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A rapid and simple screening method to identify conditions for enhanced stability of modular vaccine candidates

Alemu Tekewe [a], Natalie K. Connors [a,*], Frank Sainsbury [a], Nani Wibowo [a], Linda H.L. Lua [b], Anton P.J. Middelberg [a]

[a] Centre for Biomolecular Engineering, Australian Institute for Bioengineering and Nanotechnology, The University of Queensland, St Lucia, QLD 4072, Australia
[b] UQ Protein Expression Facility, The University of Queensland, St Lucia, QLD 4072, Australia

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ABSTRACT

Virus-like particles (VLPs) and capsomere subunits have been developed as safe and effective vaccine candidates in the fight against infectious diseases. These bioengineered structures are suitable platforms for repetitive high density presentation of foreign epitope modules. However, due to the module's diverse physicochemical properties, modularisation of capsomeres and VLPs requires tailoring of the physicochemical environment specific to each module to maintain protein stability against aggregation. Here, we report a high-throughput technique for screening buffer components to stabilize capsomeres, based on light scattering analysis. This screening method was applied to modular capsomeres presenting peptide epitopes from the rotavirus spike protein VP8 subunit domain, engineered as a next-generation rotavirus vaccine candidate. Among various additives tested, non-ionic detergents, such as Triton X-100, Tween-80 and Tween-20, were able to stabilize modular capsomeres, either alone or in combination with L-arginine, as confirmed with high-resolution size exclusion chromatography. Results demonstrate that tailoring the nature of the environment surrounding self-assembling proteins using small organic molecules can enhance the bioprocessing of modular vaccine capsomeres. The developed screening method potentially provides a powerful approach for rapid tailoring of processing conditions specific to antigenic modules displayed on next-generation recombinant capsomere and VLP vaccines, for low-cost vaccine delivery at global scale.

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1. Introduction

Virus-like particles (VLPs) are macromolecular assemblies of the viral structural protein(s) that retain antigenic features of the authentic virus without the viral genome [1–3]. VLPs have come into focus for their promising application in vaccination [4–7] because of their high safety and efficacy profile [8]; their repetitive and high density native display of epitopes leading to their self-adjuvanting property; their ability to present foreign epitopes on the surface; and their stability compared with soluble antigens [9–12]. So far, three VLP-based vaccines have been commercialized for human use against hepatitis B virus infection, human papillomavirus-induced cervical cancer and hepatitis E virus infection [4,13]. Numerous other VLP-based vaccines against many infectious agents have shown promising results under pre-clinical evaluation using small-animal models and in clinical trials [12,14–16]. Moreover, assembly-incompetent VLP sub-units, termed capsomeres, have recently gained attention for their potential as alternative low-cost second-generation vaccine candidates to VLPs [15,17–19]. Although most capsomeres showed less immunogenic response compared with the corresponding VLPs, their reduced efficacy was significantly compensated by using effective and safe adjuvants [20]. Some capsomeres could induce almost the same level of immune response as the corresponding VLPs when formulated with safe adjuvants [15,18,19].

Despite the significant benefits of the marketed VLP-based vaccines and the promising potential of those in the developmental pipeline, many will likely remain unaffordable for resource-poor countries due to their high manufacturing and processing costs from eukaryotic expression systems [7,17,21]. Minimizing production costs has been the focus of several developments to enable low-resource countries to use the final product with affordable cost [22]. The ability to manufacture VLPs from microbially-expressed sub-units via *in vitro* assembly in a cell-free reactor, under engi-

* Corresponding author. Tel.: +61 7 3346 4195.
E-mail address: n.connors@uq.edu.au (N.K. Connors).

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neering controls, suggests huge potential for cost reduction [23]. Low-cost bacterial expression systems have been used for production of the VLP sub-units resulting in high yields [7,15,21,24,25]. For example, the murine polyomavirus major capsid protein, VP1, has been produced as a GST-VP1 fusion protein at gram-per-liter levels in *Escherichia coli (E. coli)* [7,21]. Enzyme-mediated cleavage of the GST tag followed by purification and separation by size exclusion chromatography (SEC) results in pure VP1 capsomeres that have been assembled *in vitro* to form VP1 VLPs in cell-free reactors [24,26].The production of assembly-incompetent VP1 capsomeres using prokaryotic expression has also been reported [15,18]. Current research focuses on the modularization of foreign antigenic epitopes into VLPs or capsomeres to target specific diseases. Middelberg *et al.* [15] have developed VP1 VLPs that were able to display J8-peptide from the M-protein of Group A streptococcus; such antigen-loaded modular VLPs showed protection in a mouse model following unadjuvanted nasal delivery [15,27]. The modular capsomeres containing single [15,18] or multiple modules [18] of influenza M2e peptide antigen gave protection in mice when formulated with Alhydrogel® [28].

The insertion of modules into the VLP subunits can be well tolerated, [15,18,27]; however results can be dependent on the physicochemical properties of modules. Thus, modularization may require tailoring of the processing conditions specific to each module to maintain protein colloidal stability. The stability of proteins is a major concern, particularly for the field of vaccination, where lower efficacy can be a result of poor vaccine stability [29]. A number of approaches for stabilization of proteins at different stages of processing are described in the literature [30,31]. Among those the simplest and the most common method is tailoring the environment surrounding the proteins [31]. This can be achieved by optimizing solution conditions such as pH, ionic strength [31,32] or by adding stabilizing additives [32]. Several additives [29,33–40] have been studied for their potential to stabilize proteins at different stages of processing, formulation or upon storage.

