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Integrated purification of monoclonal antibodies directly from cell culture medium with aqueous two-phase systems

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ABSTRACT

The purification of monoclonal antibodies anti-CD34 produced in hybridoma cells was accomplished by aqueous two phase extraction, using an integrated process that allowed to clarify and partially purify the produced mAb in just one step. The feasibility of using polyethylene-glycol (PEG)/dextran systems was studied at different ionic strengths (0–300 mM NaCl) and at different pH values (pH 3, 4 and 7). The effect of molecular weight (MW) of PEG (3350 and 6000 Da) was also evaluated. For all the conditions studied, it was observed that antibodies partition preferentially to the PEG-rich phase, cells to the interface and soluble proteins to the bottom dextran-rich phase. The best recovery yield was obtain with an ATPS composed by 7% PEG 6000 Da, 5% dextran 500,000 Da, 150 mM NaCl at pH 3. In this system, it was possible to recover 84 ± 6.5% IgG with 0.1 ± 0.2 % of cells in the top phase.

1. Introduction

The biopharmaceutical market has been steady increasing since Humulin, a recombinant human insulin produced in *Escherichia coli*, was approved for the treatment of diabetes in 1982. Later, in 1986, human tissue plasminogen activator (tPA) became the first therapeutic protein from recombinant mammalian cells to obtain market approval [1]. Also, in 1986, the FDA-approved the first therapeutic monoclonal antibody (mAb), Orthoclone OKT3, produced *in vivo* by hybridoma cells. The global biopharmaceuticals market, estimated at US$199.7 billion globally in 2013, has been projected to reach US$497.9 billion by 2020 [2]. Most of these biopharmaceuticals target diseases such as cancer, transplant rejection, rheumatoid arthritis, Cohn's disease, asthma, cardiovascular and infectious diseases. Amongst the different product segments, monoclonal antibodies (mAbs) constitutes the largest product segment in the global biopharmaceuticals market, with an estimated share of 25.6% in 2013, and accounting for US$51.1 billion [2].

One of the most challenging aspects of the manufacturing of mAbs is related to the high therapeutic doses required and has led to an increasing demand for high quantities of pure mAbs [3]. Upstream processing of mAbs has been considerable improved in the last years with antibody titers and cell densities reaching impressive records. Downstream processing (DSP) however has not evolved at the same pace and is currently considered the bottleneck in the manufacturing of mAbs [4,5]. In fact, the DSP train is currently unable to cope not only with the high titers, with unit operations like chromatography running into binding capacity limitations, but also with the large cell densities which are challenging the traditional solid–liquid technologies (centrifugation and filtration). One way of unlocking these bottlenecks is by process integration through extraction in aqueous two-phase systems (ATPS).

ATPS is an aqueous liquid–liquid system that results from the incompatibility between two aqueous solutions with structurally different components, which can be for example two polymers (e.g. polyethylene glycol – PEG and dextran), or a polymer and a salt (e.g. PEG and phosphate) above a critical concentration [6–8]. The advantages of using this type of purification methodology includes: (i) the stabilization of proteins due to rapid mass transfer and mixing of polymers; (ii) the separation of key components can be made highly selective; (iii) it enables the processing of solid containing streams; (iv) it is easy to scale-up; (v) it is possible to operate in a continuous mode; (vi) it is cost effective; (vii) the phases of the system are in aqueous form and, finally, (viii) it allows process integration [9,10]. Despite these compelling advantages, only a few biological products have been purified by this technique at large scale [11–13]. Nonetheless, ATPS has become a powerful non-chromatographic unit operation that can allow at the same time the clarification, concentration and partial purification of the target protein [14,15], and that has been successfully applied in the purification process of several biopharmaceutical

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products including mAbs, other proteins, virus-like particles and plasmid DNA [16–19].

In 1996, Ziljstra and co-workers proposed an integrated process based on PEG/dextran systems for both hybridoma cell culture and IgG purification. In this process, the dextran-rich phase supported the growth of the hybridoma cells while the secreted antibody was to be recovered in the PEG-rich phase. In the absence of any affinity ligand, both cells and IgG partition to the lower phase [10]. An affinity ligand, triazine dye mimetic green, coupled to the PEG molecule was further employed to improve the recovery of the IgG product in the PEG-rich phase (top phase). Nevertheless, it was observed that the integration of ATPS in culture medium changed the partition of hybridoma cells from the bottom phase to interface [11]. Latter, in 2009, Platis and co-workers following a similar process integration strategy, applied a PEG 1500/phosphate system for the separation two mAbs from an unclarified transgenic tobacco crude extract. Both mAbs partitioned to the bottom phase with recovery yields higher than 84%, at pH 5 [20].

In this work, the feasibility of using a PEG/dextran system for the simultaneous clarification and capture of mAbs from hybridoma cell cultures has been addressed. A serum-containing cell culture medium has been used to optimise the best extraction conditions for mAbs and the optimised conditions have further been applied for the extraction of the same mAb but from a serum-free cell culture medium.

