Transcutaneous subunit vaccine delivery. A combined approach of vesicle formulations and microneedle arrays

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Chapter 5

Preparation and characterization of diphtheria toxoid-loaded elastic vesicles for transcutaneous immunization

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Adapted from Journal of Drug Targeting 2008;16(7):555 -563.

Introduction

Although vaccination *via* the needle has led to tremendous advances in the control of many infectious diseases [1], there is an increasing demand for non-invasive vaccine delivery. Pediatric vaccine program is the best example where a high number of antigens need to be introduced in a short period of time, leading to complex antigen mixtures and many injections. One of the promising routes for non-invasive vaccine delivery is the transcutaneous route, as the skin is a highly immune active organ and easily accessible. Human skin is composed of three layers, from the surface down, the *stratum corneum*, the epidermis and the dermis. The epidermis has a dense population of bone marrow-derived dendritic cells, referred to as Langerhans cells. These Langerhans cells, which travel to the draining lymph nodes after activation, are highly efficient antigen presenting cells [2, 3]. They are the targets of transcutaneously delivered vaccines.

A major hurdle for successful transcutaneous immunization (TCI) is the barrier function of the skin, which resides in the uppermost layer, the *stratum corneum*. This layer is composed of dead, cornified, tightly packed keratinocytes embedded in lipid lamellar regions. This layer is considered to be only minimally permeable for high-molecular-weight substances, such as antigens [4], while chemical penetration enhancers and also physical means, such as iontophoresis, only have shown success in delivering small molecules across the skin barrier [5]. Therefore, the greatest challenge in TCI is improving the transport of antigens across the *stratum corneum*. Recently, several studies have reported that the use of antigen-containing occlusive patches induced strong immune responses in an animal model and human volunteers. [6-8]. However, successful vaccination largely depends on the antigen type and very high antigen doses have been used in these occlusive patch studies. Therefore, there is still an urgent demand to improve the delivery of antigens into the skin.

One promising approach for transcutaneous delivery of antigens is the use of vesicular formulations, especially elastic vesicles that have highly deformable bilayers [9, 10]. For instance, hepatitis B surface antigen (HBsAg)-loaded elastic liposomes applied onto intact mouse skin elicited robust systemic and mucosal antibody responses against HBsAg [11]. Transfersomes® composed of soybean phosphatidylcholine, sodium cholate and sodium dodecyl sulfate have also been used in TCI studies. Successes were achieved with these

vesicles in combination with antigens such as human serum albumin [12], gap junction proteins [13] and tetanus toxoid [14] in murine models. Depending on the antigen type, dose, immunization schedule, presence of co-stimulatory factors and vesicle composition, immunization with antigen formulations based on elastic vesicles can induce effective immune responses with serum IgG levels comparable to those obtained after subcutaneous injection.

A new generation of elastic vesicles were introduced 10 years ago [15], consisting of the bilayer-forming surfactant L-595 (sucrose-laurate ester) and the micelle-forming surfactant PEG-8-L (octaoxyethylene-laurate ester). These elastic vesicles were able to penetrate pores much smaller than their diameter [16]. Recently, it was suggested that these elastic vesicles may act as carrier systems; after non-occlusive application elastic vesicles and bound fluorescent label followed the same route of penetration into the *stratum corneum* and intact vesicle structures have been visualized in the *stratum corneum* [17]. Furthermore, transport studies of small drugs such as pergolide, lidocaine, ketorolac and rotigotine [18-21] demonstrated that higher transport rates of these drugs, compared to the same drug in buffer solutions or with rigid vesicles, can only be achieved when drug molecules are physically associated with the elastic vesicles. The drug-loaded elastic vesicles were applied non-occlusively since the trans-epidermal osmotic gradient is believed to be the driving force for the transport [22]. As these vesicles act as drug carrier systems for low-molecular-weight drugs, they may hold promise as antigen delivery systems by enhancing antigen partitioning and transporting to the deeper layers of the *stratum corneum*. If intact antigen-loaded vesicles are in the *stratum corneum* close to the viable epidermis, the antigen might be released and diffuse into the viable epidermis and, after reaching the Langerhans cells, trigger an immune response.

