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Stabilization of tetanus toxoid formulation containing aluminium hydroxide adjuvant against freeze-thawing

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ABSTRACT

Exposure to subzero temperature leads to loss of vaccine potency. This can happen due to degradation of adjuvant surface and/or inactivation of the antigen. When adsorbed on aluminium hydroxide and subjected to freeze-thawing, tetanus toxoid was desorbed from the gel matrix and the preparation was found to lose its antigenicity. Analyses showed that the gel particles were denatured after freezing. When freeze-thawing was carried out in the presence of glucose, sorbitol and arginine, the degradation of gel particles was inhibited. A higher fraction of the protein could be retained on the gel. However, the antigenicity of these preparations was quite low. In the presence of trehalose, the protein could be partially retained on aluminium hydroxide. Being a cryoprotectant, trehalose was also able to inhibit the freezing-induced denaturation of tetanus toxoid, which resulted in retention of antigenicity of the adjuvanted toxoid.

1. Introduction

Tetanus toxoid is a formaldehyde inactivated, attenuated form of the 150 kDa protein, tetanus toxin, which consists of two subunits (50 and 100 kDa) joined together by two disulphide linkages (Eisel et al., 1986). Tetanus toxoid provides immunity against tetanus, a devastating disease which remains a major public health problem in developing countries. According to the World Health Organization (WHO), tetanus was responsible for causing 2,13,000 deaths in 2002, ~85% of which were neonatal fatalities (Weekly epidemiological records, 2006). Immunization with tetanus toxoid has been identified as a key strategy to eradicate this disease.

Cold chain is a frequently used system of transporting and storing biopharmaceuticals within the recommended temperature range (2–8 °C), from the place of manufacture up to the time of administration (Miller and Harris, 1994). Temperature-labile biopharmaceuticals must be transported in a manner that ensures that the integrity of the products is not compromised. Monitoring and recording the temperature variation during transit are thus essential components of cold chain. Temperature monitoring devices are usually pre-validated single-use devices which report temperature variation during transport and are available for auditing by regulatory authorities. Recent studies in some developed and developing countries have found widespread freezing at many stages of the vaccine distribution system (Hazelton et al., 2002; Matthias et al., 2007; Nelson et al., 2004). Freezing was found to occur during all stages of the distribution chain except during storage at ambient temperature (Nelson et al., 2004). Since vaccines may quickly lose their potency if transported or stored outside the prescribed temperatures, it is essential to maintain an uninterrupted cold chain. If the cold chain is disturbed, the vaccine gets exposed to either low (freezing) or high temperature. Both can alter the stability of the vaccine. Improperly adjusted refrigeration equipment, poor compliance with cold chain procedures, inadequate monitoring and poor understanding of the dangers of freezing result in the inactivation of the vaccine in the cold chain.

Aluminium-salt adjuvants are widely used to increase immunogenicity of vaccines (Clapp et al., 2011; Marrack et al., 2009). The conventional mechanism of adjuvanticity of aluminium salts is hypothesized to be via depot formation of aluminium salt particles at the site of injection and slow release of the antigen from the depot (Clausi et al., 2008). Recently, some other mechanisms for immunopotentiation by adjuvants have been proposed. These include their role in activating complements and dendritic cells, and selectively inducing chemokines, thus regulating the type 2 immune response (HogenEsch, 2002). Adjuvants have also been shown to be important in delivering the antigens to dendritic cells (Romero Méndez et al., 2007). When vaccines formulated with the adjuvants are frozen or lyophilized, losses of efficacy are often reported. This loss of potency is usually attributed to the aggregation of adjuvant particles during processing. Freeze-concentration of buffer salts induces modifications in adjuvant surface chemistry and crystallinity, which in turn favour aggregation (Clausi et al., 2008).

A series of small molecules have been used to stabilize protein formulations, both in the solid and solution states. These

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molecules, often referred to as compatible solutes, have mostly been found to interact with the solvent and modulate the microenvironment around the protein molecule, thus stabilizing it against various stress conditions (Hamada et al., 2009; Lee and Timasheff, 1981). During freezing of a protein solution, ice crystals are formed which alter the solute concentrations in solution. This change in equilibrium affects the stability of the protein. Osmolytes, mostly disaccharides, amino acids, polyamines, etc., act as 'cryoprotectants' and inhibit the denaturation of the protein molecule against freezing-induced stress by the mechanism of preferential exclusion (Arakawa et al., 1990). Stabilization of several attenuated pathogens against freezing, drying and other stresses in the presence of small molecules has been reported (Amorij et al., 2007; Gupta et al., 1996; Roser, 1992; Roser et al., 2010). In case of vaccines which are supplied to users through 'cold chain', the stability problem is twofold. It has been seen in many cases that exposure of the vaccine to suboptimal temperatures during transit leads to degradation of both the antigen as well as the adjuvant (Chen et al., 2009). This is not a 'resource-limited' problem; it is seen in developed as well as developing countries. The antigen and the adjuvant are exposed to freezing temperature, which leads to degradation of both the protein and the gel matrix, resulting in a vaccine with reduced efficacy and in some cases, with no activity at all. Clausi et al. have studied freezing-induced agglomeration of Alhydrogel™ (Clausi et al., 2008). They have shown that degradation of the adjuvant can be prevented by incorporating glass-forming excipients such as trehalose. It was proposed that formation of a glassy matrix by trehalose does not allow further ice formation, thus protecting the adjuvant. The problem that we have tried to address in this work is to search for a stabilizing agent that can provide protection to both the protein and the adjuvant against freezing-induced denaturation. We found that glucose is superior to trehalose in protecting the adjuvant alone but cannot prevent the inactivation of the antigen during freeze-thawing.

