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Note

Immobilisation of vaccines onto micro-crystals for enhanced thermal stability

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Abstract

The thermal instability of many vaccines leads to the wastage of half of all supplied vaccines. In this note, we report the application of a novel technology: protein-coated micro-crystals (PCMC) to improve the thermostability of a model vaccine (diphtheria toxoid, DT). The latter was immobilised onto the surface of a crystalline material (L-glutamine) via a rapid dehydration method, resulting in the production of a fine free-flowing powder. The PCMC consisted of thin, flat crystals with an antigen loading of 3.95% (w/w). The DT-coated glutamine crystals and free DT (the controls) were incubated at different temperatures for a defined time period (4 °C, RT and 37 °C for 2 weeks and 45 °C for 2 days), after which the crystals were suspended in buffer and intramuscularly administered to mice. Incubation of DT (free and crystal-coated) at room temperature and at 37 °C for 2 weeks did not result in any change in the antibody response compared to DT that had always been stored properly (i.e. in the refrigerator). In contrast, incubation of free DT at 45 °C resulted in a reduced IgG response, indicating thermal instability of free DT at that temperature. The antibody response was not reduced, however, with the crystal-coated DT. These preliminary studies show that PCMC is a promising technology for the thermal stabilisation of vaccines.

Keywords: Vaccine; Thermal instability; Heat; Stability; PCMC

Vaccination is estimated to save approximately 3 million lives every year worldwide; yet another 3 million lives are still lost to vaccine-preventable diseases, mainly due to the thermal instability of vaccines, which leads to the wastage of half of all supplied vaccines worldwide (WHO et al., 2000; Brandau et al., 2003). Wastage is especially acute in developing countries; however, improper storage has also been reported in temperate countries and in the developed world (Setia et al., 2002; Bell et al., 2001).

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The creation and maintenance of the cold-chain is a significant cost to immunisation programs (Milstien et al., 1997); in addition, maintenance of the cold-chain is thought to be a 'Herculean task' in developing countries due to erratic power supplies and difficulties associated with the repair and maintenance of equipment (Arya, 1994). This highlights the urgent need to develop vaccine formulations that are stable both at room temperature and ideally at elevated temperatures.

To overcome the problems associated with the cold-chain, different means of enhancing the thermal stability of vaccines have been explored, such as the production of dried vaccine formulations via lyophilisation, spray drying, spray freeze-drying (Maa et al., 2004) and Xerovac (Worrall et al., 2000) technologies and improved formulation strategies including the use of: optimal pH (Lancz and Sample, 1985), excipients such as sugars and amino acids, proteins (e.g. albumin, gelatin), surfactants (e.g. Tweens), formaldehyde, chelators (e.g. EDTA), salts (Salo and Cliver, 1976), such as magnesium chloride (Melnick et al., 1963), and the substitution of deuterium oxide for water (Wu et al., 1995). In spite of research in this area, thermal instability of vaccines remains a major problem and the majority of vaccines used in immunisation programs worldwide still require maintenance of the cold-chain.

In this paper, we report the use of a novel technology: protein-coated micro-crystals (PCMC) to stabilise the vaccine onto a crystalline support in an attempt to improve vaccine thermostability.

Protein-coated micro-crystals represent a novel particle engineering approach for the formulation of a wide range of biomolecules, including proteins, peptides, DNA/RNA and vaccines. PCMC consist of a core crystalline material, such as a sugar, amino-acid or salt on which the biomolecules are immobilised (Parker et al., 2003). The preparation of PCMC involves dissolution of the appropriate crystal-forming carrier together with the given biomolecule in aqueous solution. Rapid dehydration of the two components is facilitated by the addition of the aqueous solution to a water-miscible organic solvent, resulting in the immediate formation of the PCMC with the biomolecule immobilised on the surface of the crystalline core carrier (via a crystal-lattice mediated self-assembly process). These particles can then be filter-dried to form a free-flowing powder (Ross et al., 2002). Particle morphology, size and biomolecule payload are tuned via appropriate choice of dehydration conditions (Ross et al., 2004).

To prepare vaccine coated micro-crystals, an appropriate aliquot of an aqueous solution of diphtheria toxoid (DT, a model antigen, supplied by Aventis, Pasteur, France) at an initial concentration of 14.5 mg/ml was diluted with an appropriate amount of H_2O and added to a saturated solution of L-glutamine (Sigma–Aldrich, UK), the crystalline core carrier material. 4.5 ml of this aqueous solution was slowly dispensed from a 10 ml Hamilton syringe attached to a 79400 series syringe pump (Cole-Parmer Instrument Company, UK) into 45 ml of 2-propanol saturated glutamine solution (antisolvent) under vigorous stirring. This resulted in the formation of DT/L-glutamine crystals with a theoretical DT loading of 5% (based on work with model proteins). The solvent was then removed from the crystals using a Millipore filtration system (Millipore Ltd., UK) to produce a free-flowing crystalline powder. A control sample of pure L-glutamine crystals without the addition of DT was also produced by this method to determine the effect of DT inclusion on crystal size and morphology.

