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Integration of cell harvest with affinity-enhanced purification of monoclonal antibodies using aqueous two-phase systems with a dual tag ligand

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ABSTRACT

Monoclonal antibodies (mAbs) are currently the most important class of recombinant protein therapeutics in the biotechnological and biopharmaceutical industry with more than 250 therapeutic mAbs currently undergoing clinical trials. High titer producing cultures and complex mixtures containing high cell densities, together with an increasing growing demand for highly pure mAbs is making recovery and purification processes hot targets for improvement and opens important technological challenges in mAbs manufacturing platforms.

This work explores the use of an affinity dual ligand based on a choline binding polypeptide tag (C-LytA) fused to the synthetic antibody binding Z domain (LYTAG-Z) in aqueous two-phase systems (ATPS) composed of phase forming polymers able to bind to the choline binding site of C-LytA (polyethylene glycol -PEG- and thermosensitive polymers -EOPO) for mAbs selective extraction. Integration of harvesting and ATPS affinity extraction steps were evaluated with ATPS proving to be an alternative strategy for integrating the clarification and the primary recovery of mAbs. An extraction yield of 89% and a clarification higher than 95% were achieved using a system composed of 7% PEG 3350 and 6% dextran 500,000.

1. Introduction

Monoclonal antibodies (mAbs) are established medicines that are being safely produced by big pharma companies in quantities up to several tons per year. However, despite their effectiveness and safety as therapeutic agents, mAbs are amongst the most expensive drugs on the market – the annual cost for a cancer patient receiving antibody treatment can reach $35,000, making this treatment considerably expensive for many patients [1]. Given their low potency and increasing market potential, large amounts of pure mAbs are thus required. This increasing product demand has been challenging biotechnologists to increase production capacity, improve purification technologies and reduce manufacturing costs [1,2].

Upstream processing of mAbs has been considerable improved in the last years with antibody titers and cell densities reaching impressive records, while downstream processing (DSP) is currently considered the bottleneck in the manufacturing of mAbs. Indeed, it has been stated that industrial scale production viability strongly depends on the separation and purification techniques [3]. Up to now, protein A affinity chromatography has been considered the most effective technology in the downstream processing of mAbs, though it accounts for more than 50% of the entire downstream processing costs [4]. Improved resins have been developed allowing higher flow rates, cleaning easiness and increased capacity, however their cost is still considered high for the generation of preclinical and early phase clinical batches [5]. Along with pressure drop problems, the inability to cope with crude stocks, cells and cell debris is also a major limitation of packed-bed chromatography [6]. In fact, the current DSP technologies are unable to cope not only with the high titers, but also with the large cell densities, which are challenging traditional solid–liquid technologies, resulting in poor yields [7].

Novel affinity-based separations have been emerging from the development of synthetic ligands, including peptides obtained by

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combinatorial libraries and artificial ligands generated by *de novo* process designs [6], although, so far with limited applicability in big pharma companies. Non-chromatographic alternatives including tangential flow filtration, high gradient magnetic fishing, aqueous two-phase extraction, precipitation and crystallization have also been described [8–12].

Aqueous two-phase systems (ATPS) allow for the simultaneous purification, concentration, and clarification of bioproducts by direct extraction of the target product from the cell culture media in a system composed by an aqueous solution of two polymers or a polymer and a salt [13–16]. ATPS can combine a high biocompatibility and selectivity with an easy and reliable scale up and capability of continuous operation [17–20].

Taking advantage of this alternative technology that can combine cell clarification and purification in one step, this paper explores the use of an affinity dual ligand, the LYTAG-Z, which is composed of a choline binding polypeptide tag (C-LytA) fused to the synthetic antibody binding Z-domain, for mAbs purification from complex media using PEG and EOPO containing ATPSs. C-LytA is the C-terminal region of the *Streptococcus pneumoniae* LytA amidase and enables the attachment of this enzyme to the phosphocholine residues present on the teichoic acids of the cell wall surface [21]. Structurally, C-LytA is built up from six conserved β-hairpins that form four choline-binding sites [22]. This module's affinity for choline and choline structural analogs (e.g. ternary amines) has been used for the purification of C-LytA-tagged proteins by adsorption to amine-based chromatographic resins (e.g. DEAE cellulose) followed by elution with choline [23]. Several recombinant proteins fused to C-LytA, such as GFP and β-galactosidase, have been purified in PEG containing ATPSs by preferential partition of the fused protein to the PEG-rich phase, followed by the subsequent elution to the PEG-poor phase using choline [24].

In this paper, C-LytA was fused to the Z domain, a synthetic IgG-binding peptide based on the B-domain of protein A [25] and used as a dual affinity ligand for mAbs purification from complex media through aqueous two-phase extraction. The feasibility to process unclarified cell cultures is further investigated in order to integrate cell harvesting and initial purification in one single step, envisaging process integration. Once the two-phase system is generated, it is expected to obtain LYTAG-Z-antibody complexes in the PEG rich-phase, while cells and other impurities partition to the opposite phase.

