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Stabilization of tetanus toxoid formulation containing aluminium hydroxide adjuvant against agitation

Vipul A. Solanki, Nishant K. Jain, Ipsita Roy*

Department of Biotechnology, National Institute of Pharmaceutical Education and Research (NIPER), Sector 67, S.A.S. Nagar, Punjab 160062, India

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ABSTRACT

The aggregation of tetanus toxoid leads to reduced bioavailability of the vaccine and failure of immunization programmes in many parts of the globe. One of the main reasons for denaturation and aggregation of tetanus toxoid formulations is agitation of the protein during transport. We have identified that agitation leads to collapse of the gel matrix of aluminium hydroxide which is used as an adjuvant in these preparations. This results in desorption of the toxoid from the matrix, which then loses its antigenicity due to agitation-induced denaturation of the protein. We show that incorporation of some compatible osmolytes like sorbitol, glucose and arginine, but not trehalose, is able to protect the adjuvant matrix from degradation, and retain the integrity of the vaccine preparation in terms of its antigenicity.

1. Introduction

Tetanus toxoid is a formaldehyde-inactivated, attenuated form of tetanus toxin. The protein consists of two subunits (50 kDa and 100 kDa) joined together by two disulphide bonds (Eisel et al., 1986). Exposure to mechanical, thermal and chemical stresses is reported to affect the stability of the protein (Chang and Gupta, 1996; Ho et al., 2002; Schwendeman et al., 1995). Conformational changes caused by these stresses lead to interaction between buried hydrophobic clusters of the protein and finally aggregation (Costantino et al., 1996).

Agitation-induced stress is commonly used to judge the physical stability of a protein formulation (Eppler et al., 2010). Agitation can cause an increase in the interaction between protein molecules. It also creates an air–water or glass (vial)–water interface wherein the hydrophobicity of air (or glass) initiates aggregation by exposing the hydrophobic residues of the protein to air or glass (Bam et al., 1998; Eppler et al., 2010; Mahler et al., 2009; Marin et al., 2010). The response of a protein to shaking or agitation varies with the nature of the protein. Proteins which can interact strongly via hydrophobic moieties with the hydrophobic air/glass–water interface are unfolded faster. For formulations in solution phase which need to be transported to regions with extreme climate, agitation-induced aggregation is a major area of concern. The formation of aggregates can be either reversible if only physical forces are involved or irreversible. In case of adjuvanted vaccines like tetanus toxoid, the effect of agitation on the integrity of the matrix structure constitutes an additional parameter determining the efficacy of the final preparation (Shi et al., 2004). Osmolytes or compatible solutes have been used quite commonly for stabilization of proteins against a variety of stress conditions (Arakawa et al., 2001; Lee and Timasheff, 1981). These are thought to interact with the solvent and alter the environment around the protein molecule, thus stabilizing them against unfolding and subsequently denaturation, when exposed to a variety of harsh environmental factors (Wang, 2005). Non-ionic surfactants have been suggested as stabilizers of proteins against shearing-induced stress since they diminish the unfolding of proteins (Eppler et al., 2010; Marin et al., 2010). However, osmolytes such as sugars have found use as stabilizer in case of agitation of recombinant human granulocyte colony stimulating factor (Pavisić et al., 2010). In case of toxoid, in addition to the protein (antigen), the stability of the adjuvant matrix is a second factor to be taken care of. We and others have reported earlier that sugars such as glucose (Solanki et al., 2011) and trehalose (Clausi et al., 2008) were able to protect aluminium hydroxide gel against freezing-induced denaturation. We have checked the stabilizing effect of some compatible osmolytes on agitation-induced denaturation of adjuvanted tetanus toxoid.

* Corresponding author. Tel.: +91 172 2292 061; fax: +91 172 2214 692.
E-mail address: ipsita@niper.ac.in (I. Roy).

