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Nano-sized Soluplus® polymeric micelles enhance the induction of tetanus toxin neutralising antibody response following transcutaneous immunisation with tetanus toxoid

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ABSTRACT

The use of Soluplus® polymeric micelles as a novel adjuvant for tetanus toxoid (TTxd) in transcutaneous immunisation was evaluated. TTxd was added to Soluplus® polymeric micelles to form TTxd-Soluplus® nano-aggregates with a size of 68 nm. Non-adjuvanted TTxd commonly induces very poor antibody response by the transcutaneous route. However, in this study, the use of TTxd-Soluplus® resulted in a significant increase in the antibody response to TTxd, which was similar to that induced in the presence of CPG-oligodeoxynucleotides (CPG-ODNs) adjuvant. The toxin neutralising potency of the immune sera induced by TTxd-Soluplus® was also much stronger than that from TTxd alone, in a passive transfer experiment in mice. Soluplus® also enhanced the immunogenicity of the toxoid when TTxd-Soluplus® was stored at 4 °C for 4 weeks, but not at higher temperatures. Confocal microscopy imaging showed a much higher uptake of TTxd in the epidermis and dermis layers of the skin when it was associated with Soluplus®, suggesting that the mechanism for Soluplus® adjuvanticity is through enhanced uptake of the TTxd through the skin. Overall, our findings demonstrated that Soluplus® is an effective novel adjuvant for transcutaneous immunisation.

1. Introduction

Transcutaneous immunisation (TCI) is non-invasive and pain-free compared to the commonly used injectable systems [1]. It targets the dense population of immune cells, such as the Langerhans cells, in the different layers of the skin and holds great promise for increased compliance of future global vaccination programmes and reduced risk of needle-borne diseases [2]. Induction of protective responses by TCI has been demonstrated in animal models [3–11] and in clinical trials for various vaccine antigens [12–16].

Despite its large surface area, skin delivery of drug/vaccine molecules is challenging due to the formidable barrier of the stratum corneum (SC) which enables lipophilic molecules with molecular mass <500 Da to passively penetrate, whilst transport of relatively large hydrophilic molecules (peptides, proteins and DNA) is especially difficult [17]. Therefore, successful TCI often requires administration of large amounts of antigen, strong mucosal adjuvant, such as cholera toxin [18], prolonged immunisation time [19], disruption of skin by abrasion, tape stripping [20,21], use of microneedles [22–25] or external stimulations, such as low frequency ultrasound [26]. However, some of these skin abrasion techniques are fairly aggressive and can potentially cause infection due to prolonged SC disruption. Furthermore, they do not always enhance penetration of hydrophilic macromolecules and could be difficult to translate successfully to clinical use.

A promising technology for TCI enhancement is the use of nano-carriers for vaccine delivery, such as transferosomes, liposomes and polymeric nanoparticles [27–29], which prolong antigen retention at the delivery site, improve antigen uptake by APC and allow antigen and adjuvant encapsulation in the same particle [30]. However, TCI with nanoparticles has so far been limited with focus mainly on lipid vesicles consisting of bilayers of hydrated amphiphilic lipids or other amphiphilic compounds, especially cationic liposomes which have been extensively explored as protein carriers [31] and DNA-based vaccines [32] as they can carry

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both membrane-associated and water soluble antigens. Although a number of nano-carriers, including liposomes, have been tested in clinical trials as adjuvants/immunopotentiators, the use of liposomes is hampered by a complicated fabrication process and instability of their cationic lipids [33].

The use of polymeric micelles has been investigated extensively in drug/protein delivery [34,35]. They are often formed spontaneously in an aqueous environment by amphiphilic polymers with both hydrophobic and hydrophilic moieties within the same polymer construct. Soluplus®, a polyvinyl caprolactam-polyvinyl acetate-polyethylene glycol graft co-polymer, is a novel commercial excipient commonly used to improve oral bioavailability of poorly soluble drugs [36]. It is a non-ionic amphiphilic graft co-polymer which forms nano-sized polymeric micelles in aqueous solution. The fabrication process of Soluplus® polymeric micelles is devoid of organic solvents or heat which could potentially denature biologically active compounds during formulation. This is particularly important in the preparation of vaccine formulations, offering an advantage of Soluplus® over other approaches for nano-carriers in TCI. It is cheap to produce and has been safely used in rats and dogs for oral delivery of anti-inflammatory and prostatic hyperplasia drugs [36–38]. To our knowledge, Soluplus® has not been utilised in transcutaneous delivery of vaccine and this study describes the results of the evaluation of Soluplus® as an adjuvant for TCI with tetanus toxoid (TTxd).

2. Materials and methods

2.1. Preparation of TTxd-Soluplus® formulation

TTxd-Soluplus® formulations were prepared as follows: Soluplus® was dissolved at 10 mg/ml in 5 mM Tris-HCl buffer (pH 7.4). TTxd at 5 mg/ml in Tris-HCl buffer was added to Soluplus® solution at a Soluplus®:TTxd ratio (w/w) of 7:1. The mixture was stirred constantly (20 rpm) at RT for 2 h before use in TCI studies.