2. Experimental

2.1. Chemicals

Monobasic and dibasic potassium phosphate were obtained from Pancreac (Darmstadt, Germany). PEG 3350, sodium chloride, dextran 500,000, polyacrylic acid sodium salt (NaPAA) 8000 and other analytical grade reagents were purchased from Sigma Aldrich (St. Louis, MO, USA). Coomassie-Bradford reagent was supplied by Thermo Scientific (Rockford, IL, USA). Ethylene oxide/propylene oxide co-polymers (EOPO) (Breox 50A140, Breox 50A225 and Breox 50A380) were kindly donated by ILCO Chemikalien GmbH (Erkelenz, Germany). Their main difference relies in their viscosity, which is 133, 217 and 387 cSt, respectively, at 40 °C.

2.2. Biologicals

Human immunoglobulin was obtained from Octapharma (Lachen, Switzerland) as a commercially available solution for therapeutic administration (Gammanorm®), in which the active substance has a concentration of 165 mg/mL, and at least 95% corresponds to immunoglobulin G (IgG).

CHO cell supernatant was produced and delivered by IcoSagen (Tartumaa, Estonia). This supernatant contains a humanized monoclonal antibody, from IgG1 sub-class. This antibody is derived from mouse anti-hepatitis C virus subtype 1b NS5B (nonstructural protein 5B) monoclonal antibody 9A2 expressed in mouse Hybridoma culture. cDNA of antibody variable regions was isolated and cloned into the human IgG1 constant region-containing antibody expression vector. CHO cells were grown in a mix of two serum-free growth media, the CD CHO Medium (Gibco®, Carlsbad, CA) and the 293 SFM II Medium (Gibco®). Final concentration of IgG is around 0.300 g/L.

Hybridoma cells were grown in a mixture of two different media, composed of 25% (v/v) of Dulbecco's modified Eagle's medium (DMEM) high glucose (Gibco®) and 75% (v/v) of CD Hybridoma medium (Gibco®). The IgG produced is a mouse anti-human CD34+ antibody. DMEM contains 4.5 g/L D-glucose and 4 mM GlutaMAX™. After resuspension of DMEM powder in milli-Q water, 3.5 g/L of $NaHCO_3$ and 1% antibiotics (v/v) (100 U/mL penicillin and 100 μg/L streptomycin) (Gibco®) were added. The DMEM medium was also supplemented with fetal bovine serum (FBS) with ultra-low IgG content (Gibco®) at a final concentration of 10% (v/v). CD Hybridoma medium, an animal origin-free, protein-free and chemically defined formulation, was supplemented with 8 mM de GlutaMAX™ and 1% antibiotics (v/v) (100 U/mL penicillin and 100 μg/L streptomycin) (Gibco®). Both media were sterilized with a 0.22 μm filter. The cell density present in the cell culture was around 5×10^6 cells/mL.

The LYTAG-Z fusion protein (245 aa, 28.39 kDa, theoretical pI 4.88) was expressed in *Escherichia coli* and purified by Biomedal S.L. (Seville, Spain) using the *Cascade*™/LYTAG technologies.

2.3. ATPS formulation

Four different types of ATPS were formulated: PEG/phosphate, PEG/dextran, EOPO/dextran and EOPO/NaPAA. The different ATPS were formulated by weighting the corresponding quantities of the needed reagents. Stock solutions were prepared for 50% (w/w) PEG 3350, 40% (w/w) phosphate and 25% (w/w) dextran 500,000. Each ATPS system was thoroughly studied using a factorial design and the best formulation found for each ATPS was: 12.5% (w/w) PEG and 12.5% (w/w) potassium phosphate; 7% (w/w) PEG and 6% (w/w) dextran; 10% (w/w) EOPO and 10% (w/w) dextran; 10% (w/w) EOPO and 10% (w/w) NaPAA. The corresponding biological sample (pure human serum antibodies, clarified CHO cell culture supernatant or unclarified Hybridoma cell culture media) was loaded at 20% (w/w). The LYTAG-Z ligand (600 mg/L) was also loaded at 20% (w/w) of the system, to assure a molar ratio of antibody to LYTAG-Z of 1:10. The system was completed to 2 g with milli-Q water. The ATPS were afterward mixed by vortex and phases' coalescence was achieved letting the systems settle for 30 min. Control systems were prepared without LYTAG-Z and used to assess the effectiveness of the dual ligand. Blank systems were prepared without biological sample and LYTAG-Z and used to evaluate and discount the interference of the phase forming components in the analytical methods.

2.4. Cell counting

The total number of cells present in the top and bottom phases was counted using a hemocytometer under an optical microscope Leica DMLB (Wetzlar, Germany) by means of trypan blue dye (Gibco®) exclusion method. Each phase was also visualized in an Olympus CKX41 microscope (Tokyo, Japan) with digital images acquisition done with a CellSens Entry software (Tokyo, Japan) and treated with ImageJ software [26].