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Optimization and miniaturization of aqueous two phase systems for the purification of recombinant human immunodeficiency virus-like particles from a CHO cell supernatant

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ABSTRACT

Virus-like particles (VLPs) are promising candidates for a new generation of biopharmaceuticals, with a high impact in gene therapy, vaccination and also in the construction of delivery vehicles. Despite the growing interest in these particles, their production is currently limited by the low capacities and throughputs of classical downstream processing technologies.

Aqueous two-phase extraction (ATPE) is a promising bioprocessing technique allowing clarification, concentration and purification to be accomplished in a single step. ATPE also combines a high biocompatibility with a simple and reliable scale-up and can also be performed in a continuous mode of operation.

In this work, ATPE conditions for the purification of a Human Immunodeficiency Virus (HIV) VLP were screened and optimized in mL scale batch conditions. Polyethylene glycol (PEG)–salt (potassium phosphate, ammonium sulfate and trisodium citrate) and polymer–polymer (PEG–dextran) systems were investigated, among which the PEG–ammonium sulfate system demonstrated the higher partition coefficient ($K = 4.4$). This parameter was then compared with the obtained in a continuous microfluidic setting, performed by flowing both immiscible phases through a 100 width × 20 μm wide microchannel. The batch optimization results showed good agreement with the continuous miniaturized extraction, both in terms of K ($K = 3.9$ in microfluidic scale) and protein purity. These novel findings show that PEG–ammonium sulfate ATPE is a promising system for primary HIV-VLP recovery and demonstrate the potential of a miniaturized ATPE for massive parallelization (scale-out) at the preparative scale or integrated in analytical miniaturized systems.

1. Introduction

Virus-like particles (VLPs) are macromolecular structures resulting from the expression of viral proteins which spontaneously reassemble into the viral form without the nucleic acid content [1]. These particles cannot self-replicate, and thus they may serve as potential immunogens for vaccination. Furthermore, the structural proteins of VLPs are repetitive and present a high density display of epitopes leading to stronger and more effective immune responses [2]. VLPs can also be used as gene or biomolecule delivery tools [3]. However, there are still barriers to achieving high recovery efficiencies and maintaining the product quality after the downstream processing. The separation techniques currently used for purification of VLPs take advantage of the relatively large size of the particles and are based on gradient ultracentrifugation, ultrafiltration, precipitation or size exclusion chromatography (SEC) [4]. However, all these methods share general drawbacks and are in general labor-intensive, time-consuming and not easily amenable to scaling-up [5]. In particular, sucrose and cesium chloride density-gradient ultracentrifugation provided low yields, retained impurities and is very difficult to implement at an industrial scale [6,7]; precipitation with polyethylene glycol (PEG), ammonium sulfate or calcium phosphate has a low selectivity towards VLPs [5,8]; and traditional membrane-based tangential flow filtration techniques, such as ultrafiltration and microfiltration, suffer from membrane fouling issues and the retention and co-concentration of large molecular weight contaminants [5,9].

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Aqueous two phase systems (ATPS) are a promising alternative to address these challenges. ATPSs are formed when two immiscible compounds, such as two polymers or a polymer and a salt are mixed in an aqueous solution above a certain critical concentration, and spontaneously separate into two immiscible phases. These systems provide a biocompatible environment since they have a high water content, low interfacial tensions and some polymers also have a protein stabilizing effect [10], resulting in an efficient separation without denaturation or loss of biological activity. These systems have already been successfully applied to the separation of proteins from cell debris or to the purification of a target bioproduct from a crude feedstock [11,12], including antibodies [12], viruses [13], intact cells [14], VLPs [15,16], inclusion bodies [17], and plasmid DNA (pDNA) [18].

The separation effect arises from the differences in the physicochemical properties of the immiscible phases. Different solutes may have different affinities for the top and bottom phases. This behavior is often evaluated by the partition coefficient (K), defined in Eq. (1).

$$K = \frac{C_T}{C_B} \quad (1)$$

C_T and C_B are the equilibrium concentrations of the solute in the top and bottom phases, respectively.

The partition coefficient of a certain solute in ATPS depends on multiple factors either extrinsic to the target molecule such as (i) polymer molecular weights/size, (ii) concentration of the phase forming components, (iii) ionic strength, (iv) pH or (v) the use of an additional salt, such as NaCl, reported to promote the salting out effect of the desired protein to the top phase [19,20] or intrinsic to the target molecule, such as the (vi) size of the target molecule, its (vii) hydrophobicity or its (viii) isoelectric point, which is related to the global charge at a given pH.