For the stability study, TTxd and TTxd-Soluplus®, prepared as above, were incubated at various temperatures for 4 weeks before being used for TCI.

2.2. Physico-chemical characterisation of Soluplus®-TTxd formulations

TTxd-Soluplus® formulations were characterised on the day of preparation for hydrodynamic diameters, polydispersity indices (PDI) and zeta potential using photon correlation spectroscopy (PCS) (Zetasizer Nano-ZS, Malvern Instruments) at 25 °C.

2.3. Animals and immunisation

Female Sprague Dawley rats (6–8 week-old; Charles River), were anaesthetised using isoflurane, where individual animals had face mask connected to the anaesthesia machine. Animals were laid on their back and a restricted area of the abdomen (~3–4 cm^2) was shaved and depilatory cream (Nair) applied for one min before being thoroughly removed with a damp cloth. Residual cream was removed using 75% alcohol and skin cleaned with distilled water. The skin was hydrated for 5 min then blotted dry before applying 50 μl containing 20 μg of TTxd, TTxd-Soluplus® or TTxd and CPG. During the procedure, animals were kept under deep anaesthesia for 45 min to prevent grooming. At the end, the skin was thoroughly washed with tap water. Animals received booster immunisations on days 14 and 28 with the same dose and formulation and skin was monitored throughout the experiment for signs of irritation. Animals were terminally bled on day 35 and serum samples prepared and stored at −20 °C until tested.

2.4. Statistical analysis

The Wilcoxon Rank-Sum Test was used for comparing the responses between groups in the same experiment and the 2-tailed unpaired Student's *t*-test (for data combined from different experiments). $p < 0.05$ was considered statistically significant.

3. Results

3.1. Physical Characterisation of TTxd-Soluplus® and TTxd

Preparation of Soluplus® in Tris-HCl resulted in a colourless solution. The addition of TTxd did not change the physical appearance and the TTxd-Soluplus® solution remained clear. All three colloidal solutions TTxd, Soluplus® and TTxd-Soluplus® were in the nano-size range (Table 1). The hydrodynamic size of TTxd was 42 nm - approximately 20 nm smaller than Soluplus® and TTxd-Soluplus® formulations. Upon addition of TTxd to Soluplus®, the hydrodynamic size (68 nm) remained similar to Soluplus® alone (65 nm). PDI is a ratio indicating homogeneity of the particle size of a colloidal solution, where a small PDI indicates a narrow size distribution. TTxd alone yielded a polydispersed colloidal solution with a high PDI of 0.54, whilst upon addition of TTxd to Soluplus® the PDI was 0.04, indicating the solution had a narrow size distribution. This is supported by the particle size distribution of TTxd-Soluplus® presented in Fig. 1 as a single peak. The formation of TTxd-Soluplus® also resulted in a significant change in the zeta potential of TTxd: from highly negative (−14.93 mV) to near neutral (−2.21 mV) after addition to Soluplus® (Table 1). Furthermore, to ascertain that TTxd was incorporated into the micelles, TTxd-Soluplus® preparation was centrifuged to remove the micelles and the supernatant tested for the presence of free TTxd by ELISA (Suppl. Fig. 1). The results showed that while TTxd and TTxd-Soluplus® formulations had the same level of TTxd content, it was clear that no free TTxd was present in the supernatant of the TTxd-Soluplus® after centrifugation, indicating that all the TTxd was encapsulated into the micelles.

3.2. Immunogenicity of TTxd administered by the transcutaneous route and effect of Soluplus®

The immunogenicity of TTxd and the adjuvant effect of Soluplus® were assessed in 3 different experiments (Fig. 2A). TCI with TTxd alone induced a weak anti-TTxd IgG response (mean IgG titre of 713) after a single dose. The antibody titre increased to 1017 and 2391 after the 1st and 2nd booster dose, respectively. However, this increase was not statistically significant ($p > 0.05$) suggesting that TTxd alone, at the dose used, is poorly immunogenic. Animals immunised with TTxd-Soluplus® mounted a much higher response after all immunisations. The level of response was almost double that induced by TTxd alone after the primary dose (mean IgG titre = 1375 vs 713; $p > 0.05$) and was ~10-fold higher after the 1st boost ($p < 0.05$). Similarly, a 2nd boost with TTxd-Soluplus® induced a 19-fold higher anti-TTxd IgG response compared with the response in the TTxd group (48,760 vs 2567; $p < 0.05$), indicating the adjuvanticity of Soluplus® for TTxd by TCI. The

Table 1
Characterisation of the freshly prepared TTxd and TTxd-Soluplus® (n = 3).

Sample	Size (nm) Mean ± SD	PDI Mean ± SD	Zeta potential (mV) Mean ± SD
TTxd	42 ± 2	0.54 ± 0.03	−14.93 ± 2.76
Soluplus®	65 ± 0	0.03 ± 0.01	−1.59 ± 0.11
Soluplus®-TTxd	68 ± 1	0.04 ± 0.01	−2.21 ± 0.94

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