The aim of the current study was to develop DT-containing elastic vesicle formulations for TCI. DT was chosen as a model antigen, since it has been used in murine models for TCI studies in combination with mucosal adjuvants [23, 24], physical methods such as electroporation [25] and local hyperthermia [26]. However, DT alone cannot induce protective immunity when applied on intact mouse skin because of the low intrinsic immunogenicity of plain DT and/or the insufficient amount of DT delivered *via* the skin [23]. One approach to improve the delivery of DT through the skin and make it more immunogenic is utilizing carrier systems such as elastic vesicles. As elastic vesicles act as carrier, physical association of DT with the

vesicles is expected to be crucially important. Therefore, in the current study, the vesicle composition, DT concentration, pH and buffers were optimized to obtain high DT association levels as well as good colloidal stability of the DT-vesicle formulations.

Materials and methods

Materials

Diphtheria toxoid (MW: 58 kDa; isoelectric point about 4.7), horse anti-DT and horse radish peroxidase conjugated anti-DT were provided by the NVI (Netherlands Vaccine Institute, Bilthoven, The Netherlands). Sucrose-laurate ester (L-595; 30% mono-, 40% di-, and 30% triesters, mean MW 734) was kindly supplied by Mitsubishi Kasei (Tokyo, Japan). Octaoxyethylene-laurate ester (PEG-8-L; mean MW 552) was a gift from Lipo Chemicals (Paterson, New Jersey, US) and sodium bistridecyl sulfo succinate (TR-70; mean MW 585) was a gift from Cytec B.V. (Rotterdam, The Netherlands). Tween 80 was purchased from Merck (Darmstadt, Germany). Tween 20, lyophilized bovine serum albumin (BSA) and Folin Ciocalteu's phenol reagent were obtained from Sigma (Zwijndrecht, The Netherlands). Chromogen 3, 3', 5, 5'-tetra-methylbenzidine (TMB) and the substrate buffer were purchased from Biosource B.V (Etten-Leur, The Netherlands). All other chemicals used were of analytical grade and all solutions were prepared with distilled water.

Methods

Preparation of the vesicle dispersion and DT-loaded vesicle formulations

Elastic vesicles used in this study consisted of the bilayer-forming surfactant L-595, the micelle-forming surfactant PEG-8-L, and the stabilizer TR-70 in the molar ratio of either 5:5:1 or 7:3:1. The 5:5:1 and 7:3:1 ratios were selected as in previous studies these ratios resulted in stable dispersions containing medium elastic (7:3:1) or very elastic (5:5:1) vesicles [15-17]. For clarity, all vesicle compositions will be indicated below only by the molar ratio between the two main components L-595 and PEG-8-L. Vesicles were prepared using a modified sonication method [10]. In brief, the surfactants were dissolved in ethanol, while TR-70 was dissolved in an ethanol/isopropanol mixture. The solutions were mixed in an appropriate ratio. The organic solvents were then evaporated overnight in a vacuum centrifuge and the remaining surfactants were dispersed in 0.01 M acetate buffer (Ac, sodium acetate buffer, 8.4 mM

$H_2C_2O_4$ and 1.6 mM $Na_2C_2O_4$, pH 4.0), citrate buffer (CB, 3.2 mM $H_3C_6H_5O_7$ and 6.8 mM $Na_3C_6H_5O_7$ adjusted by adding 0.1 M HCl, pH 5.0) or phosphate buffer (PB, 7.7 mM Na_2HPO_4 and 2.3 mM NaH_2PO_4, pH 7.4). After adding DT, the final concentration of total surfactant was 10% w/w. DT with the same buffer was added to the vesicle dispersion to a final concentration of 1.7 mg/ml. The selected concentration of DT derives from the dose and volume of formulation needed in future *in vivo* studies. Subsequently, vesicle dispersions were sonicated for 3×5 s using a Branson Sonifier 250 (Branson Ultrasonics, Danbury, UK) with 3 mm micro tip at 70 mW energy output. DT has lest solubility in buffer at pH 4.7 due to its isoelectric point (pI, unpublished data). It was found that solubility of DT in CB is below 1.7 mg/ml at pH 4.5 (visible precipitation) and above 1.7 mg/ml at pH 5.0. Therefore, to obtain a DT-containing vesicle formulation at pH 4.5, the surfactants mixture after vacuum centrifuge was first rehydrated in CB at pH 5.0. After adding DT and sonication, a small amount of 0.1 M HCl was added to the DT-vesicle suspension, lowered the pH to 4.5. This resulted in a stable DT-containing vesicular formulation. The final DT concentration was kept constant for all the formulations. Stability studies were carried out from day 1 till day 14 after preparation. During these studies the formulations were stored at 4 ºC.