2. Materials and methods

2.1. Materials

Tetanus toxoid solution (2550 Lf/unit with antigenic purity of 1572 Lf/mg of protein nitrogen) was obtained as a gift from Shantha Biotechnics Ltd., Hyderabad, India. Bovine serum albumin (BSA, fraction V), picrylsulphonic acid (2,4,6-trinitrobenzene sulphonic acid, TNBSA), Ellman's reagent [5,5′-dithio-bis (2-nitrobenzoic acid), DTNB] and aluminium hydroxide gel (13 mg/ml, Alhydrogel™, Cat. No. A8222) were purchased from Sigma-Aldrich, Bangalore, India. Anti-tetanus toxoid mouse monoclonal antibody (sc-58053) was obtained from Santa Cruz Biotechnology, California, USA. Goat anti-mouse IgG HRP (horse radish peroxidase) conjugated antibody and TMB/H_2O_2 (substrate for HRP) were products of Bangalore Genei, Bangalore, India. All other reagents and chemicals used were of analytical grade or higher.

2.2. Methods

2.2.1. Adsorption of tetanus toxoid on preformed aluminium hydroxide gel

The protocol followed for adsorption here has been modified from that described earlier (Gupta and Rost, 2000). Aluminium hydroxide gel (13 mg Al/ml of gel matrix) (10 ml) was added to the mixing vessel along with 1 ml of physiological saline (0.9% NaCl) and the contents were stirred (60 rpm) continuously at 4 °C. Dialyzed tetanus toxoid solution (6 mg/ml, 7.8 ml) was added to the gel solution and the solution was stirred (60 rpm) overnight at 4 °C. The aluminium hydroxide adsorbed tetanus toxoid thus obtained was stored at 4 °C till further use. At all stages, the pH of the reaction mixture was maintained at 5.9–6.2 with 5 N NaOH. For estimation of the amount of tetanus toxoid adsorbed on the adjuvant surface, the adsorbed preparation was centrifuged at 16,000 × *g* for 30 min at 4 °C. The amount of protein present in the supernatant was determined by the Bradford method, using bovine serum albumin (BSA) as the standard protein, and subtracted from the amount of protein initially added to the gel to calculate the amount of tetanus toxoid adsorbed on the gel matrix.

2.2.2. Freeze-thawing of tetanus toxoid

Aluminium hydroxide-adsorbed tetanus toxoid was subjected to freeze thawing by taking 200 μl of the suspension in 1 ml glass vials and sealing them with parafilm. The vials were kept upright at −20 °C for 12 h and then shifted to a water bath maintained at 37 °C for 2 h, this was termed as the first cycle of freeze thawing (Braun et al., 2009). The vials were re-incubated at −20 °C for 12 h to start the next cycle. This was repeated for a total of five cycles. Another set of vials was kept at −80 °C and subjected to the same treatment as above. The control sample vials were kept at 4 °C for the entire duration of freeze thaw cycles. After freeze-thawing, the solutions were centrifuged at 16,000 × *g* for 20 min at 4 °C. The amount of protein present in the supernatant was estimated by the Bradford method. The amount of protein desorbed from the matrix after freeze-thawing was calculated by subtracting the amount of protein present in the supernatant from the amount of starting protein. The amount of initially adsorbed protein taken for freeze-thawing was considered to be 100%. The freeze-thawed samples were analyzed by various analytical techniques as described in later sections. In case of freeze-thawing in the presence of osmolytes, the additives were added to the adsorbed toxoid at the desired concentrations, keeping the total volume of the reaction mixture unchanged (200 μl). Samples were also subjected to freeze-thawing in the absence of osmolytes. Control sample refers to the adsorbed toxoid incubated at 4 °C throughout the period without freeze-thawing.

2.2.3. Determination of antigenicity of tetanus toxoid

The antigenicity of tetanus toxoid samples was measured by carrying out enzyme-linked immunosorbent assay (ELISA) by following the protocol described earlier (Determan et al., 2006). Tetanus toxoid samples were serial diluted and coated on 96-well microtitre plates (high protein binding, Costar, Corning, Lowell, USA) for 18 h at 24 °C. Unbound antigen was removed with PBS-T (PBS containing 0.02% Tween 20). In a slight modification of the published protocol, unreacted sites were blocked with 0.2 M glycine (100 μl/well) for 0.5 h at 24 °C, followed by washing with PBS-T. Non-specific binding was eliminated by incubation with 2% BSA (in PBS) for 6 h, followed by washing with PBS. Mouse anti-tetanus toxoid monoclonal antibody (1:5000) was used as the primary antibody and HRP-conjugated anti-mouse antibody (1:3000) was used as the secondary antibody. The presence of tetanus toxoid was detected using tetramethyl benzidine/H_2O_2 (TMB/H_2O_2) as the substrate for HRP. The colour formed was measured at 450 nm (ELX808™ absorbance microplate reader, Biotek Instruments Inc., Winooski, USA) after terminating the reaction with 0.02 N H_2SO_4. For the aluminium hydroxide-adsorbed samples, antigenicity was determined for the whole preparation (including desorbed protein). The non-interference of adjuvant in the above process was confirmed by measuring the antigenicity of the protein alone (theoretically adsorbed on the gel matrix) and matching it with the colour generated with the adjuvant-adsorbed toxoid. Alhydrogel™ alone did not contribute to the generation of colour.

2.2.4. Determination of free amine groups (Snyder and Sobocinski, 1975)

Quantitative determination of number of free amine groups present in the samples was carried out by comparison with a standard curve using L-alanine as standard molecule. An aliquot (150 μl) of reconstituted tetanus toxoid samples was taken and diluted with 250 μl of reaction buffer (0.2 M sodium bicarbonate, pH 8.5). 250 μl of 2,4,6-trinitrobenzene sulphonic acid (TNBSA) solution (0.01%) was added and incubated at 37 °C. After incubation for 2 h, the reaction was stopped by the addition of 250 μl of 10% SDS and 125 μl of 1 N HCl. The samples were centrifuged at 13,200 × *g* for 5 min to remove suspended particles and the absorbance of the supernatant was measured at 335 nm (UV1700, Shimdazu, Kyoto, Japan). Samples were also subjected to freeze-thawing in the absence of osmolytes. Control sample refers to the adsorbed toxoid incubated at 4 °C throughout the period of freeze-thawing.

