23]. Two-fold dilution series (between 0.11 and 220 pM) of toxoids were prepared with complete medium 199 (medium 199 (Gibco-BRL) with 10% fetal calf serum, 10,000 E/l penicillin and 0.1 mg/l streptomycin), so that each well of the microtitre plate contained a mixture of 100 µl. Subsequently, 50 µl medium with 5×10^5 Vero cells per ml was added to each well. The plates were covered with a plate sealer and incubated for 6 days at 37 °C and 5% CO$_2$. To determine the viability of the cells [24], 10 µl MTT-solution (5 mg/ml tetrazolium salt in PBS) was added to each well. The plates were incubated for a further 4 h in the incubator. Then, the medium was removed, 100 µl extraction buffer (100 g/l SDS, 50% (v/v) dimethyl formamide in water and a pH of 4.7, adjusted with acetic acid) was added, the plates were covered again and incubated overnight in the incubator. Finally, the absorbance of the blue-coloured samples was recorded at 570 nm with a plate reader (Bio-kinetics reader EL312e, Bio-tec instruments).

2.9. Vaccine preparation

Diphtheria toxoids were diluted to 50 µg/ml (16.6 Lf/ml) in an adsorption mixture (1.5 mg/ml AlPO$_4$ (Adju-Phos®; Brenntag Biosector, Denmark) and 0.15 M NaCl). The samples were mixed by rotating for 24 h at 4 °C. The adsorption of diphtheria toxoid onto aluminium phosphate was indirectly checked after centrifugation of the samples by a sandwich ELISA on the supernatant, using horse anti-diphtheria toxoid serum and a horse anti-diphtheria peroxidase

conjugate [22]. The adsorption was between 50 and 80%. Before vaccination of mice, the vaccines were diluted in physiological saline solution to a toxoid concentration of 20 μg/ml (ca. 6.2 Lf/ml).

2.10. Potency determination

Mice were used to determine the potency of experimental diphtheria toxoid vaccines. An amount of 250 μl vaccine (5 μg toxoid) was subcutaneously injected in the groin of each mouse (eight mice per vaccine, NIH, female, weight 10–14 g). Beside the experimental toxoids, a reference vaccine DTa 93/1 with a potency of 4.15 IU/Lf was used for vaccination. Five groups were injected with 250 μl DTa 93/1 dilutions (containing 18, 9, 4.5, 2.3 and 1.1 Lf/ml, respectively). After 35 days animals were bled and the blood was individually collected in tubes. The blood was incubated for 2 h at 37 °C and subsequently for 2 h at 4 °C. The samples were centrifuged for 20 min at 800 × g. The supernatants were transferred into new tubes and centrifuged once again to obtain cell-free sera. Then, sera were again transferred into new tubes and complement was inactivated by heating at 56 °C for 45 min and stored at −20 °C. The amount of protecting antibodies was measured by a toxin neutralisation test using Vero cells. Two-fold dilution series of individual sera were prepared with complete medium 199 so that each well of the microtitre plate contained 50 μl. Then, 50 μl toxin DTa 79/1 (0.0005 Lf/ml) in complete medium 199 was added to the wells. The plates were incubated for 2 h at 37 °C. Subsequently, 50 μl complete medium 199 with 5×10^5 Vero cells per ml was added to each well. The plates were covered with a plate sealer and incubated for 6 days at 37 °C and 5% CO_2. The scores (the number of wells containing living cells) of each vaccine was determined by using the microscope. The living cells form an intact monolayer within 6 days. A reference curve was calculated from the scores of reference vaccine DTa93/1 and used to determine the potency of the experimental toxoids.

3. Results

3.1. SDS-PAGE

Diphtheria toxin normally appears in two structural forms: as a single chain of 58.3 kDa and in the nicked form as two fragments of 21.0 kDa (A-fragment) and 37.3 kDa (B-fragment), which are connected by a disulphide bridge [25,26]. Diphtheria toxin and 10 experimental toxoids (DTd1–DTd10; see Table 1) were analysed on an acrylamide gel after reduction of the disulphide bridges. The diphtheria toxin used in this study was almost completely nicked (lane 1). The bands of A- and B-fragment appeared in acrylamide gel at higher apparent masses (27 and 43 kDa, respectively) than expected. A number of differences were found between the toxoids (Fig. 1). Firstly, the intensity of the 58 kDa band increased with higher formaldehyde concentrations (lanes 1–11). Secondly, formaldehyde induced a number of shifts of the B-fragment on the gel, which was verified by immunoblotting and by using a B-fragment specific monoclonal Dim 25 (data not shown). The intensity of the apparent 43 kDa band was reduced with increasing formaldehyde/glycine concentrations, whereas the intensity of an apparent 39 kDa band was increased. The B-fragment was maximally shifted when formaldehyde concentrations above 32 mM were used (lanes 7–11). A similar effect was observed for the 58 kDa band and the band of the A-fragment, albeit less pronounced. Finally, increasing concentrations of formaldehyde result in broader and more diffuse protein bands.