To investigate whether electrostatic interactions play a role in DT-vesicle association, DT formulations were also prepared with 0.01 M phosphate buffered saline at pH 7.4 (PBS, PB with 153 mM NaCl) and 0.01 M citrate buffered saline at pH 5.0 and 4.5 (CBS, CB with 153 mM NaCl). To achieve vesicles with similar size, the sonication period was extended to 80-100 s at this higher salt concentration. The formulations were stored at 4 ºC and the association studies were carried out within 3 days after preparation.

Particle size and ζ-potential measurements

The vesicle size and polydispersity index (PDI) of all formulations were measured by dynamic light scattering (DLS) using a Malvern Zetasizer 3000 HSA (Malvern Ltd., Malvern, United Kingdom). All size measurements were performed at 25 °C at an angle of 90° between laser and detector. Before measuring, all samples were diluted with their original buffer.. For estimation of the surface charge of the DT-associated vesicles, 3.0 µl of vesicle formulation were diluted in 1.00 ml of 1 mM CB or PB (pH corresponding to that of the formulation) and the ζ-potential was measured by laser Doppler electrophoresis using the same device. Formulations were measured at

regular time-intervals during a period of 2 weeks after preparation to check the colloidal stability.

Size exclusion chromatography (SEC)

To determine the amount of DT associated with vesicles in the formulations, SEC was performed with a Sepharose CL-4B (Amersham, Uppsala, Sweden) column (30 cm long, cross-sectional area 0.79 cm^2). A dual λ absorbance detector (Waters 2487 EMI ISM Instrument, Etten-Leur, The Netherlands) set at 280 nm was used to monitor the separation of the free DT and vesicles-associated DT fractions. Empty vesicles and free DT in buffer solution were used to determine the collecting time windows of these fractions. For each formulation, the corresponding formulation buffer was used as eluent at a flow speed of 0.44 ml/min.

Protein and antigenicity assay

The protein recovery rate of the DT from the fractions collected after SEC were determined by a modified Lowry-Peterson protein assay [27]. The antigenicity of the DT from SEC fractions (relative to that of untreated DT) was measured by a sandwich ELISA, using horse anti-diphtheria serum and a horse anti-diphtheria peroxidase conjugate [28]. ELISA was also performed with sonicated DT, DT-vesicle formulations at pH 4.5 and pH 5.0 to study the preservation of DT antigenicity. Data were analyzed by use of the principles of parallel-line analysis of the OD-log concentration plots as described by Tierney *et al*. [29].

Fluorescence spectroscopy

DT-vesicle formulations and DT solutions before and after sonication were studied by fluorescence spectroscopy as described elsewhere [30]. In brief, DT incubated at 4 ºC for 4 h with 4 M guanidine-HCl (denatured DT) served as control [28, 31]. The selected excitation wavelength was 295 nm (band width 2.5 nm) and the emission spectra of the toxoid samples (140 µg/ml) were recorded from 300-450 nm (band width 5 nm) at 25 ºC with a fluorescence spectrometer (Perkin Elmer LS50B, Massachusetts, US). For each sample the emission spectrum was determined from five averaged scans (corrected for background fluorescence).

SDS-PAGE

DT solutions before and after sonication and DT-vesicle formulations were diluted with corresponding buffer to a concentration of 0.3 mg/ml. Non-reducing loading buffer (4x) was added to the samples. SDS-PAGE molecular weight markers (broad range; Bio-Rad, Veenendaal, The Netherlands) were used for calibration. Approximately 3 μg protein was loaded on the gel (4-20% precise protein gel, Pierce, Etten-Leur, The Netherlands) and run at 100 V. Protein bands were visualized by Coomassie brilliant blue.

Results

Effect of DT-vesicle composition on particle size

In our first series of studies the stability of the selected vesicle formulation varying in DT concentrations between 0.2 and 1.7 mg/ml DT was examined by visual inspection and DLS. The pH of the formulations was varied between 4.0 and 7.4. When the formulations were prepared at pH 4.0, pH 4.5, 5.0 and 7.4 with a DT concentration of 0.2 mg/ml, visible precipitation was observed for the 7:3 vesicles, whereas the 5:5 vesicle formulations with up to 1.7 mg/ml DT remained stable at pH values of 4.5, 5.0 and 7.4 (Table I). Using DLS, a z-average diameter of 80-110 nm was measured for the 5:5 vesicles. Therefore in the presence of DT the 5:5 vesicles appeared to be more stable than the 7:3 vesicles. The 5:5 vesicle formulations were selected for more detailed characterization at pH 4.5, 5.0 and 7.4.