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2. Materials and methods

2.1. Materials

Tetanus toxoid (2550 Lf/unit with antigenic purity of 1572 Lf/mg of protein nitrogen) was obtained as a gift from Shantha Biotechnics Ltd., Hyderabad, India. Aluminium hydroxide gel (Alhydrogel™) and bovine serum albumin (BSA, fraction V) were purchased from Sigma–Aldrich, Bangalore, India. Mouse anti-tetanus toxoid monoclonal antibody (HYB 278-01) was purchased from Santa Cruz Biotechnology, CA, USA. Goat anti-mouse IgG HRP (horse radish peroxidase) conjugated antibody and TMB/H_2O_2 (substrate for HRP) were obtained from Bangalore Genei, Bangalore, India. All other reagents and chemicals used were of analytical grade or higher and purchased from Sigma–Aldrich, Bangalore, India, Sisco Research Laboratories, Mumbai, India, etc.

2.2. Methods

2.2.1. Adsorption of tetanus toxoid on preformed aluminium hydroxide gel

Adsorption of tetanus toxoid on preformed aluminium hydroxide gel was carried out following modification of an earlier protocol (Gupta and Rost, 2000). Briefly, preformed aluminium hydroxide gel (13 mg Al/ml of gel matrix) (10 ml) was mixed with 1 ml of physiological saline (0.9% NaCl) in a vessel. Tetanus toxoid solution (6 mg/ml, 7.8 ml) which was previously dialyzed (for 7.5 h with buffer change after every 1.5 h) with phosphate buffer (10 mM, pH 7.4) to remove thiomerosal was added to the gel solution and stirred (60 rpm) overnight on a magnetic stirrer (Corning Incorporated, Lowell, USA) at 4 °C. This preparation was called aluminium hydroxide adsorbed tetanus toxoid and stored at 4 °C till further use. The pH of the preparation was kept at 5.9–6.2 with 5 N NaOH at all stages. For the estimation of the amount of toxoid adsorbed on aluminium hydroxide gel, the adsorbed preparation was centrifuged (16,000 × *g*) for 30 min at 4 °C. The resulting supernatant was analyzed for the amount of protein present by the dye-binding method (Bradford, 1976) using bovine serum albumin (BSA) as the standard protein. The amount obtained was 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. Agitation of tetanus toxoid

Aluminium hydroxide-adsorbed tetanus toxoid samples were kept for agitation at 300 rpm at 37 °C in 5 ml glass vials in an incubator shaker. Shaking speeds of 150–250 rpm have been used earlier for agitation-induced stress studies of monoclonal IgG antibodies (Eppler et al., 2010). Two different concentrations, 1 mg ml^{-1} and 3 mg ml^{-1} (0.01 M phosphate buffer, pH 7.4), of protein suspension were used for agitation studies. The vials were taken out at different time intervals. The controls were kept for the same time intervals at 4 °C without agitation. The samples were centrifuged (16,000 × *g*) for 20 min at 4 °C after agitation. The amount of protein present in the supernatant was estimated by the dye-binding method (Bradford, 1976) using BSA as the standard protein. The amount of protein precipitated was determined by subtracting the amount of protein present in the supernatant from the protein initially adsorbed on the gel.

2.2.3. Determination of antigenicity of tetanus toxoid

The antigenicity of aluminium hydroxide-adsorbed tetanus toxoid upon agitation was determined by enzyme-linked immunosorbent assay (ELISA) following slight modification of the published protocol (Determan et al., 2006). In brief, serial diluted aliquots of standard tetanus toxoid and agitated samples were coated on 96-well microtitre plates (high protein binding, Costar, Corning, Lowell, USA). After incubation for 18 h at 24 °C, unbound antigen was removed by washing the wells with PBS containing 0.02% (v/v) Tween 20. Unreacted sites were blocked with 0.2 M glycine for 0.5 h at 24 °C followed by washing with PBS containing 0.02% (v/v) Tween 20. Non-specific binding was eliminated by treating the wells with 2% (w/v) BSA for 6 h at 24 °C. Mouse anti-tetanus toxoid monoclonal antibody at a dilution of 1:5000 in 2% (w/v) BSA was used as the primary antibody. HRP-conjugated anti-mouse antibody at a dilution of 1:3000 in 2% (w/v) BSA was used as the secondary antibody. Tetramethyl benzidine/H_2O_2 (TMB/H_2O_2) was used as the substrate for HRP for the detection of tetanus toxoid. The reaction was terminated by 0.02 N H_2SO_4. The absorbance of colour formed was measured at 450 nm (ELX808™ absorbance microplate reader, Biotek Instruments Inc., Winooski, USA). Whole preparations (including desorbed protein) of aluminium hydroxide-adsorbed samples were used for determination of antigenicity. Antigenicity of the protein alone (theoretically adsorbed on the gel matrix) was measured and matched with colour generated with the aluminium-adsorbed toxoid to confirm the non-interference of adjuvant (Alhydrogel™) in the above analysis.