3.2. Primary amino groups

The diphtheria toxin molecule has 40 primary amino groups [25]. It is expected that formaldehyde causes a reduction in the number of primary amino groups, because it reacts in the first step with primary amino groups and forms in the second step cross-links with other amino acids. The
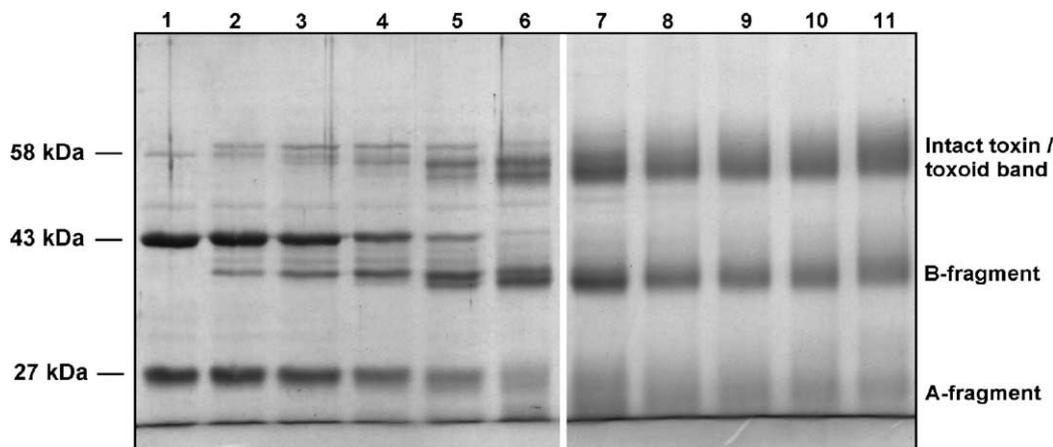

Fig. 1. SDS-PAGE of diphtheria toxin DTx (lane 1) and experimental toxoids DTd1–10 (lanes 2–11, respectively). Diphtheria toxoids DTd1–10 were prepared with increasing formaldehyde concentrations (see Table 1).
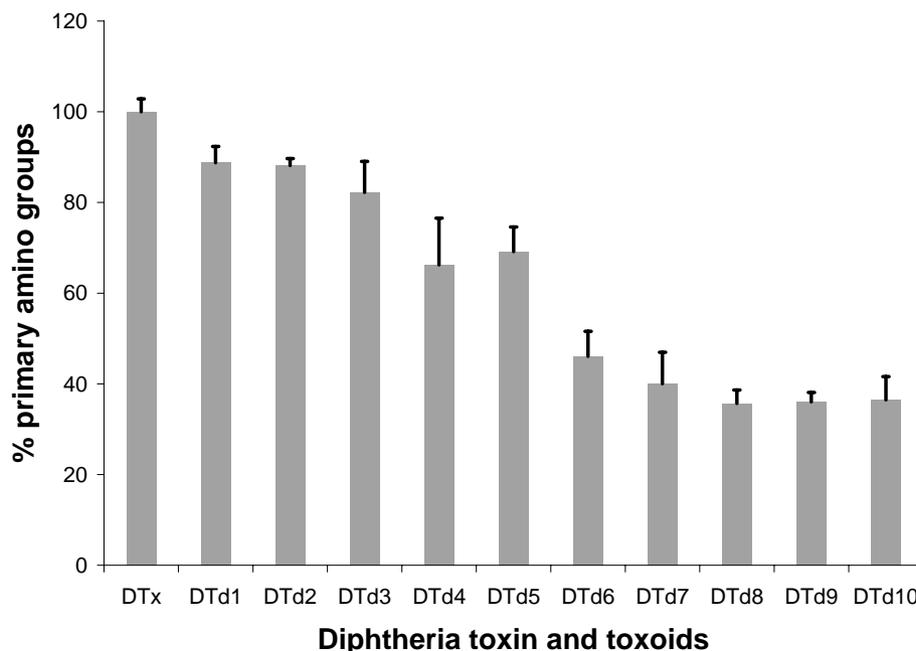
Fig. 2. The relative number of primary amino groups in diphtheria toxin and experimental toxoids determined by the TNBS assay and corrected for differences in protein concentration (mean ± S.D.; $n = 6$).

number of primary amino groups has been determined for the experimental toxoids by the TNBS assay. The results are shown in Fig. 2. With increasing formaldehyde concentration, the number of NH_2-groups was gradually reduced to a minimum level of 40% for DTd6–DTd10.

