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Downstream processing of virus-like particles: Single-stage and multi-stage aqueous two-phase extraction☆

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ABSTRACT

The demand for vaccines against untreated diseases has enforced the research and development of virus-like particle (VLP) based vaccine candidates in recent years. Significant progress has been made in increasing VLP titres during upstream processing in bacteria, yeast and insect cells. Considering downstream processing, the separation of host cell impurities is predominantly achieved by time-intensive ultracentrifugation processes or numerous chromatography and filtration steps. In this work, we evaluate the potential of an alternative separation technology for VLPs: aqueous two-phase extraction (ATPE). The benefits of ATPE have been demonstrated for various biomolecules, but capacity and separation efficiency were observed to be low for large biomolecules such as VLPs or viruses. Both performance parameters were examined in detail in a case study on human B19 parvovirus-like particles derived from *Spodoptera frugiperda Sf*9 insect cells. A solubility-guided approach enabled the design of polyethylene (PEG) salt aqueous two-phase systems with a high capacity of up to 4.1 mg/mL VLPs. Unique separation efficiencies were obtained by varying the molecular weight of PEG, the pH value and by using neutral salt additives. Further improvement of the separation of host cell impurities was achieved by multi-stage ATPE on a centrifugal partition chromatography (CPC) device in 500 mL scale. While single-stage ATPE enabled a DNA clearance of 99.6%, multi-stage ATPE improved the separation of host cell proteins (HCPs). The HPLC purity ranged from 16.8% (100% VLP recovery) for the single-stage ATPE to 69.1% (40.1% VLP recovery) for the multi-stage ATPE. An alternative two-step downstream process is presented removing the ATPS forming polymer, cell debris and 99.77% DNA with a HPLC purity of 90.6% and a VLP recovery of 63.9%.

1. Introduction

Virus-like particles (VLPs) represent a new class of bionanoparticles incorporating unique features for medical applications. VLPs are composed of one or several viral structural proteins capable of spontaneously self-assembling into capsids. Thus, they can either mimic the pathogens they derived from [1] or present antigen epitopes of foreign pathogens [2] or tumour cells [3]. Apart from diagnostic applications VLPs show increasing importance as vaccine candidates against unsolved or emerging infectious diseases and as new "designer vaccine platforms" [4,5]. Currently, there are five VLP-based products licensed for vaccination: two cervical cancer vaccines (Gardasil®, Merck & Co. and Cervarix®, GlaxoSmithKline), two hepatitis B vaccines (Recombivax HB®, Merck & Co. and Engerix-B®, GlaxoSmithKline) and a hepatitis E vaccine (Hecolin®, Xiamen Innovax Biotech Co., Ltd.). About 10 candidates entered clinical phases II or III [6] for indications such as influenza, gastroenteritis and malaria. Further research studies focus on using VLPs as vectors for targeted delivery of small molecules [7] or nucleic acids [8].

Hence, there is an increasing demand for pure clinical grade VLPs in both pharmaceutical industry and research centres for infectious diseases. Purity guidelines for human VLP-based vaccines are given by the U.S. Food and Drug Administration and the World Health Organization: DNA concentrations should be below 10 ng per dose and protein purities above 95% [9,10]. The production of VLPs has been optimized in recent years for a number of different VLPs enabling the choice between varieties of recombinant systems such as bacteria, yeast, insect, mammalian or plant cells [11]. In contrast, downstream processing of VLPs predominantly relies on

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time-consuming and hardly scalable ultracentrifugation steps [2]. Vaccine manufacturers have recently focused on replacing ultracentrifugation by chromatography or filtration steps [12,13,11]. Although this approach has significantly improved the scalability of VLP processes, low binding capacities [14,15], product loss due to irreversible interactions and process-related product alterations [16,17], long process times and high process costs [18] remain major challenges. Making vaccines more available to the developing world, responding faster to pandemics and accelerating research, development and clinical studies are main issues to be solved. An alternative unit operation for biomolecules avoiding interactions with solid phases is aqueous two-phase extraction (ATPE). ATPE has proven to be a rapid [19], low-cost [20], scalable [21], selective [22] and integrated [23] bioprocess unit operation. A high and steadily increasing number of publications has underlined the applicability of aqueous two-phase systems (ATPSs) for the purification of antibiotics, enzymes, therapeutic proteins, monoclonal antibodies and viruses [19,20,22,24–26]. ATPSs have beneficial characteristics, especially for capturing products from crude cell feedstocks [27]. Drawbacks arise due to high experimental effort during process development, limited mechanistic understanding and low capacities.