2.2.4. Scanning electron microscopy

Samples of tetanus toxoid agitated in the presence and absence of excipients were centrifuged at 4 °C and the pellet obtained was washed once with distilled water followed by centrifugation at 4 °C to eliminate interference of buffer salts. No protein was detected in the washings. The washed pellet was resuspended in minimum volume of distilled water and an aliquot (2 μl) was placed over a broken cover slip. Samples were dried under air at room temperature for 1 h and gold coated by ion sputter (E1010, Hitachi High-Technologies Corporation, Tokyo, Japan) under vacuum (10 Pa) for 30 s. Coated samples were viewed under scanning electron microscope (S-3400N, Hitachi High-Technologies Corporation, Tokyo, Japan).

2.2.5. Polyacrylamide gel electrophoresis

Denaturing polyacrylamide gel electrophoresis (SDS-PAGE) (Laemmli, 1970) was run to characterize the aggregates of toxoid. For the aluminium hydroxide adsorbed tetanus toxoid, the pellet of the agitated samples was mixed with the gel loading buffer, heated at 100 °C for 5 min (Accublock™ Digital Dry Bath, Labnet, USA) and centrifuged for 5 min and finally the supernatant was loaded on to 8% crosslinked polyacrylamide gel. The gel was stained by coomassie brilliant blue R-250 followed by destaining with a destaining solution (10% acetic acid, 7.5% methanol in water).

3. Results and discussion

3.1. Adsorption of tetanus toxoid on preformed aluminium hydroxide gel

Tetanus toxoid solution was adsorbed on aluminium hydroxide according to the protocol described (Gupta and Rost, 2000). The concentration of adsorbed tetanus toxoid on aluminium hydroxide was calculated to be 0.78 mg of protein/mg of aluminium hydroxide by dye binding method (Bradford, 1976).

3.2. Agitation of tetanus toxoid with and without additives

Agitation of aluminium hydroxide-adsorbed tetanus toxoid samples was carried out at two different concentrations, 1 mg ml^{-1} and 3 mg ml^{-1} (0.01 M phosphate buffer, pH 7.4), of protein suspension. The amount of protein desorbed after different time intervals was determined by the dye-binding method (Bradford, 1976). Osmolytes were added to the agitated suspensions at concentrations of 0.05 and 0.15 M. A significant amount of the protein was

Table 1
Determination of amount of tetanus toxoid desorbed from the adjuvant following agitation at 300 rpm for 120 min when the initial amount of tetanus toxoid was 200 μg. Values represent mean (±SEM) of three independent experiments.

Condition	Protein desorbed (μg)
No excipient	142.7 ± 10.8
50 mM trehalose	132.9 ± 2.3
150 mM trehalose	127.9 ± 5.0
50 mM sorbitol	138.1 ± 4.8
150 mM sorbitol	129.2 ± 2.3
50 mM arginine	138.3 ± 3.2
150 mM arginine	135.9 ± 2.6
50 mM glucose	131.5 ± 2.9
150 mM glucose	125.5 ± 1.0

desorbed after 120 min of agitation (Table 1). This desorbed protein underwent aggregation and could not enter the denaturing polyacrylamide gel (Fig. 1). No significant difference was detected between the amount of protein desorbed in the presence and absence of stabilizers (Table 1). This could be due to the inability of the additives to inhibit desorption of tetanus toxoid from aluminium hydroxide gel. To confirm this, SDS-PAGE of the whole samples was carried out. In case of the control sample (agitated in the absence of any additive), the desorbed protein aggregated following agitation for 120 min and was thus unable to enter the gel (Fig. 1a). In the presence of either sorbitol or trehalose, the desorbed protein was able to enter the crosslinked gel, indicating that the protein had not aggregated. In the presence of glucose or arginine, the desorbed protein was able to enter the gel although the amount of gel-permeable tetanus toxoid was less in this case (data not shown). Thus, although the additives were unable to prevent desorption of the toxoid from the adjuvant, they were successful in keeping the desorbed protein in the soluble form.