2.2.5. Determination of free sulphydryl groups (Riddles et al., 1983)

Reconstituted tetanus toxoid samples (1.25 ml each) were taken and an equal volume of reaction buffer (0.2 M sodium phosphate, pH 8.0, containing 2 mM EDTA) was added. The stock solution of 5-5′-dithiobis-(2-nitrobenzoic acid) (DTNB, Ellman's reagent) was prepared in the reaction buffer at 4 mg/ml. Ellman's reagent (50 μl) was added to the tetanus toxoid samples and incubated for 15 min at 37 °C. The colour generated by the samples was read at 412 nm (UV1700, Shimdazu, Kyoto, Japan). In case of aggregates, the samples were centrifuged at 13,200 × *g* for 5 min after the incubation period to remove suspended particles before measuring the absorbance of the supernatant. Free thiols were calculated using an extinction coefficient value of 14,150 M^{-1} cm^{-1} for the TNB dianion (Riener et al., 2002). Samples were also subjected to freeze-thawing in the absence of osmolytes. Control sample refers to the adsorbed toxoid incubated at 4 °C throughout the period of freeze-thawing.

2.2.6. Scanning electron microscopy

Different freeze-thawed samples were centrifuged. The pellet obtained was washed once with distilled water and centrifuged to remove interference due to buffer salts. No protein was detected in the washing. The washed pellet was resuspended in minimum volume of distilled water. 2 μl of each sample was deposited over broken cover slip and dried under air. Dried samples were gold coated by ion sputter (E1010, Hitachi High-Technologies Corporation, Tokyo, Japan) under vacuum (10 Pa) and viewed under scanning electron microscope (S-3400N, Hitachi High-Technologies Corporation, Tokyo, Japan).

2.2.7. Particle size analysis

Particle size of the samples was estimated by measuring the dynamic light scattering of the samples on Zeta sizer (Nano ZS, Malvern Instruments). For each sample, an average of five separate readings was recorded. The adjuvanted samples were diluted twofold with distilled water before recording the values immediately in triplicate.

2.2.8. Fluorescence spectroscopy

All adjuvanted samples were diluted to a final concentration of 1.06 μM in phosphate buffer (10 mM, pH 7.4). After dilution, no scattering due to the gel matrix was observed. It has been reported that on long-term storage, phosphate anions may be adsorbed on the surface of the adjuvant, altering its antigenicity (Wittayanukulluk et al., 2004). In our case, however, similar results were obtained when experiments were carried out with the control diluted in 50 mM Tris–HCl buffer, pH 7.4, probably because of the much shorter duration of the experiments (data not shown). Samples were excited at 280 nm and the intrinsic fluorescence spectra were recorded in the range of 300–400 nm. The excitation and emission slit widths were kept at 5 nm each. Spectra obtained were corrected by subtraction of the spectrum of phosphate buffer (10 mM, pH 7.4).

3. Results

3.1. Adsorption of tetanus toxoid on aluminium hydroxide

Aluminium hydroxide is reported to be capable of adsorbing higher amounts of tetanus toxoid (0.82 mg/mg of gel) than aluminium phosphate (0.16 mg/mg of gel) (Gupta and Rost, 2000). Hence, tetanus toxoid was adsorbed on aluminium hydroxide following the protocol described in Section 2.2.1. The concentration of adsorbed tetanus toxoid on aluminium hydroxide was calculated to be 0.78 mg of tetanus toxoid/mg of aluminium hydroxide. Accelerated study conditions were employed to mimic the destabilization of vaccines under freeze-thawing conditions. Freezing of the samples was carried out at −20 °C and −80 °C, followed by thawing. The amount of protein retained on the adjuvant following freeze-thawing was determined by the Bradford method (Table 1). The total protein (adsorbed on the gel matrix) could be recovered in the supernatant, showing that complete desorption of the protein from the adjuvant surface had occurred. This could be due to two reasons, either degradation of the adjuvant or inactivation of the protein following freezing. This has been investigated further later. During exposure to the stress of freeze-thawing, tetanus toxoid is desorbed from the adjuvant. It is important to determine whether this desorbed protein remains antigenic, as also the residual tetanus toxoid.

3.2. Loss in antigenicity

Antigenicity of tetanus toxoid adsorbed on aluminium hydroxide was determined by tetanus toxoid-specific ELISA using a slight modification of the published protocol (Determan et al., 2006), as described in Section 2.2.3. After five cycles of freeze-thawing at −20 °C, the loss in antigenicity of the total preparation was found to be 75% (Table 1). The residual antigenicity (25%) is due to the fraction of tetanus toxoid which did not lose the functional conformation following desorption from the matrix. As the freezing temperature was lowered to −80 °C, the loss in antigenicity was 60% (Table 1). After ten cycles, the loss in antigenicity remained almost the same (Table S1, supplementary information). Aluminium hydroxide-adsorbed tetanus toxoid, which was stored at 4 °C and not subjected to freeze-thaw cycles, did not exhibit any loss in antigenicity.

