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Accelerating the clinical development of protein-based vaccines for malaria by efficient purification using a four amino acid C-terminal 'C-tag'

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ABSTRACT

Development of bespoke biomanufacturing processes remains a critical bottleneck for translational studies, in particular when modest quantities of a novel product are required for proof-of-concept Phase I/II clinical trials. In these instances the ability to develop a biomanufacturing process quickly and relatively cheaply, without risk to product quality or safety, provides a great advantage by allowing new antigens or concepts in immunogen design to more rapidly enter human testing. These challenges with production and purification are particularly apparent when developing recombinant protein-based **vaccines** for difficult parasitic diseases, with ***Plasmodium falciparum* malaria** being a prime example. To that end, we have previously reported the expression of a novel protein vaccine for malaria using the ExpreS^2 *Drosophila melanogaster* Schneider 2 stable cell line system, however, a very low overall process yield (typically <5% recovery of hexa-histidine-tagged protein) meant the initial purification strategy was not suitable for scale-up and clinical **biomanufacture** of such a vaccine. Here we describe a newly available affinity purification method that was ideally suited to purification of the same protein which encodes the *P. falciparum* reticulocyte-binding protein homolog 5 – currently the leading antigen for assessment in next generation vaccines aiming to prevent red blood cell invasion by the **blood-stage** parasite. This purification system makes use of a C-terminal tag known as 'C-tag', composed of the four amino acids, glutamic acid – proline – glutamic acid – alanine (E-P-E-A), which is selectively purified on a CaptureSelect™ affinity resin coupled to a camelid single chain antibody, called NbSyn2. The C-terminal fusion of this short C-tag to *P. falciparum* reticulocyte-binding protein homolog 5 achieved >85% recovery and >70% purity in a single step purification directly from clarified, concentrated Schneider 2 cell supernatant under mild conditions. Biochemical and immunological analysis showed that the C-tagged and hexa-histidine-tagged *P. falciparum* reticulocyte-binding protein homolog 5 proteins are comparable. The C-tag technology has the potential to form the basis of a current good manufacturing practice-compliant platform, which could greatly improve the speed and ease with which novel protein-based products progress to clinical testing.

1. Introduction

The production of recombinant antigen remains central to the development of many types of subunit vaccines, and especially for those seeking to induce antibody (Draper et al., 2015). Such antigen may take numerous forms, ranging from a relatively simple peptide to soluble monomeric protein through to more com-

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plex oligomeric scaffolds (Li et al., 2016) or larger virus-like particles (VLPs) (Wu et al., 2015; Brune et al., 2016). Following purification, the classical approach to antibody induction by subunit vaccination has been the delivery of the protein or VLP antigen formulated in a chemical adjuvant (Coler et al., 2009; de Cassan et al., 2011), with notable success in humans including hepatitis B virus surface antigen (HBsAg) and bacterial toxoids (tetanus and diphtheria). These approaches are further exemplified by ongoing efforts to develop a highly effective vaccine against infection, disease or transmission caused by the *Plasmodium falciparum* human malaria parasite (Halbroth and Draper, 2015). In this case multiple stages of the parasite's complex lifecycle are susceptible to functional antibodies – including sporozoites, merozoites, infected red blood cells, gametocytes and sexual stages within the mosquito.

Current subunit vaccine strategies are seeking to improve on the modest levels of efficacy reported for the RTS,S/AS01 malaria vaccine – based on the recombinant HBsAg VLP technology and which targets the pre-erythrocytic circumsporozoite antigen (Rts, 2015). One leading approach will involve future trialling with a multi-antigen, multi-stage formulation by combining with other effective vaccine components against the pathogenic asexual blood-stage of infection (Goodman and Draper, 2010) and/or the subsequent sexual/mosquito stages (Nikolaeva et al., 2015). The blood-stage vaccine component would seek to protect against death and clinical disease, whilst contributing to reduced transmission through control and clearance of blood-stage parasitemia. The mainstay approach in this arena has involved targeting merozoite proteins involved in the red blood cell (RBC) invasion process. Although historical candidates have suffered from substantial levels of polymorphism leading to induction of strain-specific antibody responses (Remarque et al., 2008), a new generation of targets are being identified which are relatively highly conserved and yet susceptible to neutralising antibodies raised by vaccination. Currently the most advanced of these candidates is the *P. falciparum* reticulocyte-binding protein homolog 5 (PfRH5) (Drew and Beeson, 2015). Antibodies raised by vaccination of animals can cross-inhibit all *P. falciparum* lines and field isolates tested to date (Douglas et al., 2011; Williams et al., 2012; Bustamante et al., 2013; Reddy et al., 2014), also with higher efficiency than other historical target antigens (Williams et al., 2012). Importantly, PfRH5 is reported to be essential (Hayton et al., 2008; Baum et al., 2009), and forms a critical non-redundant interaction with its receptor, basigin (CD147), during invasion (Crosnier et al., 2011). Moreover, the relatively high degree of PfRH5 sequence conservation is associated with low-level immune pressure following natural infection (Douglas et al., 2011; Villasis et al., 2012; Tran et al., 2014), as well as functional constraints linked to basigin binding and host RBC tropism (Hayton et al., 2008, 2013; Wanaguru et al., 2013). Vaccination of *Aotus* monkeys also showed significant efficacy against a stringent heterologous strain blood-stage *P. falciparum* challenge, where protection was strongly associated with anti-PfRH5 serum IgG antibody concentration and in vitro growth inhibition activity (GIA) measured using purified IgG (Douglas et al., 2015).