3.3. Analysis of antigenicity

The antigenicity of the preparation was determined as per the reported protocol using enzyme-linked immunosorbent assay (ELISA) (Determan et al., 2006). Within 30 min of agitation at 300 rpm, the loss in antigenicity of the control tetanus toxoid sample was more than 80% (Fig. 2a–h). Osmolytes were added to the agitated suspensions at concentrations of 0.05 and 0.15 M. In the presence of sorbitol, the effect on the retention of antigenicity was quite dramatic (Fig. 2a and b). At a protein concentration of 1 mg ml^{-1}, the protective effect of both 0.05 and 0.15 M sorbitol was evident. After 120 min of agitation, when the control sample (without excipient) lost more than 88% antigenicity, the residual antigenicity was more than 86% in the presence of 0.05 M sorbitol, which increased to 91% in the presence of 0.15 M sorbitol. At a higher concentration of protein, sorbitol was less effective in maintaining the antigenicity of the preparation. The residual antigenicity was 58% in the presence of 0.05 M sorbitol in case of 3 mg ml^{-1} suspension, which increased to almost that of the original value at 0.15 M sorbitol. The excipient-free adsorbed toxoid had residual antigenicity of 13% after 120 min at this protein concentration. Similar results were obtained when agitation of adsorbed tetanus toxoid was carried out in the presence of glucose (Fig. 2c and d) and arginine (Fig. 2e and f). In case of trehalose, on the other hand, the results looked less promising. At both concentrations of protein, retention of antigenicity was not observed after agitation in the presence of either 0.05 or 0.15 M trehalose (Fig. 2g and h). As has been seen in case of tetanus toxoid and other antigens, efficacy of the vaccine can be retained even if the antigen is not adsorbed on the adjuvant (Chang et al., 2001; Flebbe and Braley-Mullen, 1986; Kanra et al., 2003; Romero Méndez et al., 2007; Noe et al., 2010). Thus, the stabilizer has dual roles; it should be able to stabilize