3.3. Determination of free amine groups

The change in the amount of free amine groups was determined by the standard 2,4,6-trinitrobenzene sulphonic acid (TNBSA) assay (Snyder and Sobocinski, 1975). A comparison of the number of free amine groups in the control and aggregated protein can give an idea of the chemical changes in the protein subjected to stress conditions and the nature of the aggregates formed. TNBSA reacts with free amine containing molecules and generates an orange coloured derivative that can be detected at 335 nm. The number of free amine groups in case of the control sample was 0.56 nmol/μg protein. After subjecting the adsorbed toxoid to freeze-thawing at −20 °C and −80 °C, this number was found to decrease (Table 2). No colour was seen when the adjuvant alone was subjected to the same analytical protocol. Since treatment with TNBSA reagent removes all non-covalent interactions, this decrease indicates that

Table 1
Amount of adsorbed protein and residual antigenicity after freeze-thawing of tetanus toxoid for five cycles. All experiments were carried out at least in triplicate and the average values are shown.

Sample	Adsorbed protein (%)		Residual antigenicity (%)	
	−20 °C	−80 °C	−20 °C	−80 °C
Without freeze-thawing	100.0		100.0	
No additive	0.97 ± 0.19	0.58 ± 0.19	25.0 ± 0.8	40.0 ± 0.2
0.05 M Glucose	32.16 ± 6.82**	47.37 ± 11.30*	46.0 ± 0.5***	46.0 ± 0.4***
0.15 M Glucose	73.68 ± 2.92***	86.94 ± 8.77***	31.5 ± 0.7**	39.0 ± 0.1**
0.05 M Sorbitol	25.15 ± 1.56***	15.20 ± 2.34**	29.5 ± 0.5**	34.0 ± 0.5***
0.15 M Sorbitol	52.02 ± 0.97***	33.92 ± 7.4*	70.3 ± 0.8***	21.0 ± 0.9***
0.05 M Arginine	12.48 ± 3.9*	52.44 ± 12.67*	17.5 ± 0.8**	23.5 ± 0.5***
0.15 M Arginine	69.79 ± 2.53***	76.02 ± 10.13**	35.0 ± 0.5***	40.0 ± 0.6
0.05 M Trehalose	49.12 ± 8.19**	37.42 ± 5.6**	45.0 ± 0.7***	63.5 ± 0.3***
0.15 M Trehalose	61.01 ± 11.31	35.87 ± 4.09***	71.0 ± 0.4***	48.9 ± 0.5***

* $p < 0.05$ v/s freeze-thawing without additive.
** $p < 0.01$ v/s freeze-thawing without additive.
*** $p < 0.001$ v/s freeze-thawing without additive.

covalent bonds formed via amine groups are involved in the aggregation of the adsorbed toxoid when it is subjected to iterative cycles of freeze-thawing. Reduction in the number of free amine groups following freeze thawing shows that amine groups are involved in crosslinking and this could be through the participation of formaldehyde in the reaction pathway.

3.4. Estimation of free thiol groups

The amount of sulphydryl groups was calculated from the molar extinction coefficient of TNB anion ($\varepsilon = 14{,}150\ M^{-1}\ cm^{-1}$) (Riener et al., 2002). Any alteration in the concentration of free thiol groups can be detected by Ellman's assay using Ellman's reagent, 5,5′-dithio-bis (2-nitrobenzoic acid) (Riddles et al., 1983). No colour was seen when the adjuvant alone was subjected to the same analytical protocol. Even without a free cysteine, disulphide-bonded proteins can undergo aggregation through disulphide exchange via β-elimination (Costantino et al., 1994). Tetanus toxoid has an isoelectric point of 5.8 (Tafaghodi et al., 2006) and is known to have 10 cysteine residues, of which 4 are involved in the formation of disulphide bonds (Bizzini et al., 1970). By estimating the concentration of free thiol groups in aggregates, the nature of the aggregates can be understood. The number of free thiol groups was found to decrease when aluminium hydroxide adsorbed tetanus toxoid was subjected to freeze thawing. The number of free thiol groups in case of the control sample was 45.44 nmol/mg protein. After subjecting the adsorbed toxoid to freeze-thawing at −20 °C, this number was found to decrease to 41.8 nmol/mg protein. There was no change in the number of free thiol groups with increase in the number of freeze-thaw cycles (Table S2, supplementary information). At −80 °C, there was a marginal decrease in the number of free thiol groups following freeze-thawing (Table 2). The number of free thiol groups was determined to be 42.8 nmol/mg protein. Thus, covalent linkages involved in the formation of aggregates may also be partly mediated by disulphide bridges.

3.5. Effect of osmolytes on the freeze-thaw stability of aluminium hydroxide-adsorbed tetanus toxoid

Aluminium hydroxide-adsorbed tetanus toxoid was subjected to freeze-thawing cycles as described earlier, in the presence of 0.05 and 0.15 M of four different osmolytes. In the absence of osmolytes, all the protein was found to be desorbed from the gel matrix following freeze-thawing. However, this preparation still retained some antigenicity (Table 1), presumably because of the residual soluble protein that is still a part of the desorbed preparation. It may be noted that the antigenicity of all samples measured includes both adsorbed and desorbed fractions.

When tetanus toxoid was subjected to freeze-thawing at −20° and −80 °C in the presence of glucose, the amount of protein retained on the matrix was found to increase with increase in the concentration of glucose (Table 1). The antigenicity of the total preparation was also found to increase. After five cycles of freeze-thawing in the presence of 0.05 M glucose at −20 °C, 32% of the protein was retained on aluminium hydroxide (Table 1) as compared to no retention in the absence of the additive. The amount of protein retained on the matrix was found to increase to 47% when freezing was carried out at −80 °C. In the presence of 0.15 M glucose, the amount of protein retained was higher. The antigenicity of the aluminium hydroxide-adsorbed preparation however showed

Table 2
Estimation of free amine groups and thiol groups after freeze-thawing of tetanus toxoid for five cycles. All experiments were carried out at least in triplicate and the average values are shown.