3.3. Conformational stability

The effect of formaldehyde concentration on the conformational stability was studied by denaturing the toxoids by guanidine–HCl, which was monitored by fluorescence spectroscopy. It has been demonstrated that the toxoid is more resistant to denaturation than the toxin [21,22]. Under physiological conditions, the six tryptophans of diphtheria toxin and toxoids showed an average fluorescence emission maximum around 335 nm. Denaturation causes an increased exposure of Trp residues to the aqueous surroundings, resulting in a shift of the maximal emission to higher wavelengths, in this case to about 353 nm. Fig. 3 shows the denaturation curves of diphtheria toxin and the experimental toxoids. Increasing formaldehyde concentrations yielded toxoids that were more resistant to unfolding. However, the resistance to denaturation slightly declined and the denaturation curves became less steep when formaldehyde concentrations were used above 64 mM, e.g. in toxoids DTd9 and DTd10.

3.4. Circular dichroism

Far-UV and near-UV CD spectra were taken from diphtheria toxin and toxoids to compare their secondary and tertiary structure. Representative spectra are shown in Fig. 4.

Substantial differences between the experimental toxoids were observed in the near-UV CD spectra (Fig. 4A). Firstly, a shift in the maximal intensity was observed in the toxoids DTd5–DTd10 from 275 to 280 nm, respectively. Secondly, a sharp transition was observed at a formaldehyde concentration of around 16 mM (Fig. 5). These changes indicate that the reaction of diphtheria toxin with formaldehyde-induced perturbations of the tertiary structure. In contrast, the far-UV CD spectra of toxin and toxoids were essentially the same, indicating that the secondary structure was unaffected by the detoxification process (Fig. 4B).
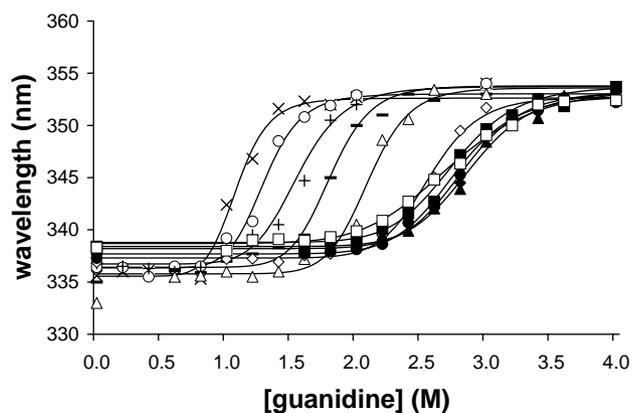

Fig. 3. Denaturation of diphtheria toxin DTx (×) and experimental toxoids DTd1–10 (○, +, −, △, ◇, ●, ▲, ◆, ■, □, respectively) monitored by fluorescence. Denaturation causes a shift in the emission maximum from 335 to 353 nm. The denaturation midpoints are 1.0 M for DTx, and 1.3, 1.5, 1.8, 2.1, 2.5, 2.8, 2.8, 2.8, 2.7 and 2.7 M, DTd1–DTd10, respectively.
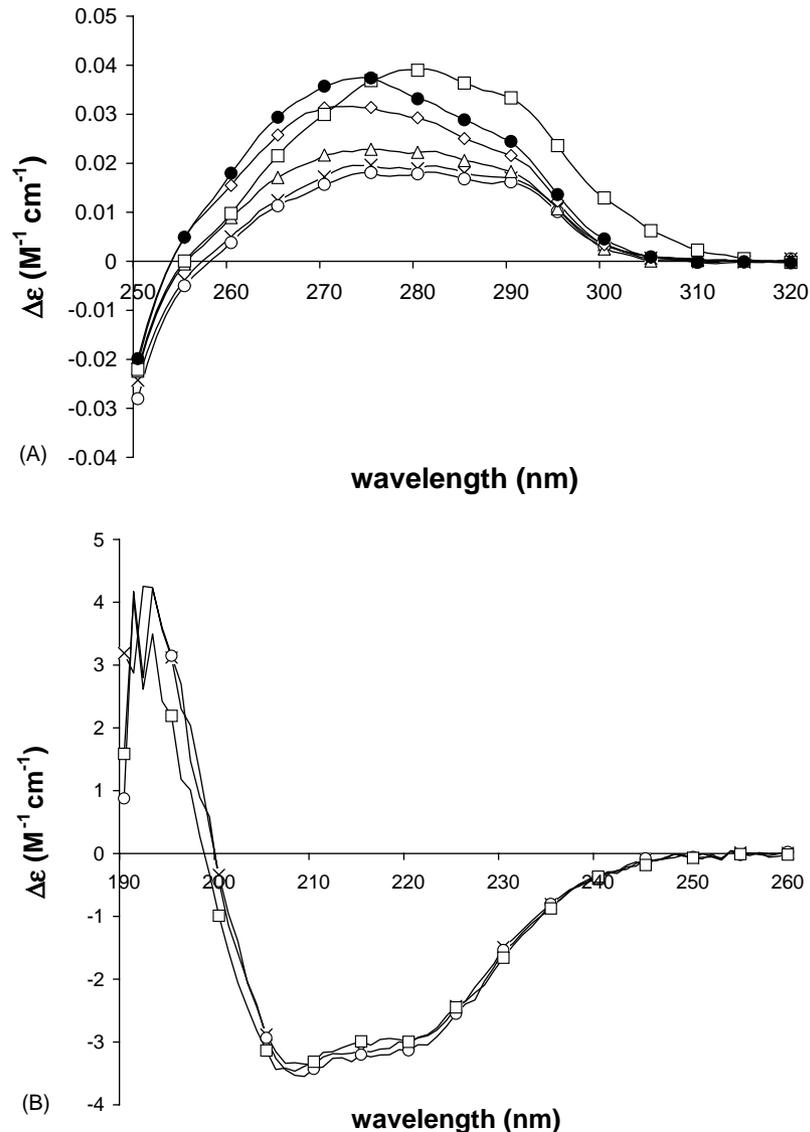
Fig. 4. (A) Circular dichroism spectra in the near-UV region obtained for DTx (×) and experimental toxoids DTd1, 4, 5 and 10 (○, △, ◇, ● and □, respectively). (B) Far-UV CD spectra obtained for DTx (×) and experimental toxoids DTd1 and 10. (○, and □, respectively).