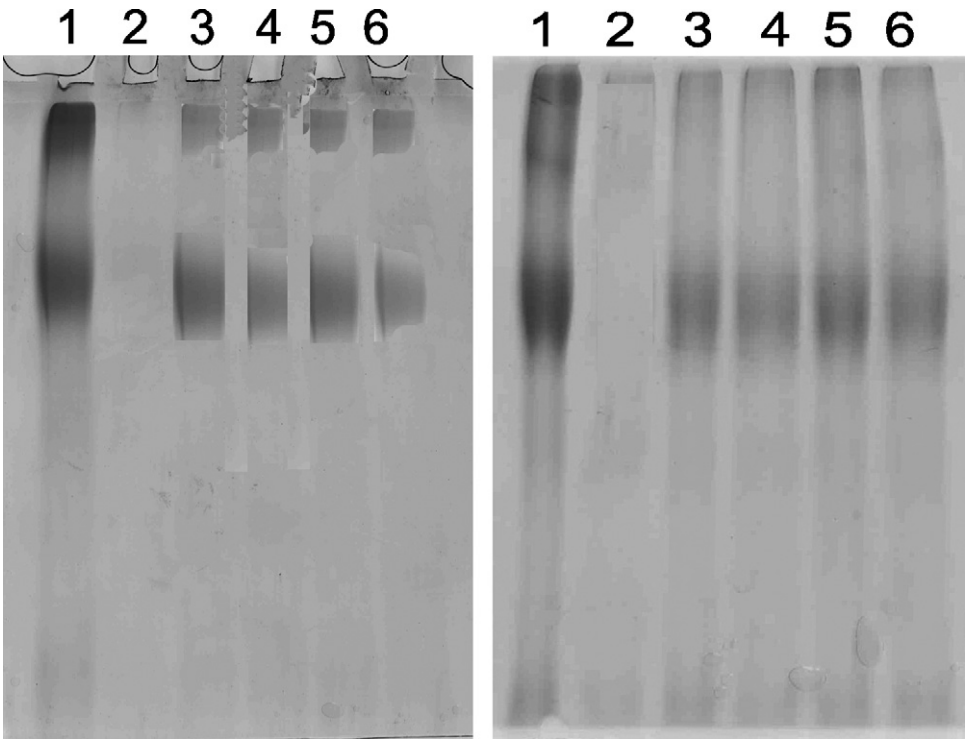

Fig. 1. SDS-PAGE of aluminium hydroxide-adsorbed tetanus toxoid after agitation at 1 mg ml^{-1} (A) and 3 mg ml^{-1} (B) concentrations in the absence and presence of additives. Electrophoretic analysis was carried out using standard protocol (Laemmli, 1970). Lane 1, native tetanus toxoid; lane 2, adsorbed tetanus toxoid agitated for 120 min; lane 3, adsorbed tetanus toxoid agitated for 120 min in the presence of 0.05 M sorbitol; lane 4, adsorbed tetanus toxoid agitated for 120 min in the presence of 0.15 M sorbitol; lane 5, adsorbed tetanus toxoid agitated for 120 min in the presence of 0.05 M trehalose; and lane 6, adsorbed tetanus toxoid agitated for 120 min in the presence of 0.15 M trehalose.