The purification of VLPs using ATPS was first reported in 1989 for the concentration and purification of the outer envelope protein gp70 of the feline leukemia virus and the gag protein p27 using dextran-sulfate and polyvinyl alcohol, and where the two proteins were more than 40-fold purified [21]. Also, the bovine leukemia virus outer envelope protein gp51 achieved 15-fold purification in the system bottom phase using a dextran–PEG system [22]. The infectivity titer in an dextran–PEG ATPE of HIV-1 was retained in the interphase to about 48%, as measured by infectivity [23]. More recently, intracellular and extracellular double-layered rotavirus-like particles from insect cell culture were separated using PEG–phosphate systems with an overall recovery of 85% and a purity increase of 30–55 times comparing to the initial medium has also been described [15]. An initial extraction of a recombinant viral coat protein from cytoplasm of *Escherichia coli* using PEG–phosphate system with a $\ln K > 2.5$ [24], and an initial extraction step of B19 particles to the bottom phase of PEG 1000–magnesium sulfate system with a yield around 90%, while removing cell debris and 31% of total protein to the system's top phase, interface and sediment [16], were also described.

Miniaturized techniques taking advantage of soft-lithography processes and microfluidics have recently emerged as effective tools for expediting bioprocess design in ATPE [25]. These miniaturized processes are based on a number of parallel streams of immiscible phases flowing in continuous in a microchannel under a laminar flow regime. They are cost-effective since a large number of variables can be evaluated in parallel, reducing the sample/reagent volumes required, and promote lab-scale process similar to the continuous large-scale processes. Such technique has been applied to the purification of biomolecules such as monoclonal antibodies [26], bovine serum albumin [27], membrane proteins from crude cell extract [28], and recombinant proteins from a cell lysate [29]. The main advantages of this design are (i) the possibility of adjusting the length and width of the separation channel in order to speed up the corresponding purification and separation process by decreasing diffusion times; (ii) the simple operation; (iii) the very low amount of required reagents, of the order of 1–100 μL; and (iv) the absence of major manual preparation steps [25].

The goal of this study is to perform the screening and optimization of ATPE conditions for the extraction and purification of a VLP from Human Immunodeficiency Virus (HIV) produced in Chinese Hamster Ovary (CHO) cell cultures with a covalent bond to a GFP tag (HIV-GFP VLP) using different polymer–salt and polymer–polymer ATPS and to compare the obtained partition and purity results to a miniaturized continuous microfluidic approach.

2. Materials and methods

2.1. Chemicals and biologicals

PEG with molecular weights of 1000, 1500, 2000, 3350, 6000, 8000, 10000 and 20000 Da, dextran with molecular weights of 40000, 100000, 162000, 298000 and 500000 Da, potassium phosphate monobasic anhydrous, potassium phosphate dibasic anhydrous, sodium phosphate monobasic anhydrous, sodium chloride (NaCl), ammonium sulfate, citric acid, trisodium citrate dehydrate, glycine and propylene glycol monomethyl ether acetate (PGMEA) 99.5% were purchased from Sigma–Aldrich. SU-8 negative photoresist 2015 formulation was purchased from Microchem. Polydimethylsiloxane (PDMS) was purchased from Dow-Corning (Midland, MI, USA) as a Sylgard 184 silicon elastomer kit.

CHO cell medium supernatant expressing HIV-GFP VLP (pH 7.6) was formulated and provided by Icosagen® (Tartumaa, Estonia). The culture medium comprised a mixture of CDCHO and 293SFM Life Technologies commercial serum-free mediums, supplemented with GlutaMAX™.

2.2. ATPS preparation in batch conditions

Stock solutions of PEG 50% (w/w), dextran 25% (w/w), potassium phosphate, ammonium sulfate and trisodium citrate 40% (w/w) were prepared using ultrapure water, obtained from a MilliQ purification system from Millipore (Billerica, MA, USA). ATPS were prepared by weighting the appropriated mass amounts of the phase forming components (PEG, dextran and salt stock solutions), sodium chloride, supernatant sample and MilliQ water to a total final system mass of 1.5 g, in order to achieve the desired final mass fractions of each component (% w/w).

The components concentrations in the system final composition were screened in order to optimize the partition coefficient for HIV-GFP VLPs: between 8% and 29% (w/w) for PEG, 14–16.5% (w/w) for dextran, 10–14% (w/w) for potassium phosphate, 12.5–14.5% (w/w) for ammonium sulfate, 12–15% (w/w) for trisodium citrate, 0–6% for sodium chloride, and between 15% and 30% (w/w) for CHO supernatant containing HIV-GFP VLPs.

All system components were thoroughly mixed in a vortex mixer (Ika, Staufen, Germany) and centrifuged at 4000*g* for 5 min at room temperature in a fixed angle rotor centrifuge (Eppendorf, Hamburg, Germany), assuring total phase separation. The volumes of top and bottom phases were measured and samples from both phases were taken for protein analysis and/or fluorescence measurements.

2.3. Microchannel fabrication

The microfluidic device was fabricated using standard soft lithography methodologies similar to those used by Soares and co-workers [30]. In summary, the microchannel was designed in AutoCAD 2013 software. The pattern was transferred to a hard