3.5. Biosensor analysis

Four anti-diphtheria toxin monoclonal antibodies (Dim5, Dim 25, Dim 27 and Dim 33) were used in biosensor analysis to measure real-time binding of the experimental toxoids. Each of these monoclonal antibodies has a different specificity. Dim 5 and Dim 33 bind to the A-fragment of diphtheria toxin, whereas Dim 25 and Dim 27 recognise the B-fragment. Competition studies with biosensor analysis have shown that none of the antibodies mutually influence their binding to diphtheria toxin, which means that they recognise a different epitope (unpublished results). In the binding studies, toxin DTx and toxoids DTd1–DTd10 showed nearly the same binding for monoclonal Dim 5 (Fig. 6A). The same holds true for Dim 25 (Fig. 6B). This indicates that the epitopes, recognised by these antibodies

were largely preserved for each of the toxoids. On the other hand, Dim 27 and Dim 33 (Fig. 6C and D) showed a decrease of maximal binding for toxoids that were prepared with increasing formaldehyde concentrations. The toxoids DTd6–DTd10 were not bound at all by these monoclonals. There are at least two explanations for reduction of the maximal binding: (i) the absolute epitope concentrations were decreased and/or (ii) the epitopes were modified in such a way that they were still recognised by the antibodies, but with a lower affinity. To investigate these options, binding experiments with toxin and toxoids were performed with the four monoclonals to obtain the association and dissociation constants (k_a and k_d, Table 2). There were no large differences between these constants of the toxin DTx and the toxoids DTd4 and DTd5, which have a lower maximal binding to the antibodies Dim 27 and Dim 33. This indicates that
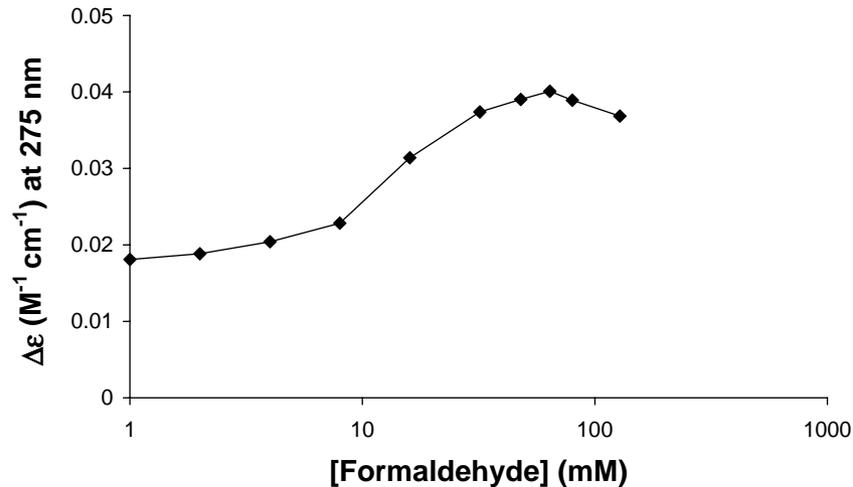
Fig. 5. The molar extinction difference ($\Delta\varepsilon$) of the experimental toxoids DTd1–10 measured by CD analysis at 275 nm. Diphtheria toxin (DTx) had a molar extinction difference of 0.019.