Colloidal stability of 5:5 DT-loaded vesicles

The stability of the 5:5 vesicles was evaluated by measuring the particle size of DT-loaded (1.7 mg/ml DT) and empty vesicles by DLS (Table I). The z-average mean diameter of the vesicles ranged from 80 to 110 nm. The PDI of chosen formulations was less than 0.3, which indicates a moderately homogenous size distribution. The vesicles were colloidally stable at 4 ºC during a period of at least 14 days after preparation. Interestingly, the size of DT-loaded vesicles was marginally larger than that of the empty vesicles at pH 4.5, while the DT-loaded and empty vesicles at pH 5.0 and pH 7.4 did not differ in size.

Table I. Characteristics of DT-vesicle formulations in buffers with different pHs. Data represent mean ± SD of three batches.

L595:PEG-8-L:TR-70 = 5:5:1 (Molar ratio)						
pH	Buffer**	[DT] mg/ml	Size (nm)*			ζ-potential at day 1 (mV)
			Day 1	Day 7	Day 14	
7.4	PB	0	96.1 ± 2.8	92.2 ± 4.0	91.9 ± 2.9	-73.7 ± 3.4
7.4	PB	1.7	96.9 ± 2.1	95.8 ± 1.0	96.5 ± 2.3	-75.9 ± 3.6
5.0	CB	0	84.1 ± 2.4	85.2 ± 2.1	84.4 ± 2.5	-86.9 ± 3.4
5.0	CB	1.7	79.7 ± 2.1	80.3 ± 2.7	79.6 ± 2.9	-83.3 ± 3.5
4.5	CB	0	84.5 ± 4.9	85.1 ± 5.1	84.3 ± 4.4	-84.2 ± 1.4
4.5	CB	1.7	103.5 ± 7.2***	118.3 ± 14.9	114.7 ± 5.0	-77.7 ± 3.6***
4.0	Ac	0	80.2 ± 4.4	N.P.**	N.P.	N.P.
4.0	Ac	0.2	88.8 ± 5.7	-**	-	-

*The PDI ranged from 0.2 to 0.3.

**N.P.: Not performed; -: Visible precipitation.

***: Significantly different from the corresponding formulation without DT (p<0.05).

As the isoelectric point of DT is about 4.7, a pH shift from 5.0 to 4.5 will change the net charge of DT molecules from negative to slightly positive, which may affect the interaction between DT and the vesicles. Therefore, the surface charge of DT-loaded vesicles was measured using laser Doppler electrophoresis (Table I). The ζ potential of the vesicles varied between -74 mV (vesicles at pH 7.4) and -87 mV (vesicles at pH 5.0). The DT-loaded vesicles prepared at pH 4.5 have a less negative surface potential than the vesicles without DT. This difference in surface potential is probably due to the presence of association between DT and vesicles. At pH 5.0, no difference in ζ potential was observed for vesicles prepared in the presence and absence of DT, indicating that less DT is associated to the vesicles at this pH. The association between DT and vesicles as a function of pH and ionic strength was examined in more details.

Association of DT with 5:5 vesicles

pH-dependence (pH selection)

In these studies, SEC in combination with Lowry-Peterson and ELISA assays were used to obtain information about the DT-vesicle association in the formulations. The pH of the DT-vesicle formulations was 4.5, 5.0 in CB and 7.4 in PB, and the DT concentration was 1.7 mg/ml. DT's association to the vesicles exceeded 70% at pH 4.5 and was below 20% at pH 5.0 and 7.4 (Fig.

1a). The recoveries measured by Lowry-Peterson and ELISA assays were very similar (Fig. 1a and 1b), indicating that associated DT had maintained its antigenicity. At pH 4.5 almost 80% of the protein was measured to be associated with the vesicles by ELISA.