The yield of DT–PCMC crystals recovered was 80% of the theoretical amount. The actual loading of DT on the PCMC was determined by Bradford assay—10 mg of the micro-crystals was dissolved in 0.8 ml of water, followed by mixing with 0.2 ml of Bradford reagent (Biorad, Hertfordshire UK) and incubation at room temperature for 5 min. The optical density (OD) of the mixture was read at 595 nm using a spectrophotometer and the DT concentration was calculated according to standard solutions of the toxoid. DT loading on the PCMC was found to be 3.95% (w/w). However, both the recovered yield and actual protein loading of DT onto the L-glutamine crystals can be improved by optimisation of the formulation strategy.

Scanning electron microscopy (SEM) of DT/L-glutamine PCMC (Fig. 1a) and L-glutamine crystals (Fig. 1b) carried out on a SEM500 (Philips Ltd., Netherlands) revealed that the incorporation of DT onto the glutamine crystal surface reduced the overall crystal size (on average less than 10 μm), as well as the relative dimensions of the crystal faces. In addition, the crystal shape is less clearly defined due to the incorporation of DT.

Investigations with regard to the thermal stability of the vaccine were conducted by incubating the DT

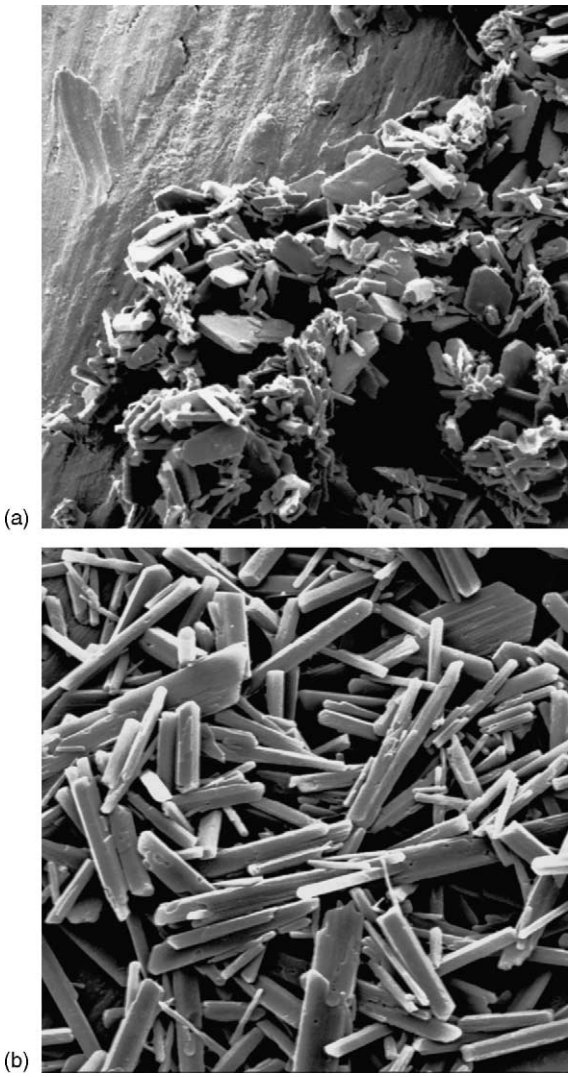

Fig. 1. (a) DT/L-glutamine and (b) L-glutamine crystals (no DT).

formulations at different temperatures, followed by in vivo experiments in mice to determine the magnitude of the immune responses generated. Specifically, DT-coated crystals (powder) and controls (free DT solution) were incubated at 4 °C, at room temperature and at 37 °C for 2 weeks, and at 45 °C for 2 days. Following incubation, the appropriate quantity of DT-coated glutamine crystals was suspended in phosphate buffered saline (5 μg of DT per 50 μl of suspension). Each 50 μl aliquot of the different formulations (containing 5 μg DT) was intramuscularly administered to the right hind leg of female Balb/c mice ($n = 5$). Booster doses of the same formulations (stored in the refrigerator until they were subjected to heat incubations as described for the first administrations) were intramuscularly administered 4 weeks later. To determine the primary and secondary responses to DT, the mice were bled from the tail vein on days 21 and 42 and the levels of antigen-specific antibody in the serum were determined using indirect ELISA assays, as described previously (Bramwell et al., 2004).