Table 2
Apparent association (k_a) and dissociation constants (k_d) for complexes between monoclonal antibodies and diphtheria toxin (DTx) and toxoids (DTd4 and DTd5)

Antibody	Sample[a]	k_a $(1\,M\,s^{-1})$[b]	k_d $(1\,s^{-1})$[b]	K_a $(1\,M^{-1})$[b]
Dim 5	DTx	$1.6 \pm 0.4 \times 10^5$	$7.7 \pm 0.4 \times 10^{-3}$	$2.1 \pm 0.5 \times 10^7$
	DTd4	$1.4 \pm 0.3 \times 10^5$	$5.6 \pm 0.7 \times 10^{-3}$	$2.4 \pm 0.6 \times 10^7$
	DTd5	$1.5 \pm 0.4 \times 10^5$	$5.5 \pm 0.3 \times 10^{-3}$	$2.7 \pm 0.8 \times 10^7$
Dim 25	DTx	$2.9 \pm 0.4 \times 10^5$	$1.7 \pm 0.1 \times 10^{-4}$	$1.7 \pm 0.3 \times 10^9$
	DTd4	$3.0 \pm 0.5 \times 10^5$	$2.0 \pm 0.1 \times 10^{-4}$	$1.5 \pm 0.3 \times 10^9$
	DTd5	$3.3 \pm 0.9 \times 10^5$	$1.8 \pm 0.2 \times 10^{-4}$	$1.8 \pm 0.5 \times 10^9$
Dim 27	DTx	$1.6 \pm 0.1 \times 10^5$	$8.2 \pm 0.8 \times 10^{-4}$	$2.0 \pm 0.6 \times 10^8$
	DTd4	$0.9 \pm 0.2 \times 10^5$	$9.2 \pm 0.8 \times 10^{-4}$	$1.0 \pm 0.1 \times 10^8$
	DTd5	$1.0 \pm 0.4 \times 10^5$	$10.5 \pm 0.5 \times 10^{-4}$	$1.0 \pm 0.2 \times 10^8$
Dim 33	DTx	$5.7 \pm 2.2 \times 10^4$	$2.4 \pm 1.1 \times 10^{-4}$	$2.4 \pm 1.4 \times 10^8$
	DTd4	$3.6 \pm 2.3 \times 10^4$	$4.0 \pm 2.2 \times 10^{-4}$	$9.0 \pm 0.7 \times 10^7$
	DTd5	$1.2 \pm 1.6 \times 10^4$	$7.7 \pm 5.5 \times 10^{-4}$	$1.6 \pm 0.2 \times 10^7$

[a] Described in Table 1.
[b] Mean values $\pm$ S.D. ($n = 5$).

the epitope concentration was reduced, but not the affinity for the remaining epitopes.

3.6. Cytotoxicity

The residual toxicity was measured with the Vero cell assay. Toxin DTx and toxoids DTd1–DTd4 showed a measurable cytotoxicity, the extent of which declined with increasing formaldehyde concentration. No residual toxicity was detected for toxoids prepared with formaldehyde concentrations above 16 mM (DTd5–DTd10; Table 3).

3.7. Potency

The neutralising capacity of sera obtained from mice immunised with an experimental toxoid vaccine was used to determine the potency. Fig. 7 shows the results of the po-

tency test. Remarkably, diphtheria toxin (0.7 IU/Lf) had a much lower potency than the reference toxoid DTa 93/1 (4.1 IU/Lf). When increasing the formaldehyde concentration, the potency of the toxoids was gradually enhanced up to a plateau level (ca. 11 IU/Lf) for formaldehyde concentrations higher than 32 mM.

Table 3
The cytotoxic concentration of diphtheria toxin (DTx) and experimental toxoids (DTd1–DTd10)

Name	Cytotoxic concentration (pM)[a]
DTx	0.3
DTd1	0.87
DTd2	1.7
DTd3	7
DTd4	55
DTd5–DT10	>220