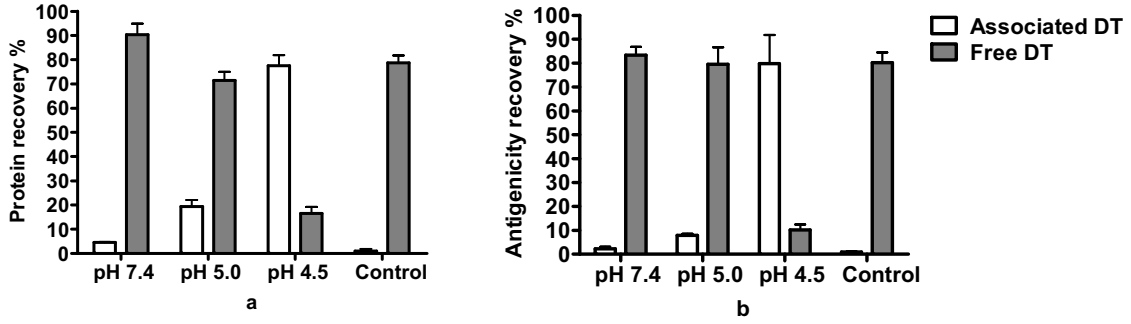

Figure 1. Effect of pH on association of DT and vesicles (5:5). Protein recovery (a) and antigenicity recovery (b) were determined after SEC fractionation of DT-vesicles at pH 4.5, 5.0 and 7.4. DT solution (1.7 mg/ml) in citrate buffer at pH 5.0 was used as control. Data shown are mean + SD (n=3).

Ionic strength (buffer selection)

Using SEC and subsequently the Lowry-Peterson protein assay, it was shown that the association of DT with the vesicles was independent on the ionic strength at all pH values (Fig. 2). This indicates that the ionic strength has little influence on the DT-vesicle association. Considering the shorter sonication time required for the preparation procedure and the low salt concentration leading to less remainder when applying the formulation non-occlusively onto the skin, DT-vesicles prepared in CB at pH 4.5 were chosen for further studies.

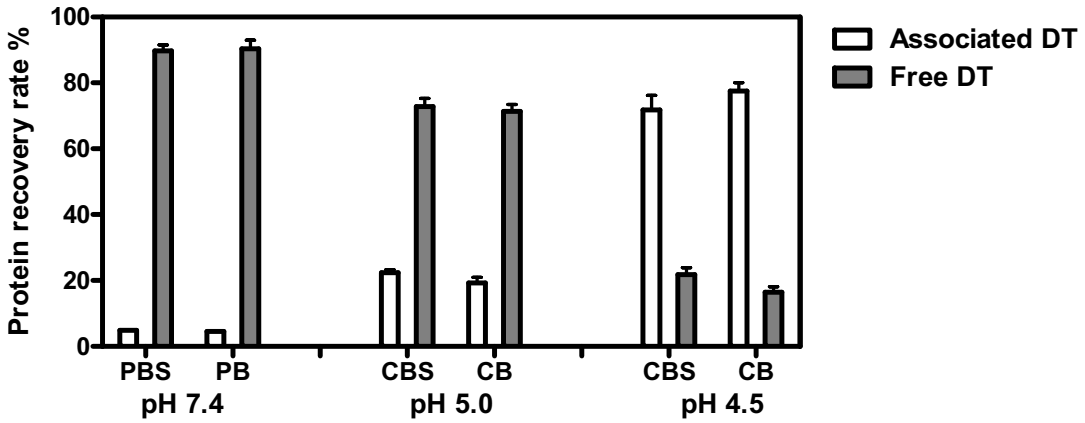

Figure 2. Influence of ionic strength on the DT-vesicle association (5:5 vesicles with 1.7 mg/ml DT). Protein recovery as detected by Lowry-Peterson protein assay was compared between PB and PBS, between CB and CBS at pH 5.0 and 4.5. Data shown are mean + SD (n=3).