2. Experimental

2.1. Materials

PEG with molecular weights (MW) of 3350 and 6000 Da were purchased from Sigma (St. Louis, MO, USA) as a 50% solution in water. Dextran with a MW of 500 000 Da was obtained from Sigma as a 20% solution. Tris(hydroxylmethyl)aminomethane (Tris), sodium chloride, sodium azide were purchased from Sigma–Aldrich (St. Louis, MO, USA). Sodium phosphate monobasic anhydrous and sodium phosphate dibasic were obtained from Panreac Quimica Sau (Barcelona, Spain), and hydrochloric acid from Fluka (Buchs, Switzerland). All other chemicals were of analytical grade.

Human IgG for therapeutic administration (product name: Gammanorm) used to prepare IgG stock solutions was purchased from Octapharma (Lachen, Switzerland), as a 165 mg/mL solution containing 95% of IgG. Fetal bovine serum (FBS) with an ultra-low content of IgG was obtained from Gibco® (Carlsbad, CA, USA). Mouse hybridoma cell line HB-12346™ (AC133.1) that secretes an antibody that reacts with a subset of hematopoietic progenitor cells derived from human bone marrow, fetal bone marrow and liver, cord blood and adult peripheral blood, were obtained from American Type Tissue Collection (Rockville, MD, USA) [21].

2.2. Methods

2.2.1. Cell culture

Mouse hybridoma cells were obtained by fusing lymph node cells, from New Zealand Black mice immunized with purified CD34 positive human progenitor stem cells, with Sp2/0 Ag14 mouse myeloma cells [22]. The produced antibody is an IgG1 with kappa light chains.

Cells were cultured in Dulbeccós modified Eaglés medium (DMEM) obtained from Gibco®, at an initial cell density of 5×10^5 cells/mL. DMEM was formulated to contain 4.5 g/L D-glucose, 1.5 g/L sodium bicarbonate and 4 mM L-glutamine (Gibco®), and was supplemented with 10% (v/v) ultra-low IgG FBS, 1% (v/v) penicillin (50 U/mL) and 1% (v/v) streptomycin (50 μg/mL), all obtained from Gibco®. The number of live and dead cells was determined using a hemocytometer under an optical microscope using the trypan blue dye exclusion method.

Hybridoma cells were also adapted to grow in a chemically-defined serum free medium, CD Hybridoma from Gibco®, using a sequential adaptation strategy, where the initial percentage of DMEM/FBS was successively decreased to 75%, 50%, 25% and finally 0% with a concomitant increase in the percentage of the serum-free media from 0% to 25%, 50%, 75% and finally to 100%. The CD Hybridoma medium was supplemented with 4 mM L-glutamine, 3.7 g/L sodium bicarbonate and 1% (v/v) antibiotics (50 U/mL penicillin and 50 μg/mL streptomycin).

For the extraction studies, cells were grown in 175 cm^2 T-flasks, at 37 °C, 5% CO_2 during 7 days in both DMEM/FBS and CD Hybridoma culture media.

2.2.2. Aqueous two phase extraction

Batch biphasic systems were prepared by weighting the PEG and dextran from stock solutions of 50% and 20% dextran, respectively, in order to achieve a final concentration of 7% PEG and 5% dextran [23]. A stock solution of 1 M sodium phosphate buffer, at pH7, was used as buffering agent at a final concentration of 10 mM. The systems had a pH around 7, and in order to obtain systems at lower pH values, HCl was added to the medium. A stock solution of 1.5 M sodium chloride was used to adjust the ionic strength. Systems were prepared with 0 mM, 150 mM and 300 mM NaCl. All chemical components were thoroughly mixed in a vortex. The hybridoma cell culture was then transferred directly from culture to the systems, without any previous centrifugation step, to a final concentration of 20% and was gently mixed with the phase forming components. The systems were incubated for 2 h at room temperature. Phase volumes were then determined and the total number of cells present in the top and bottom phase were counted using a hemocytometer under an optical microscope using trypan blue dye exclusion method. The number of cells in interface was calculated by the difference of cells in top and bottom phase to the total number of cells added initially to the systems ($7–8 \times 10^6$ cells for the DMEM/FBS culture and around $4–5 \times 10^6$ cells for the CD Hybridoma culture). The top and bottom phase were separated and store at −4ªC for further analysis (HPLC and Bradford assay).

2.2.3. Analytical methods

2.2.3.1. IgG quantification. The quantification of IgG in top and bottom phase from the different ATPS prepared was performed by protein G affinity HPLC using a Poros protein G affinity column from Applied Biosystems (Foster City, CA, USA) in an AKTA™ Purifier 10 from GE Healthcare (Uppsala, Sweden) [24]. The adsorption buffer was composed by 50 mM phosphate, 150 mM NaCl at pH 7.4 and the elution buffer by 12 mM HCl, 150 mM NaCl, pH 2–3. The absorbance was monitored at 215 nm.

2.2.3.2. Total protein quantification. Total soluble protein present in the top and bottom phase was quantified by the Bradford method [25] using the Coomassie assay kit supplied by Pierce (Rockford, IL, USA). The assay was set up in 96-well polystyrene microplates. Bovine serum albumin (BSA) was used as protein standard for calibration curve. The absorbance was measured at 595 nm in a Spectra Max 340PC microplate reader from Molecular Devices (Sunnyvale, CA, USA).