Journal of Chromatography A, 1464 (2016) 1–11

Contents lists available at ScienceDirect

Journal of Chromatography A

journal homepage: www.elsevier.com/locate/chroma

High-throughput downstream process development for cell-based products using aqueous two-phase systems

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ARTICLE INFO

Article history:
Received 14 June 2016
Received in revised form 9 August 2016
Accepted 10 August 2016
Available online 11 August 2016

Keywords:
High throughput screening (HTS)
Downstream processing of cell-based products
Label-free cell separation
Aqueous two-phase systems (ATPS)
High-throughput flow cytometry

ABSTRACT

As the clinical development of cell-based therapeutics has evolved immensely within the past years, downstream processing strategies become more relevant than ever. Aqueous two-phase systems (ATPS) enable the label-free, scalable, and cost-effective separation of cells, making them a promising tool for downstream processing of cell-based therapeutics. Here, we report the development of an automated robotic screening that enables high-throughput cell partitioning analysis in ATPS. We demonstrate that this setup enables fast and systematic investigation of factors influencing cell partitioning. Moreover, we examined and optimized separation conditions for the differentiable promyelocytic cell line HL-60 and used a counter-current distribution-model to investigate optimal separation conditions for a multi-stage purification process. Finally, we show that the separation of CD11b-positive and CD11b-negative HL-60 cells is possible after partial DMSO-mediated differentiation towards the granulocytic lineage. The modeling data indicate that complete peak separation is possible with 30 transfers, and >93% of CD11b-positive HL-60 cells can be recovered with >99% purity. The here described screening platform facilitates faster, cheaper, and more directed downstream process development for cell-based therapeutics and presents a powerful tool for translational research.

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1. Introduction

High-throughput screening (HTS) technologies are state-of-the art in various scientific disciplines, such as basic research, diagnostics, and biotechnology [1–4]. In the past decade, HTS-technologies have gained increasing importance in downstream process development of biopharmaceuticals, such as protein- and DNA-based drugs, and are currently available for most platform technologies, including column chromatography, crystallization, and aqueous two-phase systems (ATPS) [4–11]. These technologies have facilitated faster, cheaper, and more directed downstream process development. In combination with modeling and simulation data, they present a powerful tool to elicit mechanisms underlying separation and design purification strategies for novel targets.

As cell-based therapeutics are entering the market, new scalable downstream processing strategies are required. For clinical applications, cell-based products need to be provided in high purities, since certain contaminants can cause severe adverse effects, such as teratoma formation or graft-versus-host-disease (GvHD) [12–17]. The most widely applied cell separation methods use cell surface

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http://dx.doi.org/10.1016/j.chroma.2016.08.025
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antigens for affinity-purification. These methods, however, require large amounts of clinical-grade antibodies, resulting in extremely high costs. Antibody-labels that remain on the cell surface, pose considerable regulatory obstacles for the treatment of patients, as they can cause adverse reactions [15,16,18,19].

ATPS offer a gentle, cost-effective, label-free, and scalable means of cell purification, and are well established in downstream processing of biopharmaceuticals [20–22]. ATPS are aqueous solutions consisting of different phase forming compounds, e.g. polymers, which form two immiscible phases above a certain threshold. The two phases have distinct physicochemical properties, and biomolecules partition between them according to their physicochemical properties. It is well established that polyethylene-glycol (PEG)-dextran ATPS can be used to separate cells according to their surface properties. It has been shown that ATPS can be used to separate different cell types with high selectivity and resolution. Using multi-step partitioning, such as countercurrent distribution (CCD), even complex cell mixtures can be separated [21,23]. Nonetheless, the development of effective downstream processes requires the optimization of many different parameters, hampering widespread application of ATPS in cell purification.

In this work, an automated HTS-platform for the investigation of cell partitioning in ATPS was developed and validated. The screening was implemented on a robotic liquid-handling station (LHS), and includes all steps from ATPS preparation to cell quantification and analysis using HT-flow cytometry. In a first case study, the partitioning of the differentiable cell line HL-60 was evaluated in a number of ATPS. HL-60 was chosen since it can be partially differentiated towards neutrophil granulocytes by DMSO-treatment, and monitored using an anti-CD11b-antibody [24]. The influence of salt composition, pH, and tie-line length (TLL) on the resolution of CD11b-positive and CD11b-negative HL-60 was evaluated, and separation conditions were optimized. Using a CCD-model, we investigated optimal separation conditions for a multi-stage purification process. Overall, we show that the here described HTS-platform enables fast and directed downstream process development for cell-based products and will permit the systematic investigation of mechanisms underlying cell separation in ATPS.

2. Material & methods

2.1. Disposables

For absorbance measurements, UV-Star plates (Greiner Bio-One, Kremsmuenster, Austria) were used. For flow cytometry, 96-well U bottom plates (BD Falcon™, Franklin Lakes, NJ, USA) were used. ATPS were prepared in 1.3 mL deep well plates (Nalgene Nunc, Rochester, NY). For all other purposes, polypropylene flat-bottom microplates (Greiner Bio-One) were used.