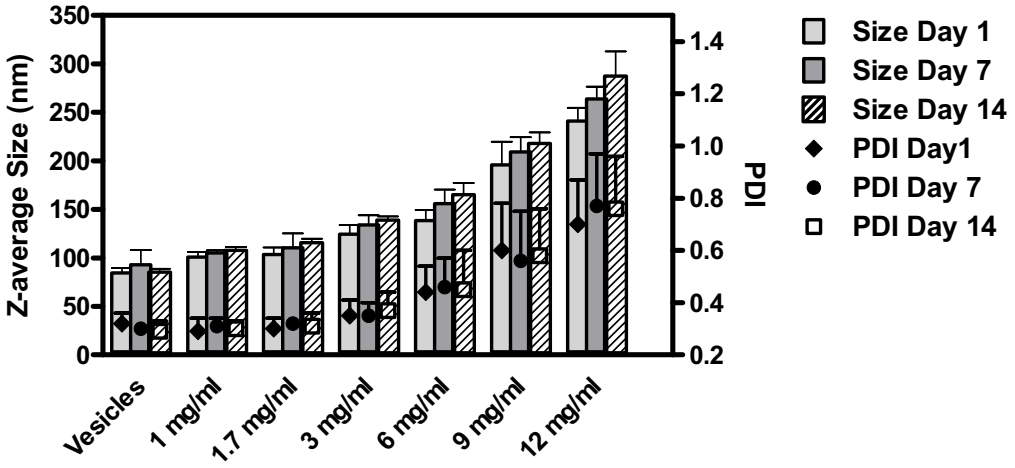

Figure 3. Effect of DT concentration on the particle size and PDI of DT-loaded vesicles (5:5) prepared at pH 4.5. Data represent mean + SD of three batches.

Loading capacity (DT concentration selection)

To study the loading capacity and stability of the 5:5 DT-loaded vesicles at pH 4.5, formulations with DT concentrations varying from 1.0 to 12.0 mg/ml in CB were prepared and evaluated for short-term colloidal stability during 2 weeks. The initial particle size increased with higher PDI at increasing DT concentrations (Fig. 3).The particle size tended to increase during storage, especially for the formulations containing high DT concentrations (Fig. 3). The ζ potential of DT-loaded vesicles (Fig. 4) became gradually less negative with increasing DT concentration, probably due to more DT association to the vesicles. To verify the ζ potential results quantitatively, the association of DT as a function of DT concentration was studied by SEC in combination with the Lowry-Peterson protein assay and ELISA. Formulation with 1 to 9 mg/ml of DT presented 75-95% association to the vesicles (Fig. 5). Similar values were seen for DT concentrations up to 3 mg/ml when the antigenicity was determined using ELISA (Fig. 5). However, formulations with 6 and 9 mg/ml DT showed a significantly lower antigenicity recovery than the detected protein recovery.

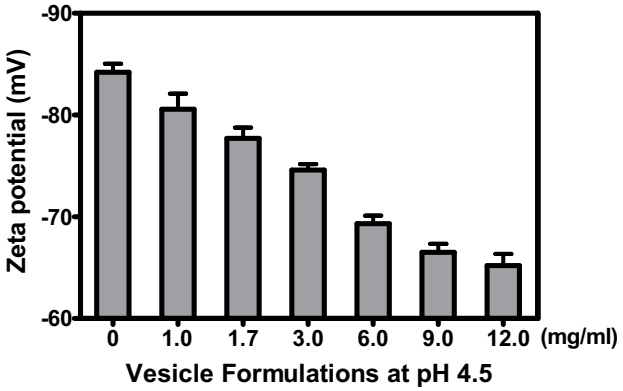

Figure 4. Z potential of empty vesicles and DT-loaded vesicles (5:5) with DT concentration from 0 to 12 mg/ml. Data represent mean + SD of three batches.

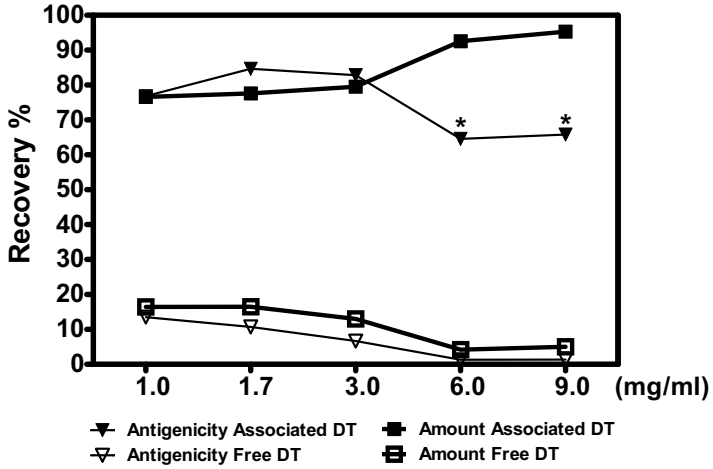

Figure 5. Effect of DT concentration (1 to 9 mg/ml) on the association of DT with vesicles (5:5) at pH 4.5. Protein recovery and antigenicity recovery were determined after SEC fractionation of the DT-vesicles. Data shown are mean of three batches. *indicates that difference between protein recovery and antigenicity recovery at DT concentration of 6 and 9 mg/ml are significant (p<0.01).