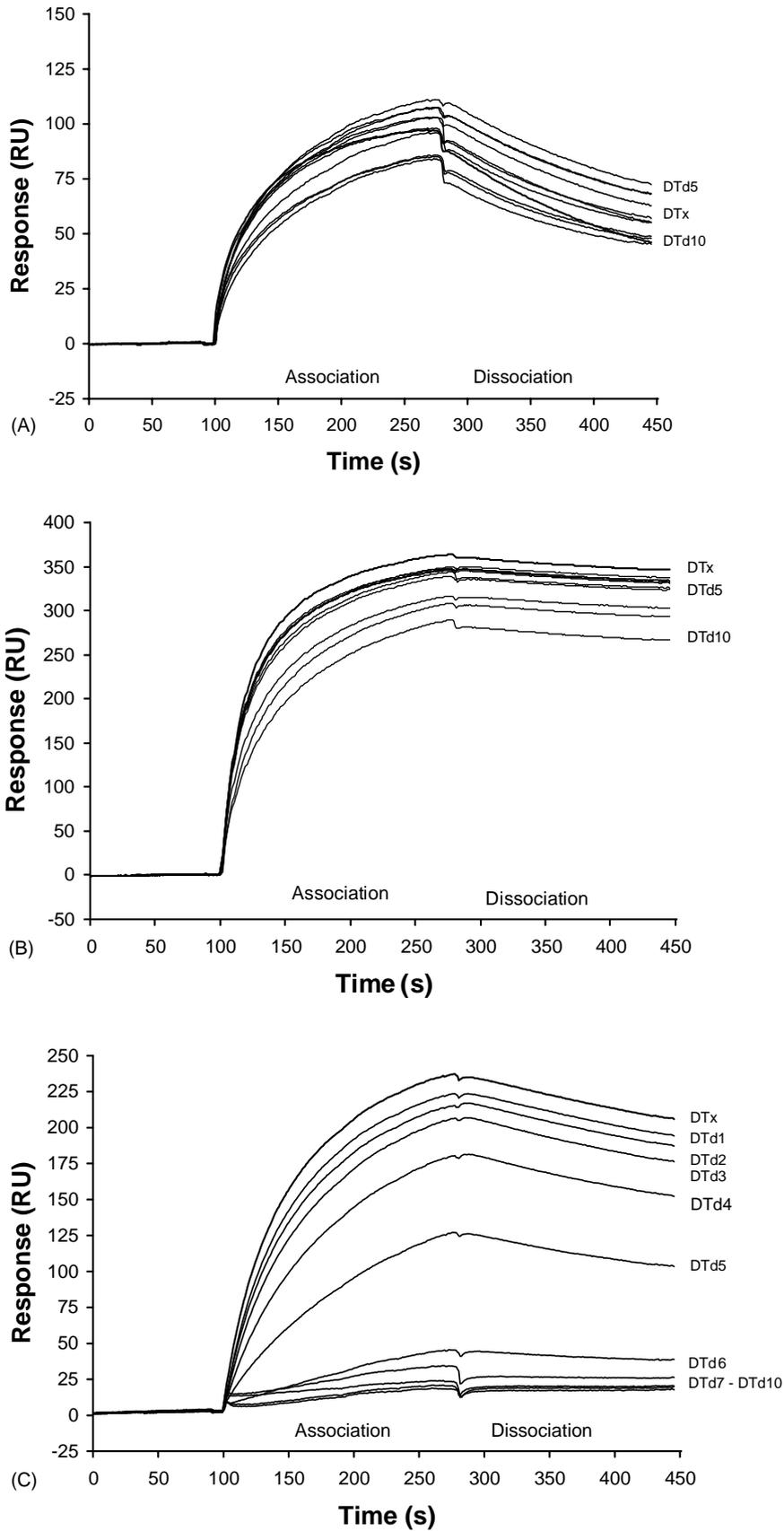[a] Determined with the Vero cell assay (see Section 2).