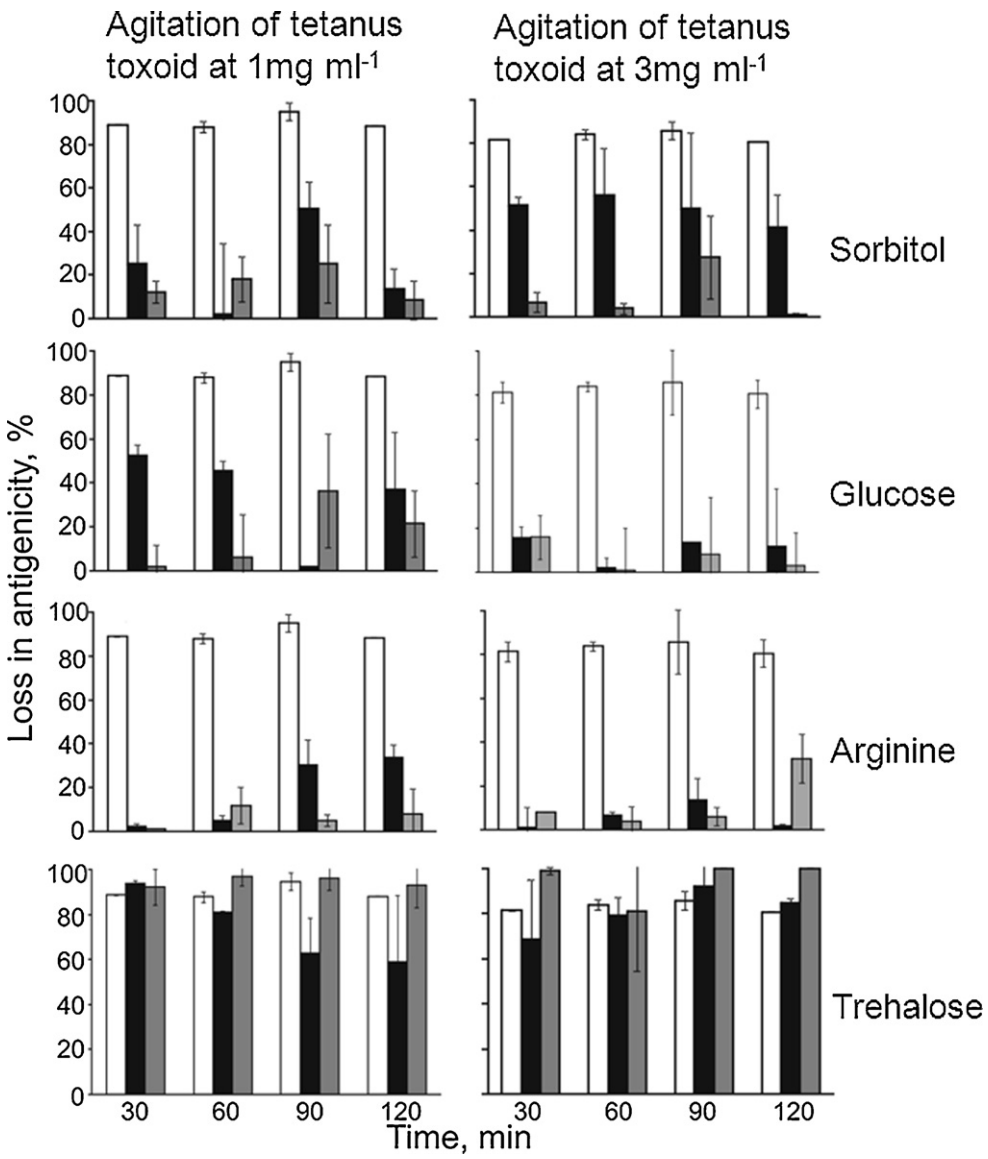

Fig. 2. Antigenicity of aluminium hydroxide-adsorbed tetanus toxoid after agitation at 1 mg ml^{-1} (left panel) and 3 mg ml^{-1} (right panel) concentrations in the presence of different excipients. Samples are shown without excipients (white bars), and in the presence of 0.05 M (black bars) and 0.15 M (grey bars) excipient. Values represent mean of three independent samples and error bars are standard errors of mean.

both the protein and the adjuvant matrix against stress conditions (Solanki et al., 2011).

3.4. Surface morphology of aluminium hydroxide gel matrix

In order to understand the reason why sorbitol and trehalose show such opposite effects in terms of potency of the formulations when SDS-PAGE and amount of desorbed protein are similar for the two preparations, the changes that occur in the aluminium hydroxide gel matrix on agitation and the effect of sorbitol and trehalose on this phenomenon were monitored by scanning electron microscopy (Fig. 3). In the absence of any osmolyte, the gel structure of aluminium hydroxide collapsed following agitation, leading to desorption of the protein from the surface of the adjuvant. This desorbed protein aggregated because of agitation-induced stress. This was seen as almost complete removal of the tetanus toxoid spots from the collapsed adjuvant surface (Fig. 3b) rather than a uniformly distributed protein pattern in case of the non-agitated control (Fig. 3a). Sorbitol was not able to inhibit desorption of the protein from the adjuvant, although it did facilitate retention of topology by the gel matrix (Fig. 3c). On the other hand, trehalose was not able to inhibit the collapse of the gel matrix to a large extent (Fig. 3d), which led to desorption of the toxoid. This indicated that sorbitol and trehalose had different effects on the adjuvant matrix following agitation. In addition, both osmolytes had differential effects on the stability of the desorbed toxoid. Sorbitol could prevent the denaturation of the desorbed protein, thus retaining its antigenicity. Trehalose, on the other hand, was unable to prevent agitation-induced denaturation of the desorbed protein. Thus, even though the toxoid did not aggregate (Fig. 1), it was denatured due to agitation-induced stress. In addition, trehalose was not successful in retaining the integrity of the gel matrix following agitation. In studies with recombinant human granulocyte colony stimulating factor, both sorbitol and trehalose were shown to inhibit aggregation of the protein following agitation (Pavisić et al., 2010). Functional studies were not reported in this case. As mentioned earlier, in order to elicit the desired immune response, adsorption of antigen on adjuvant is not necessary (Kanra et al., 2003; Romero Méndez et al., 2007; Noe et al., 2010). In these cases, it is not the fraction of antigen which is adsorbed on