The primary and secondary antibody responses to free and DT-coated micro-crystals, incubated at different temperatures, are shown in Figs. 2 and 3, respectively. Incubating free DT at room temperature and at 37 °C for 2 weeks did not change its antigenicity and the immune responses were the same as DT stored under constant refrigeration. This shows that free DT was stable at these temperatures for 2 weeks, which is in agreement with the literature reports (van Ramshorst and van Wezel, 1976; Stainer and Hart, 1978). Incubation of free DT at 45 °C for 2 days resulted in severely reduced antibody responses (Figs. 2 and 3), indicating that inactivation of DT antigen had occurred. In contrast, antibody responses to DT immobilised onto micro-crystals were not affected when the formulation had been incubated at 45 °C for 2 days, suggesting that immobilisation of the DT onto micro-crystals appears to afford protection of the antigen from thermal inactivation, when prepared and stored as a dry powder. The preparation of PCMC is very rapid (μs–ms time-scale), i.e. the rate at which the DT is dehydrated and equally the rate of formation of the micro-crystal matrix are both on this time-scale. This rapid dehydration step is thought to result in retention of the native conformation (Parker et al., 2003).

From this study, we can conclude that immobilisation of DT onto micro-crystals enhanced its heat stability when the vaccine was incubated at 45 °C for 2 days. The potential application of the PCMC technology as a means of formulating heat-labile vaccines into stable preparations has been indicated. Further work needs to be conducted with a range of heat-labile antigens, the inclusion of vaccine adjuvants and more detailed investigations into immunological responses to have a deeper understanding of this promising technology.

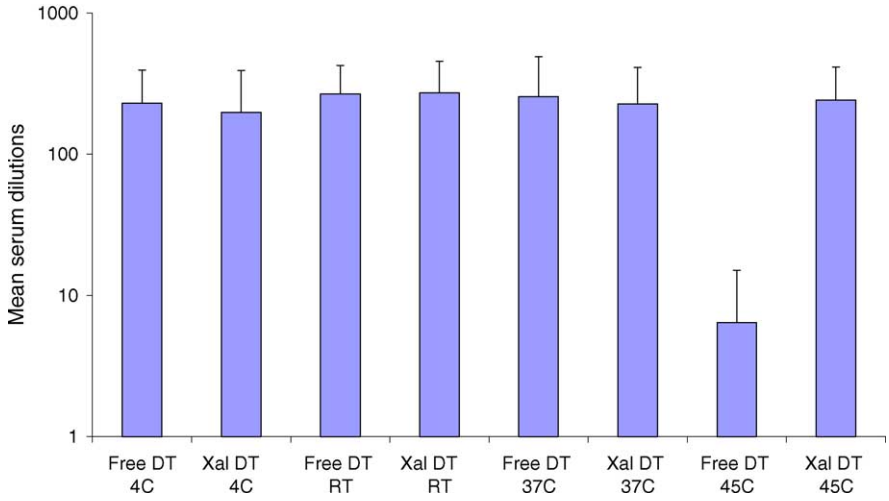

Fig. 2. The primary IgG response to free DT and to DT immobilised onto micro-crystals.

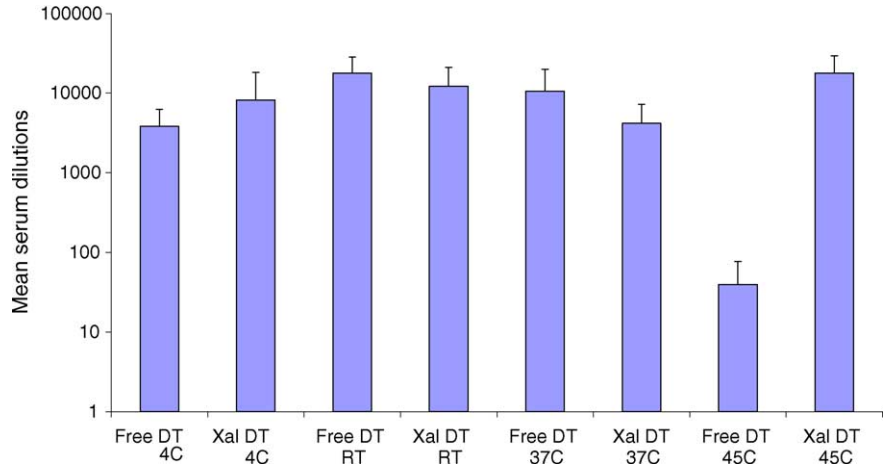

Fig. 3. The secondary IgG response to free DT and to DT immobilised onto micro-crystals.

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