Fig. 6. Biosensor analysis of the toxoids with four monoclonal antibodies. The sensorgrams show the binding and dissociation of DTx and DTd1–DTd10 to (A) the monoclonal antibody Dim 5, (B) Dim 25, (C) Dim 27 and (D) Dim 33.
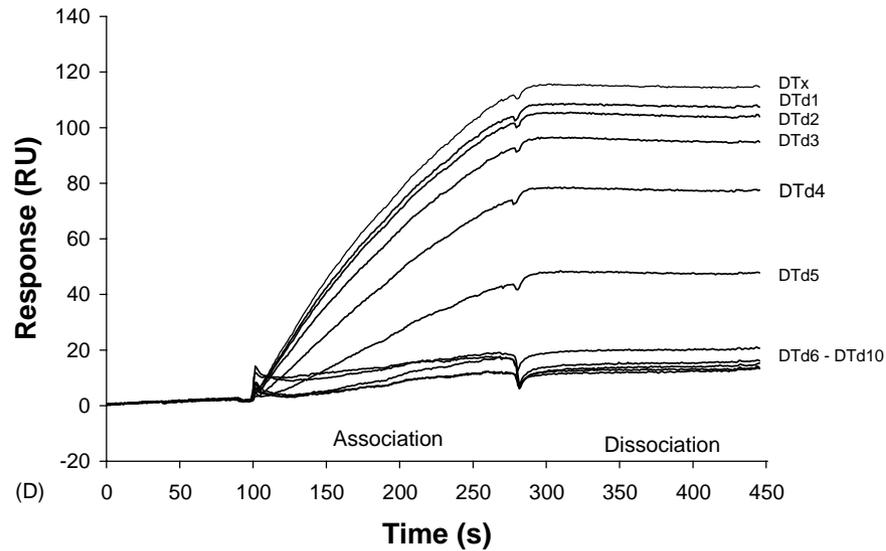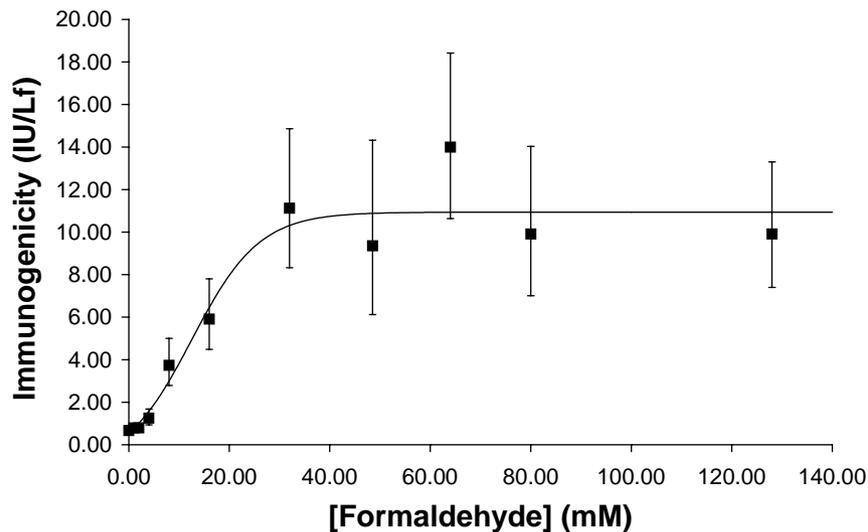
Fig. 6. (*Continued*).

Fig. 7. The immunogenicity (IU/Lf) of diphtheria toxoids vs. concentration of formaldehyde (mean $\pm$ S.E.; $n = 8$).

4. Discussion

Routine quality testing of diphtheria toxoid vaccines is required by the regulatory authorities, including potency and safety tests. These tests rely mainly on the use of animals. The question has been raised if physicochemical and immunochemical tests can be used for the quality control of diphtheria toxoid vaccines [15]. These techniques can be used to investigate the characteristics of protein antigens, such as identity, size, structure, purity and amino acid modifications. A set of methods has been selected that may be useful to monitor variation between toxoid batches. The value of each technique has been assessed in this study by using a series of toxoids, prepared with varying formaldehyde and glycine concentrations (Table 1). This study shows

that these experimental toxoids varied in immunogenicity and residual toxicity (Fig. 7 and Table 3, respectively). Most of the individual analytical methods that were applied could discriminate between these toxoids.

Electrophoretic analysis revealed three types of differences that are caused by the reaction with formaldehyde (Fig. 1): (i) a shift of the toxin bands; (ii) change in the ratio of nicked toxoid (21 and 37 kDa fragments) to apparently intact toxoid (58 kDa protein); and (iii) protein bands becoming diffuse. The effects arose successively: first the shifts, then the increased amount of apparently intact toxoid, and finally the broader protein bands. These effects have been attributed to cross-links, which can be formed within the toxin and between amino acids present in the toxoidation medium and the toxin [1,5,6,27]. Presumably, the toxin contains some

the number of primary amino groups in the toxoids should be reduced to ≤40% relative to the number for diphtheria toxin; the denaturation midpoint should be ≥2.5 M guanidine; the molar extinction difference at 275 nm determined by CD analysis should be ≥0.025 $M^{-1} cm^{-1}$; and the binding of the toxoid by anti-diphtheria antibodies Dim 27 and Dim 33 should be reduced until ≤30% of the original intensity. These criteria represent the borderline values, all of which should be met by approved toxoids based on the described parameters.

Based on the immunogenicity and toxicity test, the experimental toxoids could be divided in three categories: (i) toxoids with residual toxicity and low immunogenicity (toxoids DTd1–DTd4); (ii) borderline products such as toxoid DTd5, having no residual toxicity and inducing a relatively low protecting immune response; (iii) a group of approved toxoids,

which are safe and highly immunogenic. These three groups of vaccines were used to establish the minimum criteria the toxoids should fulfil for passing a quality control test. Fig. 8 gives a visual impression of the quality of the experimental diphtheria vaccines based on the analytical parameters listed above. If the toxoids have residual toxicity and/or a low immunogenicity, their pentagonal plots are located within the black borderline. Otherwise, the plots cross all the borders and the quality of the toxoids is satisfactory.