The earliest vaccination studies with PfRH5 used fragments of the antigen made in *Escherichia coli* that failed to induce functional antibodies (Rodriguez et al., 2008; Baum et al., 2009). Consequently, efforts focussed on protein immunogens based on the full-length PfRH5 sequence which resulted in functional neutralising antibodies (Douglas et al., 2011; Bustamante et al., 2013; Reddy et al., 2014), some of which are known to block the PfRH5-basigin interaction (Douglas et al., 2014). However, despite these successes, it proved particularly problematic to develop a process that is scalable and compliant with current good manufacturing practice (cGMP) and which could enable production of a batch of full-length PfRH5 protein for use in clinical trials. Recently, we reported the production of soluble full-length PfRH5 protein using a cGMP-compliant platform called ExpreS^2 (Dyring, 2011), based on a *Drosophila melanogaster* Schneider 2 (S2) stable cell line system (Wright et al., 2014; Hjerrild et al., 2016). Full-length PfRH5 protein was expressed from stable cell lines and purified using a C-terminal hexa-histidine (His6) tag, and induced functional antibodies following immunisation of rabbits. However, despite successful expression in this heterologous system, up to four purification steps were required, resulting in high (>95%) purity of the final PfRH5 protein but a low overall process yield, typically <5% recovery (Hjerrild et al., 2016). Consequently this purification strategy was not suitable for scale-up and clinical biomanufacture of a PfRH5 protein vaccine.

Here we describe a newly available affinity purification method that was ideally suited to purification of the PfRH5 protein. This system makes use of a C-terminal tag known as 'C-tag', composed of the four amino acids (aa), glutamic acid – proline – glutamic acid – alanine (E-P-E-A), which is selectively captured on a resin coupled to a camelid single chain antibody, termed NbSyn2 and specific for this short sequence. NbSyn2 was originally raised by immunisation of a dromedary with alpha-synuclein (De Genst et al., 2010) and further developed into a CaptureSelect™ affinity resin by BAC B.V. in the Netherlands (now Thermo Fisher Scientific). This resin can be produced to be suitable for use in clinical and commercial biomanufacture. Notably, multiple different single chain antibody-based affinity resins are now available for cGMP manufacture, and the first product purified in this manner, an adeno-associated virus gene therapy product called alipogene tiparvovec (Glybera®) for lipoprotein lipase deficiency, has been licenced in Europe (Wang et al., 2011, 2015). Here, C-terminal fusion of this short C-tag to PfRH5 achieved >85% recovery and >70% purity in a single step purification directly from clarified, concentrated S2 cell supernatant under mild conditions. The purification and biochemical analysis of the PfRH5 protein is reported, whilst a functional analysis of the antibodies induced following immunisation of rabbits shows comparable immunogenicity to His6-tagged antigen. Our data suggest that the C-tag technology could form the basis of a cGMP-compliant platform that will improve the speed and ease of process development for the biomanufacture of novel protein-based products.

2. Materials and methods

2.1. Design and cloning of PfRH5 protein vaccines

All chemicals were purchased from Sigma–Aldrich, UK unless otherwise specified. The design of the PfRH5 protein with C-terminal His6 tag has been described elsewhere, where it was reported as variant version 1.0 (Hjerrild et al., 2016). In brief, the protein encodes the full-length ectodomain of the PfRH5 antigen (aa E26-Q526) based on the sequence of the 7G8 laboratory-adapted *P. falciparum* parasite line, and all four putative N-linked glycosylation sequons (N-X-S/T) were mutated Thr to Ala – as performed for a previous PfRH5 protein vaccine produced in mammalian HEK293 cells and tested in rabbits (Bustamante et al., 2013) and *Aotus* monkeys (Douglas et al., 2015). A synthetic gene was designed based on the above 7G8 sequence for PfRH5 and codon-optimised for expression in *D. melanogaster* (GeneArt, Thermo Fisher Scientific, Germany). The construct also contained a Kozak sequence (GCC ACC) at the 5′ end, an N-terminal 18 aa Ig heavy chain binding protein (BiP) insect signal peptide (MKLCILLAVVAFVGLSLG) and a C-terminal His6 tag. This gene insert was subcloned into the pExpreS^2-1 plasmid allowing for Zeocin selection (ExpreS^{2}ion Biotechnologies, Denmark). Subsequently, the C-

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