Effect of sonication and low pH on the DT structure

From the above studies, stable DT-vesicle formulations were obtained in CB at pH 4.5 by sonication. Since DT molecules may be sensitive to environmental factors such as sonication, low pH and the presence of vesicles; ELISA, fluorescence spectroscopy and SDS-PAGE were performed to determine whether the DT structure was preserved during formulation.

DT-vesicle formulations at pH 4.5 and pH 5.0, DT solution in CB at pH 5.0 after 3×30 s sonication were measured with ELISA and compared to the control, no significant difference was observed. The conformation of DT was studied by intrinsic tryptophan fluorescence spectroscopy. Consistent with

previous findings [30], untreated DT showed an average maximum fluorescence emission at a wavelength of 335 nm, whereas the maximum fluorescence emission wavelength of DT denatured by guanidinium hydrochloride was shifted to 353 nm, caused by an increased exposure of the tryptophan residues to the aqueous surroundings. The maximal emission wavelength of the DT-vesicle formulation at pH 4.5 and pH 5.0 and the DT solutions after sonication (3×30 s) remained at 335 nm, which indicates the absence of conformational changes of DT.

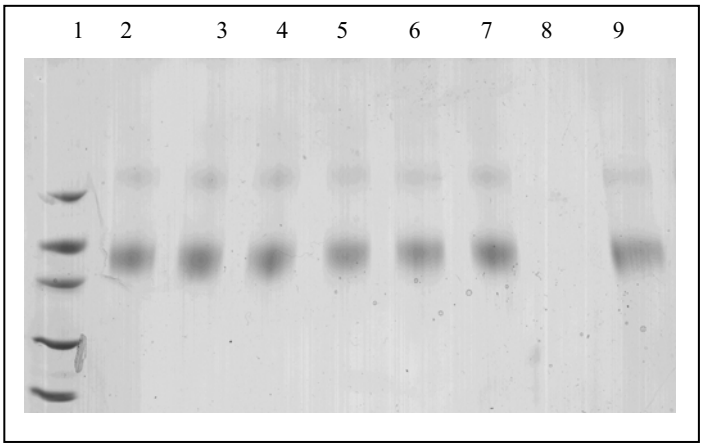

Figure 6. SDS-PAGE of DT solutions before and after sonication and DT-vesicle formulations. From lane1 to 9 are: 1) Broad range MW Marker; 2) DT in PBS as control; 3) DT in PBS after 3×10 s sonication; 4) DT in PBS after 3×30 s sonication; 5) DT vesicles in CB at pH 5.0 (3×5 s sonication); 6) DT vesicles in CBS at pH 5.0 (80 s sonication); 7) DT vesicles in CB at pH 4.5 (3×5 s sonication); 8) Empty vesicle control; 9) DT vesicles in CBS at pH 4.5 (80 s sonication).

SDS-PAGE under non-reducing conditions of DT solutions after 3×10 s or 3×30 s sonication, the DT-vesicle formulation in CB or CBS at pH 4.5 and pH 5.0 showed identical bands of similar intensities at the same positions as the untreated DT (Fig. 6). This indicates that the sonication used for formulation preparation and DT association with vesicles did not induce covalent aggregation or fragmentation of the protein.

The combined results from SDS-PAGE, fluorescence spectroscopy and ELISA point to full preservation of the DT structure after its formulation with 5:5 vesicles in CB at pH 4.5.

Discussion

In several TCI studies, DT and also other antigens derived from bacterial ADP-ribosylating exotoxins have been investigated. However, typical doses in these studies were 100 µg per vaccination [32, 33], which is much higher than

doses for injection. Therefore there is an urgent need for more efficient dermal vaccine delivery systems allowing lower antigen doses. In previous studies several groups report successful immunization *via* the dermal route using elastic vesicles [11-13]. It was also reported that surfactant-based vesicles served as adjuvants for co-administrated antigens given by injection [34] and topical immunization [35]. In the study described in this paper, the main focus was to formulate DT in colloidally stable elastic vesicle dispersions to be used for TCI.

Selection of DT-vesicle formulations

The characteristics of DT-vesicle formulations were strongly dependent on the vesicles composition and pH. As it has been reported that vesicles with L-595/PEG-8-L molar ratios of 7:3 and 5:5 are both elastic [16], these surfactant ratios were chosen to prepare DT-loaded vesicles. Our present studies revealed that the 5:5 vesicles are more stable than the 7:3 vesicles in the presence of DT. As oxyethylene headgroups are known to promote entropic stabilization [36], the higher levels of PEG-8-L in the 5:5 vesicles may count for the higher stability as compared to the 7:3 vesicles.