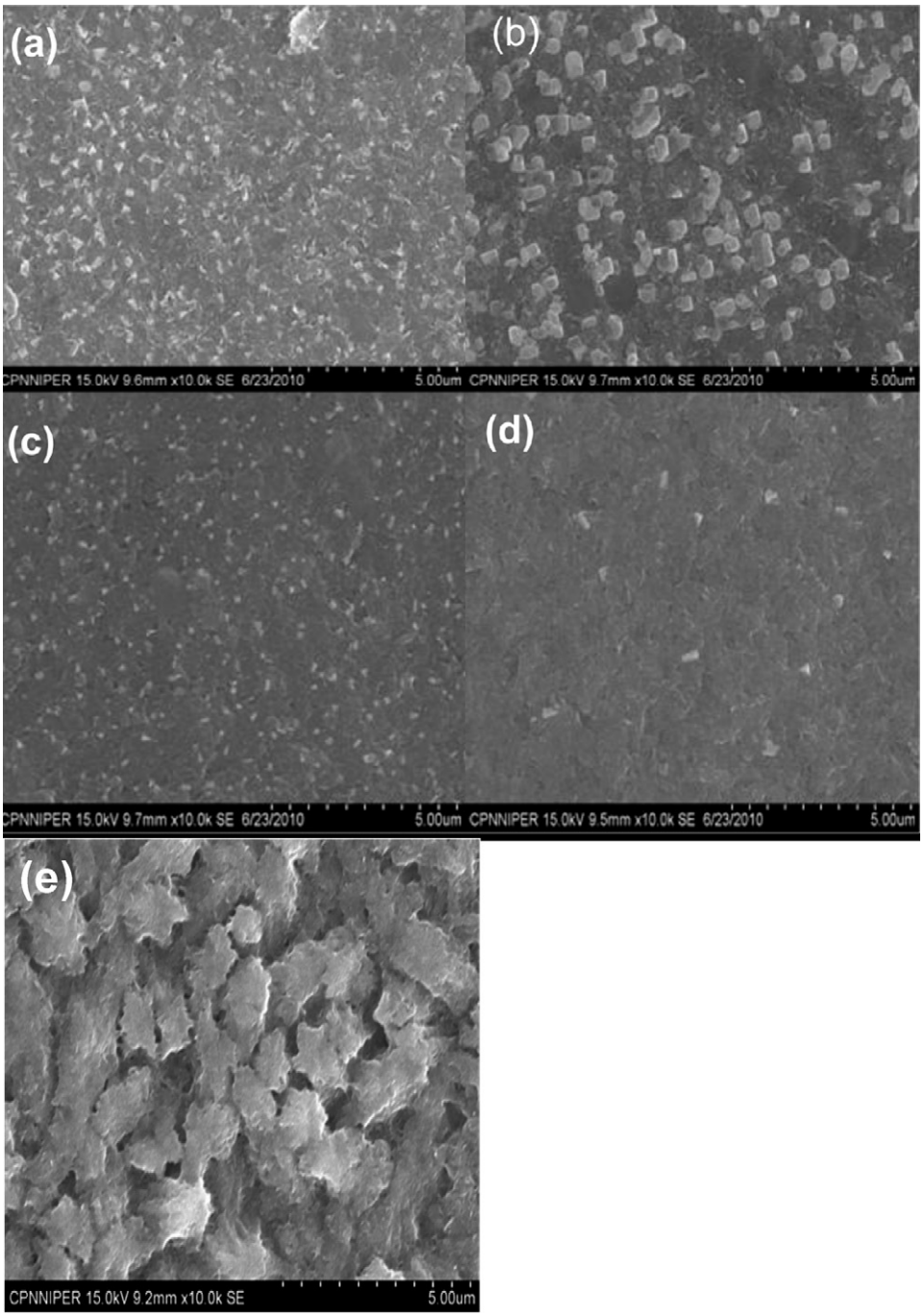

Fig. 3. Scanning electron micrographs of aluminium hydroxide-adsorbed tetanus toxoid alone (a) and agitated at 300 rpm for 120 min: alone (b), in the presence of 0.15 M sorbitol (c), and 0.15 M trehalose (d). The micrograph of aluminium hydroxide alone (e) is shown for comparison. Bar = 5 μm.

the adjuvant in the vaccine formulation, but the fraction that is entrapped in the porous structures formed by the aluminium adjuvants in the interstitial fluid upon administration to the body that determines the immunopotentiating effect of adjuvants (Romero Méndez et al., 2007). Immunopotentiation by different antigens has been achieved even via separate administration of the two components (Flebbe and Braley-Mullen, 1986). For an efficacious preparation, the foremost requirement is the maintenance of antigenicity rather than the adsorption of the antigen on the adjuvant surface. This is achieved in the presence of sorbitol in the preparation.

4. Conclusion

We have recently reported that in comparison to glucose, trehalose was less efficient in maintaining the surface morphology of the aluminium hydroxide gel during freeze-thawing of tetanus toxoid adsorbed on aluminium hydroxide (Solanki et al., 2011). A

similar phenomenon is probably at work here. Sorbitol is able to maintain the surface integrity of the gel matrix and allow the desorbed protein to be retained in a functionally active form. This is apparently not possible in case of trehalose, where the adsorbed protein is denatured and loses its antigenicity due to agitation-induced stress.

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