Sample (nmol/μg protein)	Free amine groups (nmol/μg protein)		Free thiol groups	
	−20 °C	−80 °C	−20 °C	−80 °C
Control	0.56 ± 0.03		45.4 ± 0.5	
No additive	0.35 ± 0.06*	0.39 ± 0.03*	41.8 ± 0.2**	42.8 ± 0.5*
0.05 M Glucose	0.43 ± 0.05	0.56 ± 0.02	41.3 ± 0.4**	41.3 ± 0.3**
0.15 M Glucose	0.48 ± 0.02	0.50 ± 0.06	41.9 ± 0.4**	41.9 ± 0.4**
0.05 M Sorbitol	0.41 ± 0.08	0.45 ± 0.08	40.7 ± 0.4**	40.7 ± 0.2***
0.15 M Sorbitol	0.46 ± 0.07	0.50 ± 0.03	40.0 ± 0.4***	40.0 ± 0.2***
0.05 M Arginine	0.41 ± 0.08	0.53 ± 0.05	40.7 ± 0.6**	40.7 ± 0.6**
0.15 M Arginine	0.51 ± 0.05	0.50 ± 0.03	43.4 ± 0.5*	43.4 ± 0.6
0.05 M Trehalose	0.43 ± 0.09	0.52 ± 0.06	45.3 ± 0.8	45.3 ± 0.5
0.15 M Trehalose	0.42 ± 0.04	0.47 ± 0.06	45.4 ± 0.3	45.4 ± 0.4

* $p < 0.05$ v/s control (without freeze-thawing).
** $p < 0.01$ v/s control (without freeze-thawing).
*** $p < 0.001$ v/s control (without freeze-thawing).

only a marginal increase when freeze-thawing was carried out in the presence of glucose, indicating that although glucose was able to retain tetanus toxoid on the adjuvant, it was not able to prevent the inactivation of the protein and/or adjuvant.

TNBSA assay showed that there was a reduction in the number of free amine groups of tetanus toxoid after subjecting it to five cycles of freeze-thawing at −20 °C. In the presence of 0.05 and 0.15 M glucose, there was an increase in the number of free amine groups although this did not reach the level of the control sample (0.56 nmol/μg protein). Thus, it is clear that aggregation of desorbed tetanus toxoid occurred via formation of covalent bonds involving free amine groups. Glucose was able to partially reverse this trend. This ability increased with the concentration of glucose used (Table 1). However, the antigenicity of the freeze-thawed preparation was not correspondingly high. The protective effect at −20 °C was marginally higher than at −80 °C.

Similar effects were seen with sorbitol and arginine at 0.05 and 0.15 M when tetanus toxoid was subjected to freeze-thawing at −20 °C and −80 °C. Both these osmolytes were able to partially inhibit desorption of the toxoid from the adjuvant, as seen by the levels of protein retained on the gel (Table 1). The contribution of arginine to the generation of colour during estimation of amount of protein was negligible due to dilution effects. The antigenicity of the preparation in the presence of 0.15 M arginine at −80 °C, however, was of the same level as that of tetanus toxoid subjected to freeze-thawing in the absence of any osmolyte. Estimation of the number of free amine groups by TNBSA assay showed that aggregation of the protein involved formation of covalent bonds via amine groups, which led to a reduction in their number in the control sample (without osmolyte). This trend could be partially reversed by sorbitol and arginine, similar to glucose (Table 2).

3.6. Effect of trehalose on freeze-thawing

When aluminium hydroxide-adsorbed tetanus toxoid was subjected to freeze-thawing in the presence of trehalose at −20 °C and −80 °C, the amount of protein retained on the gel matrix increased, as with other osmolytes. Interestingly, the residual antigenicity of this preparation was much higher than the toxoid preparation that had been subjected to freeze-thawing in the presence of glucose, sorbitol or arginine. It may be noted that our method of determination of antigenicity *in vitro* cannot measure the effect of adjuvant on the efficacy of the antigen. The amount of protein retained was 49% after freeze-thawing at −20 °C in the presence of 0.05 M trehalose (Table 1). This changed to 37% at −80 °C (Table 1). The amounts of protein retained were even higher when freeze-thawing was carried out at −20 °C in the presence of a higher concentration of trehalose. Thus, it appears that trehalose (at 0.05 and 0.15 M) is able to retain the protein on the gel matrix, although this effect is less pronounced than that in the presence of glucose. Simultaneously, and more importantly, being a cryoprotectant, trehalose is able to protect tetanus toxoid against freezing-induced denaturation. This is reflected in the high level of residual antigenicity of the toxoid. Analysis of free thiol groups showed that trehalose was able to prevent disulphide-mediated crosslinking of tetanus toxoid, as reflected by the retention of free thiol groups compared to the control sample (without freeze-thawing) (Table 2). Glucose, sorbitol and arginine were unable to inhibit disulphide scrambling in tetanus toxoid. Since trehalose cannot alter the redox potential of disulphide bond formation, it is likely that in the presence of trehalose, the desorbed toxoid cannot unfold and thus, disulphide crosslinking does not occur.