High-throughput screenings on liquid handling stations have proven to be a valuable tool for rapid selection of adequate ATPSs for separation tasks and for improving the mechanistic understanding of protein partitioning [28,29]. This study focuses on the effect of different ATPS characteristics on the partitioning of human B19 parvo-VLPs derived from *Spodoptera frugiperda Sf*9 insect cells and the removal of host cell contaminants such as cell debris, host cell proteins (HCPs) and DNA. Human B19 parvo-VLPs are currently investigated as prophylactic vaccine candidates against diseases attributed to parvovirus infections [30,31]. This virus infects human erythroid precursor cells in the bone marrow and may cause aplastic anaemia. An infection during pregnancy can lead to foetal anaemia, often resulting in foetal congestive heart failure (hydrops foetalis) and miscarriage. Hence, there is a need for a vaccine, a diagnostic immunoreagent and a scalable purification process with a high VLP recovery. Recent studies evaluating ATPSs for the purification of VLPs have shown that such large biomolecules can be partitioned in a stable form in either polymer- or salt-rich phases. Benavides et al. [32] observed the partitioning of human rota-VLPs derived from *Sf*9 cells into the top phase of a PEG400-phosphate system achieving an HPLC purity of 11%. Partitioning of human B19 parvo-VLPs in PEG1000-magnesium sulphate systems was investigated by Luechau et al. [34]. The outcome was either a partitioning of VLPs and the majority of host cell proteins (HCPs) in the bottom phase or the interfacial attachment of VLPs separating HCPs into top and bottom phase. Since one of the major advantages of ATPSs is the incorporation of a solid–liquid separation step the latter result of Luechau et al. using clarified cell lysate would not be a desired process design for an integrated capture step. Moreover, Luechau et al. used low pH values throughout their study, which can entail disassembly and aggregation of VLPs [34,35]. While ATPSs might have unique advantages for capturing products from crude cell lysates, separation efficiency depends predominantly on the type of operation. Multi-stage ATPE provides mostly higher separation efficiencies than single-stage ATPE due to improved concentration profiles and a higher number of theoretical plates. A promising technique for multi-stage and large scale extraction of biopharmaceutical products is centrifugal partition chromatography (CPC). CPC systems have been optimized to achieve high stationary phase retentions for ATPSs and optimized mixing between stationary and mobile phases [36]. CPC case studies with ATPSs have been performed in the past for high-resolution separations of model proteins [37], and for the purification of monoclonal antibodies [38]. In this work, we demonstrate how capacity, recovery and purity of VLPs can be optimized in an integrated ATPE step. The resulting ATPS can be easily implemented as single-step process in any laboratory scale with basic equipment or as a multi-stage process with proper extraction devices. In addition, an alternative purification process for human B19 parvoVLPs is suggested.

2. Materials and methods

2.1. Disposables

All solubility screenings on the liquid handling station were conducted in 96 well Half Area polystyrene flat-bottom plates by Greiner Bio-One (Kremsmünster, Austria). ATPSs were prepared in 1 mL-96 Deep Well polypropylene plates (Thermo Fisher Scientific Inc., Waltham, Massachusetts, USA). All UV absorbance measurements were carried out in Greiner Bio-One 350 μL-polystyrene UV Star® plates. All fluorescence measurements were conducted in black Nunc® MicroWell™ polystyrene plates (Sigma–Aldrich, St. Louis, MO, USA). For all other purposes Greiner Bio-One 350 μL-polypropylene flat bottom MTPs were used. ATPS scale-up was done in BD Falcon™ 50 mL Conical Centrifuge Tubes (Becton, Dickinson and Company (BD), Franklin Lakes, New Jersey, USA).

2.2. Chemicals and stock solutions

PEG 200 and PEG 1450 were purchased from Sigma–Aldrich (St. Louis, MO, USA). $NaH_2PO_4{}^*H_2O$ and K_2HPO_4 were obtained from VWR BDH Prolabo (Radnor, Pennsylvania, USA). All other chemicals were purchased from Merck KGaA (Darmstadt, Germany). All buffer and stock solutions were prepared with ultra pure water drawn from a water purification system from Sartorius (Goettingen, Germany). Polymer and salt stock solutions were used as follows: 70% [w/w] PEG 400, 70% [w/w] PEG 600, 70% [w/w] PEG 1000, 60% [w/w] PEG 1450, 40% [w/w] PEG 4000, 40% [w/w] NaH_2PO_4, 40% K_2HPO_4 [w/w], 35% [w/w] citric acid, 35% [w/w] trisodium citrate, 40% [w/w] ammonium sulfate, 30% [w/w] sodium carbonate. Mono- and dibasic phosphate and citrate stock solutions were combined to yield the desired pH-values: e.g. 22.73 g NaH_2PO_4 solution and 77.27 g K_2HPO_4 solution yield pH 7.4. pH values of all other stock solutions and buffer solutions were titrated with hydrochloric acid and sodium hydroxide solutions.

2.3. Human B19 parvovirus-like particles

VLPs were derived from *Spodoptera frugiperda Sf*9 insect cells. Recombinant production of human B19 parvovirus-like particles and material supply was done by the industrial partner Diarect AG (Freiburg, Germany). VLPs composed of the major capsid protein VP2 and the minor capsid protein VP1 (VP1/VP2-VLP) and VLPs assembled by solely VP2 (VP2-VLP) were used throughout this study. All experiments were performed with *Sf*9 insect cell lysates prepared according to the following procedure: Frozen (−80 °C) pellets containing 8×10^8 *Sf*9 cells were resuspended in 20 mL lysis buffer (10 mM Tris pH 7.4, 15 mM $MgCl_2$, 250 mM NaCl, 0.5% [v/v] polysorbate 80, 10% [v/v] glycerol) by shaking the suspension for 30 min on an overhead shaker. Thereafter, cells were lysed on ice by sonication with a Branson Sonicator 450D equipped with a 1/2 in. microtip horn (Branson Ultrasonics, Danbury, CT, USA): 8×15 s sonication at 80%, with 59 s cooling steps in between. Protein degradation by proteases was counteracted by adding EDTA-free SIGMAFAST™ protease inhibitor cocktail tablets (Sigma–Aldrich, St. Louis, MO, USA) to the lysis buffer. Clarification of cell lysates was carried out by centrifugation for 30 min at $10,000 \times g$ and filtration with 0.1 μm cellulose acetate filters (Sartorius AG, Goettingen, Germany). Aliquoted cell lysates were stored frozen at −30 °C. VLPs purified by several ultracentrifugation steps were supplied by the industrial partner and served as reference material for analytics.