Despite the availability of various stabilizing additives, identification of the most effective additive from a large experimental space for each target protein is laborious and time-consuming using analytical methods with low-throughput capacity [41,42]. Therefore, the use of high-throughput screening (HTS) techniques may provide opportunities for simple and rapid screening for the most effective additives. HTS techniques have become a valuable tool for speeding up process development at various stages of processing biopharmaceuticals [43,44]. They have been used effectively for rapid identification and selection of conditions for precipitation [45] and separation [46] of monoclonal antibodies from host cell proteins; for determining protein solubility [41,43] and rapid assessment of the dependence of colloidal stability on complex parameter interactions [41]; and for development of production processes [47] and quantification [48] of polysaccharide vaccine candidates. Mohr *et al.* [40] demonstrated a miniaturized HTS methodology for VLP formulation by integrating dynamic light scattering (DLS) and asymmetrical flow field-flow fractionation. DLS measurement with high-throughput capacity provides *in situ* analysis within short time periods using small amounts of protein. Although the DLS technique is a low resolution method with limitations and high sensitivity to the presence of large particles [40], it can be effectively used for HTS of protein processing conditions, particularly in cases when the size difference between different species, such as between capsomeres and soluble aggregates, is high.

The present study describes the development of a rapid, simple and effective HTS method to identify optimal processing conditions for stabilization of modular capsomeres. The method, based on DLS analysis, was used for rapid screening of subsets of buffer additives that could enhance the stability of modular capsomeres (RvVP1), presenting an antigenic module derived from the VP8 subunit domain of the rotavirus spike protein. The potential of the superior additives based on DLS analysis in stabilizing RvVP1 was further confirmed with high-resolution SEC. The screening methodology developed in this study can be applied for tailoring the physicochemical environment of modular capsomeres and VLPs that incorporate antigenic modules having diverse physicochemical properties. The identification of protein-stabilizing buffer additives in this manner highlights the need to tailor the physicochemical environment specific to each module for processing and manufacture of stable modular capsomeres and VLPs in a fast and economical way. In this regard, the method can be used as a powerful tool for further advancing the platform and adding to the speed of manufacturing of quality modular capsomeres and VLPs for low-cost vaccine delivery at global scale.

2. Materials and methods

2.1. Chemicals

The following details the reagents used as additives and their source. All additives were of analytical grade: L-arginine (L-Arg) (MP Biomedicals, LLC Solon, Ohio, USA); L-glutamic acid (L-Glu), α-cyclodextrin, β-cyclodextrin, Y-cyclodextrin, methyl-β-cyclodextrin, tween-80 (TW-80), triton X-100 (TX-100), and bovine serum albumin (BSA) (Sigma–Aldrich, MO, USA); biotechnology grade phosphate buffered saline (PBS), and isopropyl-β-D-thiogalactoside (IPTG) (Astral Scientific Pty. Ltd., Gymea NSW, Australia); Tween-20 (TW-20) (Ajax Finechem, VIC, Australia).

2.2. Plasmid construction

Vector pGEX-4T-1 (GE Healthcare Biosciences, Chalfont St. Giles, UK) with inserted murine polyomavirus VP1 sequence (pGEXVP1) was generously provided by Professor Robert Garcea (University of Colorado, CO, USA). Plasmid, pGEXVP1-S4, encoding the protein, VP1, was created previously by inserting *AfeI* restriction enzyme site at position 293 of VP1 [15]. Plasmid, pGEXVP1-S4-VP8aa1-10, encoding RvVP1 was prepared from pGEXVP1-S4; RvVP1 is the protein VP1 but containing the amino acid sequence (EMASLIYRQLLESEMASLIYRQLLESEMASLIYRQLLES) at position 293. The amino acid insert, VP8aa1-10, corresponds to tandem copies of residues 1–10 (MASLIYRQLL) of the VP8 subunit domain of the human rotavirus spike protein, with spacer amino acids. DNA sequence of the insert was prepared by PCR-based gene synthesis from a set of oligos (5′gagatggcgagcctcatct 3′, 5′cgctttcgaggagttggcgatagatgaggctcgccatctc3′, 5′gccaactcctcgaaagcgaaatggcctctctgatctaccg3′, 5′atttcagactccagcagctggcggtagatcagagaggcca3′, 5′cagctgctggagtctgaaatggcgtccctgatttaccgtc3′ and 5′ggattcgagcagttgacggtaaatcagggacgc3′), generated from DNAWorks (http: //helixweb.nih.gov/dnaworks/). Correct insertion of the desired gene sequence was verified by DNA sequencing at the Australian Genome Research Facility (Brisbane, Australia).

2.3. Protein expression and purification

pGEXVP1-S4 and pGEXVP1-S4-VP8$_{aa1-10}$ were transformed into separate *E. coli* Rosetta™ (DE3) pLysS chemically competent cells (Novagen, San Diego, CA, USA). Bacterial expression of glutathione-*S*-transferase (GST) tagged proteins, GST-VP1 and GST-RvVP1, was as previously described [7,21] except cell cultures were induced with 0.1 mM IPTG at 20 °C for expression of GST-RvVP1. GST-tagged proteins were captured by GST affinity chromatography (GSTrap™ HP 5 ml column) (GE Healthcare) as described previously for

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