Scanning electron microscopy was carried out to monitor changes in the surface topology of the adjuvant following freeze-thawing. The presence of protein adsorbed on the adjuvant can be seen as uniformly distributed white specks (Fig. 1B). A clear change was seen in the surface morphology of the adjuvant on freeze-thawing at −20 °C (Fig. 1C) and −80 °C (Fig. 1E). The intensities of the white specks were reduced, indicating desorption of the antigen from the adjuvant surface, which correlated well with the data presented in Table 1. In addition, aluminium hydroxide matrix underwent agglomeration, as seen by the appearance of clumps in the micrographs. When the adjuvant containing tetanus toxoid was subjected to freeze-thawing at −20 °C in the presence of 0.15 M trehalose, partial agglomeration of the original structure of the matrix could be seen (Fig. 1D). More importantly, a significant fraction of the protein was seen to be retained by the matrix as reflected by the presence of white specks on the adjuvant surface. Thus, trehalose was able to inhibit desorption of the protein from the adjuvant, by partially preventing the collapse of the gel structure. In addition, trehalose was also able to inhibit freezing-induced denaturation of tetanus toxoid, thus retaining its antigenicity. Trehalose is a known cryoprotectant of proteins and has been used as a stabilizing osmolyte in many freeze-drying and freeze-thawing experiments (Bozdag et al., 2005; Roy and Gupta, 2004; Wolff et al., 2008). Unlike the other osmolytes used, viz. glucose, sorbitol and arginine, trehalose exhibits a dual protective role. It is able to maintain the integrity of surface morphology of the adjuvant, thus preventing desorption of tetanus toxoid from the matrix. Additionally, and more importantly, trehalose is able to inhibit freezing-induced denaturation of tetanus toxoid, thus retaining the antigenicity of the adjuvant-containing preparation.

3.7. Particle size analysis

In order to confirm the agglomeration of aluminium hydroxide when subjected to freeze-thawing at different temperatures for different cycles, the average particle sizes of all the samples were determined in the presence and absence of 0.15 M glucose and 0.15 M trehalose (Fig. 2). Light scattering has been used earlier for determining the particle size of aluminium hydroxide adjuvant particles (Clausi et al., 2008). The average size of the aluminium hydroxide particles increased significantly when the adjuvanted toxoid was subjected to freeze-thawing. When subjected to freeze-thawing at −20 °C, the average particle size increased from 1.8 (±0.03) μm to 3.3 (±0.05) μm (Fig. 2A). In the presence of 0.15 M trehalose and 0.15 M glucose, the average particle size decreased to 1.1 (±0.06) μm and 0.6 (±0.05) μm, respectively. Interestingly, when the freezing temperature was reduced to −80 °C, the average particle size of the freeze-thawed sample did not change significantly from that of the aluminium hydroxide particles although the distribution in particle size exhibited a wider variation (Fig. 2B). This distribution could be narrowed down in the presence of 0.15 M glucose so that the average particle size returned to that in the absence of freeze-thawing. However, this was not true in the case of freeze-thawing carried out in the presence of 0.15 M trehalose (Fig. 2B). The distribution remained as broad as in the case of freeze-thawing carried out in the absence of any compatible solute although a fraction of the distribution was centred on the particle size of aluminium hydroxide gel particles before freeze-thawing. This matches the results of SEM images of aluminium hydroxide gel when freeze-thawing was carried out in the presence of 0.15 M trehalose (Fig. 1F) where agglomeration of the gel was still observed, leading to partial desorption of tetanus toxoid from the gel matrix. This kind of partitioning of particle size into two distinct fractions has been reported earlier in the case of freezing of aluminium hydroxide in the presence of trehalose (Clausi et al., 2008). The presence of 0.15 M glucose was able to reduce the particle size distribution and decrease the particle size almost to that of the control. Glucose was able to inhibit the agglomeration of aluminium hydroxide gel under more adverse conditions (lower temperature, more number of freeze-thaw cycles) than trehalose. The protective