In conclusion, the combined application of physicochemical and immunochemical techniques results in a fingerprint of the antigen. The quality of diphtheria toxoid can be predicted on the basis of values determined with the in vitro tests used. The reliability of the prediction increases as more in vitro methods are applied, because no single analytical technique can completely characterise an antigen. Furthermore,
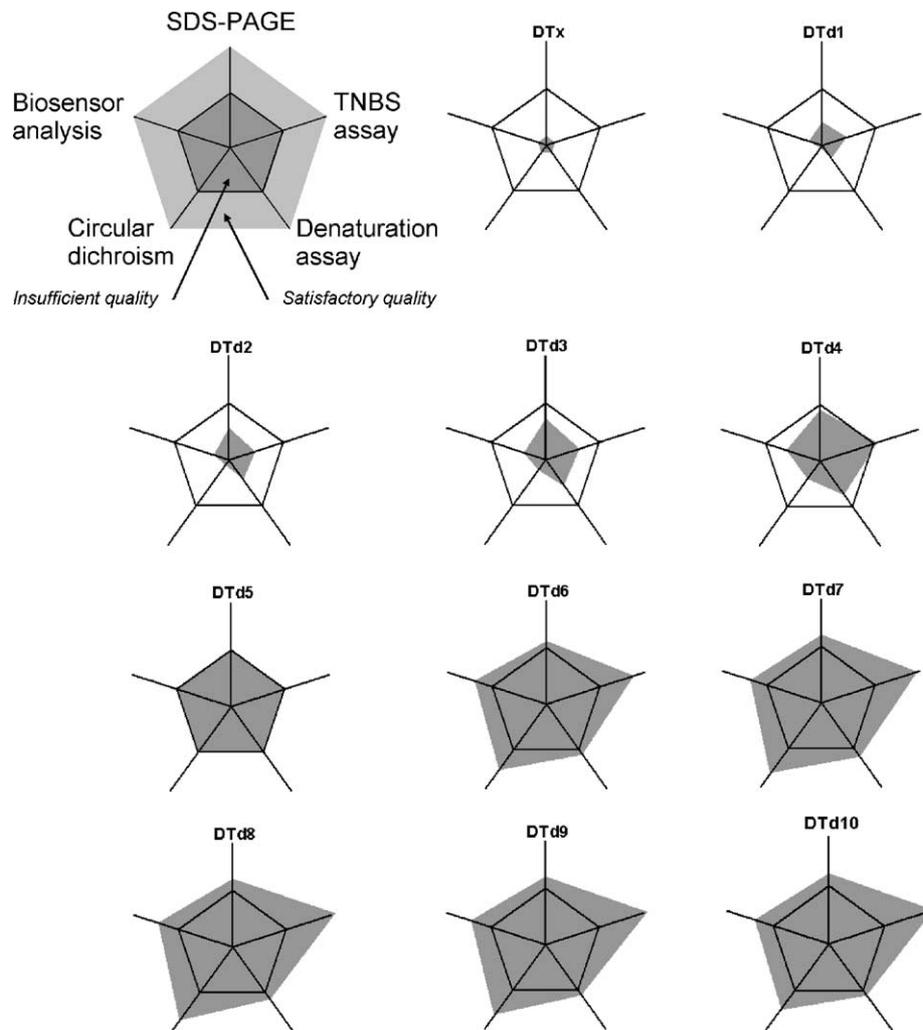

Fig. 8. Visual representation of the quality of Diphtheria toxin and experimental toxoids, as characterised by five physicochemical and immunochemical parameters (see text). If grey pentagonal plots are located within the black borderline, the quality of the toxoids is insufficient, e.g. DTx, DTd1–DTd4. DTd5 is a borderline product. The grey plots of DTd6–DTd10 cross the black borderlines, indicating satisfactory quality of these toxoids. Scales (from midpoint to limit) are for: SDS-PAGE, the intensity of the apparent 43 kDa band against the 39 kDa band (0–100%); TNBS assay, the relative amount of primary amino groups in the toxin molecule (100–40%); denaturation assay, denaturation midpoint (1.0–3.1 M guanidine); circular dichroism, $\Delta\varepsilon$ at 275 nm (0.018–0.043 $M^{-1} cm^{-1}$); biosensor analysis, relative binding intensity to Dim 33 (110 to −26 RU).

extensive validation studies have to confirm the capability of these tests for quality control. In conclusion, this study demonstrates that the quality of diphtheria toxoid vaccines can be guaranteed with physicochemical and immunochemical techniques. We expect that similar fingerprint strategies are applicable for other (toxoid) vaccines.

Acknowledgements

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