2.2. Software and data processing

The Tecan Freedom Evo 200 was controlled using Evoware 2.5 SP2 standard (Tecan, Crailsheim, Germany), and the Tecan Inifinite® Pro M200 plate reader was controlled via Magellan 7.1 SP 1 (Tecan). For advanced applications such as sampling and cell resuspension, visual basic scripts were generated and fed into Evoware. Excel 2013 (Microsoft, Redmond, WA, USA) was used as import format and for data storage. Data evaluation and visualization was performed with Excel 2013 and Matlab R2014a (The MathWorks, Natick, ME, USA). For statistical data analysis Matlab R2014a was used. BD FACSDiva 8.0 (BD Biosciences, San Jose, CA, USA) was used to control the BD LSR Fortessa Cell Analyzer and for raw data analysis. For visualization of flow cytometry data, Flow Jo V10 (Tree Star, Ashland, OR, USA) was used.

2.3. Preparation of buffers and stock solutions

All buffers and stock solutions were prepared with ultra-pure water (0.55 μS/cm) obtained from an Arium®proUV water system (Sartorius Stedim Biotech, Goettingen, Germany). Stock solutions of 2% and 20% dextran 500,000 (Pharmacosmos A/S, Holbaeck, Denmark, cat. 5510 0500 9007, Batch No.: HT3229) and 30% PEG 8,000 (Sigma Aldrich, St. Louis, MO, USA, cat. P2139, Batch No.: 059Ko121) were prepared and dissolved on a magnetic stirrer overnight. 500 mM sodium phosphate (NaPi) buffer stock solutions were prepared by using di- (Merck Millipore, Billerica, MA, USA) and monobasic sodium phosphate (Sigma Aldrich) in varying ratios, in order to obtain specific pH-values (241 mM Na_2HPO_4 and 259.4 mM NaH_2PO_4 for pH 6.6, 353 mM Na_2HPO_4 and 147.4 mM NaH_2PO_4 for pH 7.0, 430 mM Na_2HPO_4 and 70.6 mM NaH_2PO_4 for pH 7.4). 1 M NaPi buffers were prepared analogously. NaCl (Merck Millipore) was prepared as 1 M and 500 mM stock solutions. A 1 mM methyl violet 2 B stock solution (Sigma Aldrich) was prepared in ultra-pure water. All buffers and polymer solutions were filtrated (Ø 0.22 μm). Buffers were stored at room temperature and polymer solutions were stored at 4 °C. Osmolarities of the distinct ATPS were verified using a VAPRO® 5600 vapor pressure osmometer (Wescor, Logan, UT, USA), and all ATPS had osmolarities within the physiological range (290–310 mOs). FC-staining buffer consisted of phosphate buffered saline (PBS) supplemented with 2 mM EDTA (Life Technologies™, Carlsbad, CA, USA) and 0.5% bovine serum albumin (BSA) (Miltenyi Biotech, Bergisch Gladbach, Germany).

2.4. Cell culture

All cell culture reagents were purchased from Life Technologies™. The promyelocytic cell line HL-60 and the murine fibroblast cell line L929 were purchased from CLS (Cell Lines Service, Eppelheim, Germany) and propagated at 37 °C in a humidified 5% CO_2 incubator. HL-60 cells were grown in RPMI 1640 with GlutaMAX supplemented with 15% fetal bovine serum (FBS) 1% sodium pyruvate, 1% non-essential amino acids (NEAA), and 0.5% Penicillin/Streptomycin. L929 cells were grown in DMEM with GlutaMAX supplemented with 10% FBS and 1% Penicillin/Streptomycin.

2.5. Granulocytic differentiation of HL-60 cells

Granulocytic differentiation of HL-60 was induced by the addition of DMSO [24]. For each differentiation experiment, cells were seeded at a density of 2.5×10^5 cells/mL in culture medium supplemented with 1.25% (v/v) DMSO (Sigma Aldrich) and cultured for 7 days. During this time, cell growth was monitored and medium was changed after 3 days.

2.6. Cell Tracker™, antibody and viability staining

In spiking experiments and to discriminate between the two cell lines, HL-60 cells were stained with CellTracker™ Orange CMRA (Life Technologies™). 1×10^6 cells per mL were resuspended in RPMI with GlutaMAX and stained with 0.5 μM dye at 37 °C for 30 min. The cells were subsequently resuspended in culture medium, and incubated at 37 °C for 30 min to inactivate unbound dye molecules.

For detection of differentiated HL-60 cells, a FITC-conjugated rat-anti-human-CD11b antibody (Miltenyi Biotech) [24] was used at a dilution of 1:100. For antibody staining, up to 1×10^6 cells were resuspended in 100 μL of FC-staining buffer, and incubated at 4 °C in the dark for 10 min. For analysis of cell viability, cells

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