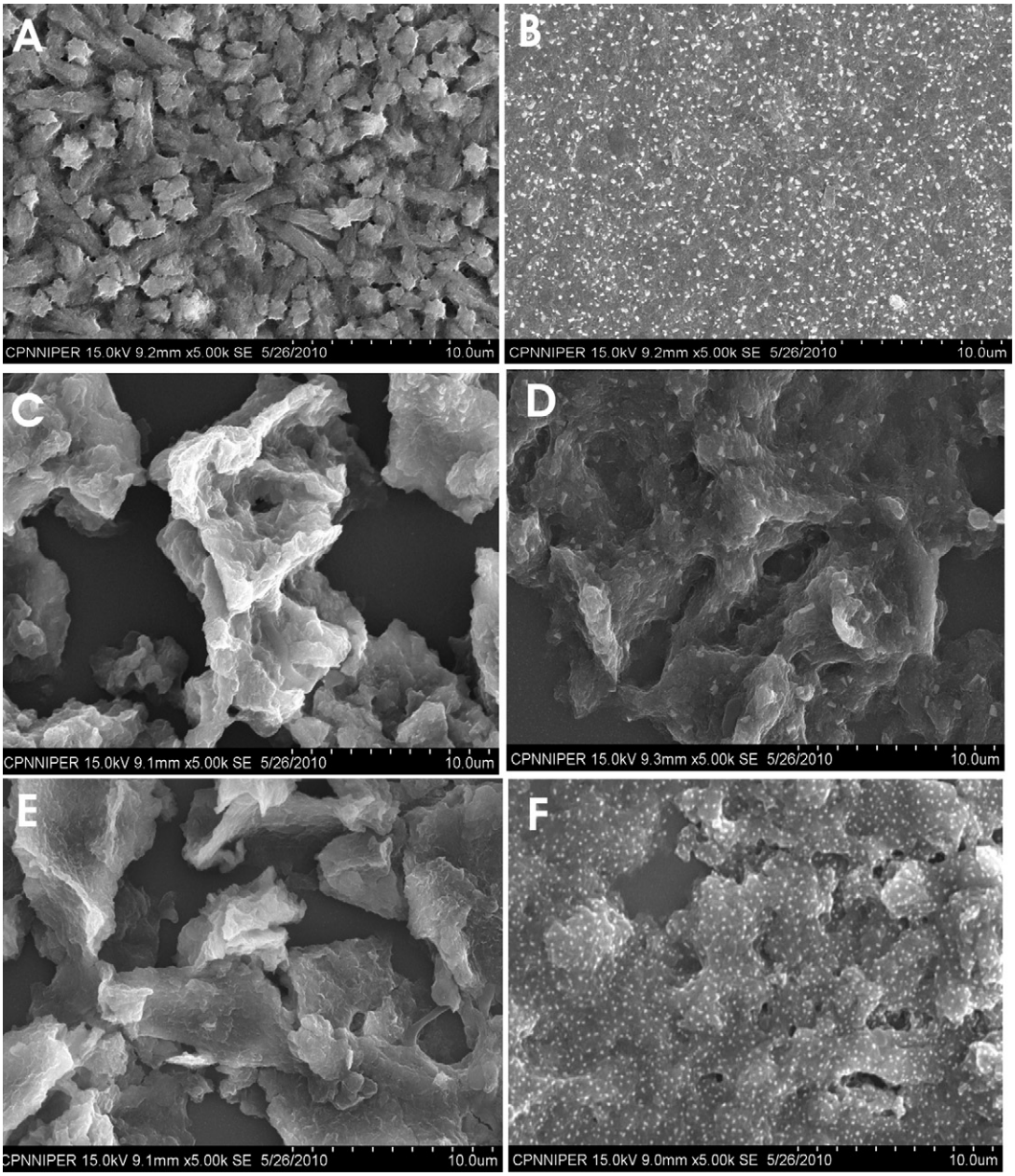

Fig. 1. Scanning electron microscopy of aluminium hydroxide-adsorbed tetanus toxoid subjected to five cycles of freeze-thawing. Samples: (A) aluminium hydroxide gel, (B) aluminium hydroxide-adsorbed tetanus toxoid, (C) aluminium hydroxide-adsorbed tetanus toxoid subjected to freeze-thawing at −20 °C, (D) aluminium hydroxide-adsorbed tetanus toxoid subjected to freeze-thawing at −20 °C in the presence of 0.15 M trehalose, (E) aluminium hydroxide-adsorbed tetanus toxoid subjected to freeze-thawing at −80 °C, (F) aluminium hydroxide-adsorbed tetanus toxoid subjected to freeze-thawing at −80 °C in the presence of 0.15 M trehalose, were reconstituted in sodium phosphate buffer (10 mM, pH 7.4) and were analyzed as mentioned in methods section. Scale (10 μm) is shown in each micrograph.

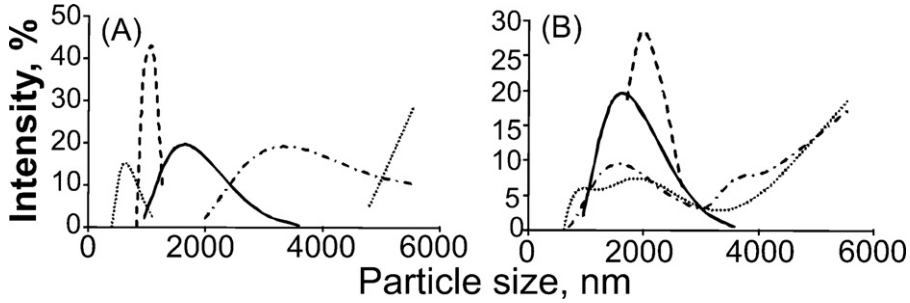

Fig. 2. Particle size analysis of aluminium hydroxide-adsorbed tetanus toxoid subjected to five cycles of freeze-thawing. Experiments were carried out in triplicate, representative distributions are shown. Samples: aluminium hydroxide-adsorbed tetanus toxoid subjected to (A) freeze-thawing at −20 °C, (B) freeze-thawing at −80 °C: aluminium hydroxide-adsorbed tetanus toxoid control incubated at 4 °C without freeze-thawing (solid line), aluminium hydroxide-adsorbed tetanus toxoid subjected to freeze-thawing (dash-dot-dash line), aluminium hydroxide-adsorbed tetanus toxoid subjected to freeze-thawing in the presence of 0.15 M glucose (dashed line), and aluminium hydroxide-adsorbed tetanus toxoid subjected to freeze-thawing in the presence of 0.15 M trehalose (dotted line).

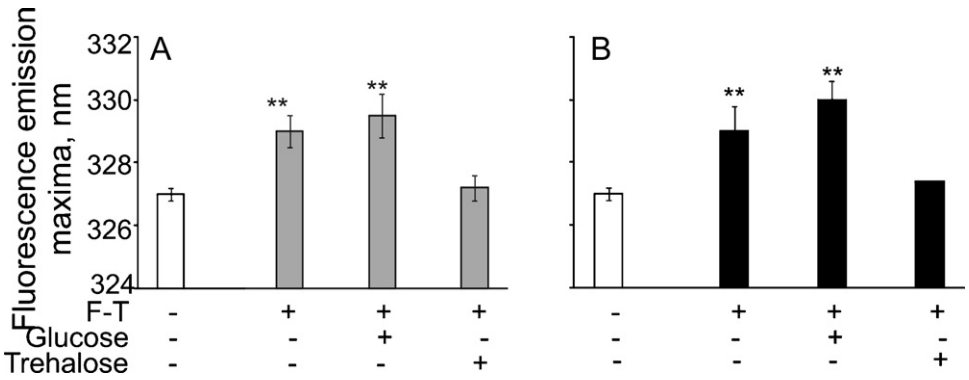

Fig. 3. Fluorescence spectroscopic analysis of aluminium hydroxide-adsorbed tetanus toxoid subjected to five cycles of freeze-thawing (F-T) at (A) −20 °C and (B) −80 °C in the presence and absence of 0.15 M glucose and 0.15 M trehalose. All samples were diluted 200 times and excited at 280 nm using a Cary Eclipse spectrofluorimeter (Varian). Negligible scattering was observed under these conditions. The emission spectra were recorded in the wavelength range of 300–400 nm after excitation at 280 nm. The spectrum of the buffer was recorded and subtracted from the other spectra. Figures represent mean (±SEM) of three independent experiments; (**) represents $p < 0.01$ with respect to control (empty bar) (not subjected to freeze-thawing).

effect of glucose however did not extend to the freezing-induced denaturation of tetanus toxoid.