Elastic vesicles may act at least partly as a carrier system. Therefore, it is crucial to associate DT with the vesicles. At pH 4.5 DT is slightly positively charged and not readily dissolved in the buffer solution. Therefore at this pH DT-membrane interactions are facilitated, resulting in more than 70% of DT associated with vesicles. It has been reported that DT undergoes irreversible conformational change at pH values lower than 4.2, which facilitates the interaction between DT and lipid membrane [31]. However, in our studies the DT vesicle formulations at pH 4.0 were not stable. Therefore the formulations at a pH lower than 4.5 were not further investigated.

For vaccination, non-occlusive application and drying of the formulation during the application period (typically 1 h) is important for the unique interactions between elastic vesicles and skin [21, 22], and only a small volume of vesicle formulations can be applied on the limited area of mouse skin (~30 µl/cm^2). For dermal delivery, antigen concentration is normally of more importance than dose. Therefore, in order to determine the highest DT concentration at which stable vesicle formulations could be prepared, vesicle formulations with a DT concentration ranging from 1 to 12 mg/ml were examined. The results indicate that vesicle dispersions at DT concentrations higher than 3 mg/ml were not optimal for several reasons, namely; i) at DT

concentrations of 6 mg/ml and higher, the PDI increased rapidly indicating less homogeneous dispersions; and ii) association studies showed that at DT concentrations up to 3 mg/ml, protein recovery and antigenicity follow the same trend, whereas at DT concentrations of 6 mg/ml and higher, the recovered antigenicity was significantly lower than the amount of protein associated with vesicles. Considering the high PDI of these formulations and the visible aggregates in some of the 12 mg/ml DT-vesicle formulations, the fraction of vesicle-associated DT from 6 and 9 mg/ml DT formulations might contain DT aggregates as well, which might reduce the accessibility of the detecting antibodies to the epitopes of DT molecules. In conclusion, vesicle dispersions at pH 4.5 and a DT concentration up to 3 mg/ml appear to be optimal for TCI.

Mechanism of association

A pH-sensitive interaction between DT and vesicles was found in the current study, which is consistent with previous studies [31]. During SEC separation, it is difficult to explain the relatively low (~80%) recovery rate of DT in CB at pH 5.0 from the column (Fig. 1). However, nearly 100% of DT was recovered from SEC in the presence of the vesicles, probably due to the solubilizing properties of vesicle components. In the SEC washout curve, a tail part following the peak of vesicles was observed, indicating the possible presence of micelles formed mainly by PEG-8-L. Since DT has a pI of about 4.7, at pH 5.0 and 7.4, DT and vesicles are both negatively charged. Under these conditions DT remained ionized in solution and hardly any DT was associated with the vesicles. In contrast, at pH 4.5 free DT tends to form aggregates, possibly due to a decrease of the net charge and increase of hydrophobicity, whereas stable DT-vesicle formulations could be obtained. This indicates that DT is stabilized by association with the vesicles. As the ionic strength of the buffer did not affect the DT-vesicle association, hydrophobic interactions may contribute largely to the association between DT and vesicles.

However, the reduction in surface potential of DT-loaded vesicles compared to the empty vesicles at pH 4.5 indicates the interaction between slightly positively charged DT and the negatively charged vesicles. Therefore, the DT-vesicle electrostatic interactions might also play a role. Probably the opposite charge of the DT and the vesicles triggers the initial (long-range) electro statically driven force. Once associated, the hydrophobic interactions may contribute significantly due to the short-range van der Waals forces.

Therefore probably the hydrophobic domain of DT is intercalated in the bilayer of the vesicles. Furthermore, when applying the DT-loaded vesicle fraction onto the SEC column again, only vesicle-associated DT but no free DT could be eluted at pH 4.5, indicating a stable interaction between DT and the vesicles (data not shown). The order of DT addition to the formulation (before or after sonication) did not affect the DT-vesicle association in CB at pH 4.5 and pH 5.0 (data not shown), indicating that energy supply into the system by sonication did not promote association or cause degradation but only served to reduce the particle size of the vesicles.

Conclusion

In the present study, stable DT-containing vesicles with a high loading capacity were developed as a potential candidate for transcutaneous immunization studies aiming at more efficient vaccination.

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