3.8. Intrinsic fluorescence spectroscopy

Any change in the tertiary fold of the protein can be monitored by changes in the fluorescence emission spectrum of the protein. The fluorescence spectra of whole aluminium hydroxide-adsorbed tetanus toxoid samples were recorded after subjecting them to freeze-thawing under different conditions. In the absence of any osmolyte, the fluorescence spectra of the desorbed toxoid exhibited a red shift, with λ_{max} shifting from 327 nm (for control) to 329 nm when the adsorbed sample was subjected to freeze-thawing at −20 °C and −80 °C (Fig. 3). This red shift is normally associated with unfolding of the protein molecule leading to exposure of the fluorophores to a more polar solvent (Lakowicz, 2006) and correlates well with the loss in antigenicity observed in case of freeze-thawing in the absence of any osmolyte. The unfolding pattern also matches well with the reduced antigenicity of the freeze-thawed preparation. When freeze-thawing was carried out in the presence of 0.15 M glucose at −20 °C and −80 °C, the fluorescence spectrum of the adsorbed toxoid continued to show this red shift (Fig. 3). Thus, glucose was not able to protect the protein against freezing-induced denaturation and thus, the structural integrity could not be regained. The loss in antigenicity observed earlier in the case of adsorbed toxoid freeze-thawed in the presence of 0.15 M glucose (Table 1) can thus be correlated to changes in the three-dimensional architecture of tetanus toxoid. On the other hand, when freeze-thawing was carried out in the presence of 0.15 M trehalose at −20 °C and −80 °C, the fluorescence spectra of the adsorbed toxoid did not exhibit this red shift any longer and reverted back to the λ_{max} of the original spectrum (of control sample without freeze-thawing) (Fig. 3). Thus, trehalose was able to inhibit the change in protein structure on freezing, leading to retention in the antigenicity of the freeze-thawed samples. This matches with the observed retention of antigenicity of the freeze-thawed antigen (Table 1), even when it is desorbed from the adjuvant surface.

4. Discussion

According to WHO guidelines, 80% of the antigen needs to be adsorbed on the adjuvant surface. However, it has been shown in a number of cases that the physical adsorption of the antigen on the adjuvant is not required for immunopotentiation (Chang et al., 2001; Flebbe and Braley-Mullen, 1986; Kanra et al., 2003; Romero Méndez et al., 2007). In fact, in some cases, separate administrations of antigen and adjuvant have provided the host organism with the required antigenicity (Flebbe and Braley-Mullen, 1986).

Aluminium hydroxide-adjuvanted products are generally not subjected to freezing (or freeze-drying) since the structure of the gel matrix collapses and forms an agglomerated structure. This is an irreversible process. The structure is also thought to disintegrate by elimination of the repulsive forces between the aluminium hydroxide particles due to increase in the local solute concentration following crystallization of ice, which forces them to flocculate (Zapata et al., 1984). In either case, the structural integrity of the gel matrix is lost, which mostly results in loss of antigenicity of the vaccine (Csizer et al., 1986; Diminsky et al., 1999). Any vaccine stabilizer thus has two different roles as far as the retention of the integrity of the vaccine is concerned. The first one is to ensure that surface morphology of the gel matrix is retained so that it does not form clumps following freeze-thawing. The second is to protect the antigen itself from the deleterious effect of freezing. In the case of freeze-drying of proteins, the role of glucose is reported to be restricted to that of a lyoprotectant. It has been suggested that glucose should be a part of a two-component system, e.g. with polyethylene glycol (PEG), where PEG provides stability to the protein during freezing stress while glucose provides protection during dehydration of the protein (Roy and Gupta, 2004). PEG has been employed to improve the stability of hepatitis B vaccine against freeze-thawing (Braun et al., 2009). Despite being a cryoprotectant, PEG is known to destabilize proteins in unfrozen solutions at higher (ambient) temperature (Arakawa et al., 1990). Thus, its use under ambient conditions needs to be avoided. In the current case, glucose was able to prevent the desorption of tetanus toxoid from aluminium hydroxide, as reflected by the higher amount of protein adsorbed on the matrix but with reduced antigenicity of the final preparation. Trehalose is thought to act as a cryoprotectant of proteins by forming an immobilization matrix around the protein, which restricts the mobility and hence reactivity of the protein (widely referred to as the *vitrification theory*) (Jain and Roy, 2009).

Aluminium hydroxide particles, with size less than 10 μm, are able to act as adjuvants (Clausi et al., 2008). In the results described in this work, the particle sizes of all gel matrices, which have been subjected to freeze-thawing, are within the prescribed range. It has been hypothesized, though not proven, that particles with smaller size are able to stimulate the immune system better (Clausi et al., 2008; Nygaard et al., 2004). This could be because of the larger surface area that they offer for the release of the antigen. Thus, the osmolyte-containing vaccine preparations, which have a smaller size, will be able to act as more potent antigens. In addition, in the presence of trehalose, a cryoprotectant, the three-dimensional folding of the antigen is retained during freezing, and the anti-

genicity of the adjuvanted formulation is the highest among all studied.

5. Conclusion

Inadvertent freezing of adjuvanted antigens results in inactivation of many vaccines. In this work, we have shown that judicious incorporation of compatible solutes can maintain the integrity of both adjuvant and antigen in case of tetanus toxoid adsorbed on Alhydrogel™ against freezing-induced stress. What is also clear is that a similar selection strategy can be adopted to select stabilizers against other kinds of stress conditions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.ijpharm.2011.05.022.

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