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RESEARCH

Continuous culture of mouse hybridoma cells for 100 Days in the Lambda MINIFOR bioreactor

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ABSTRACT

The mouse hybridoma producing the CB.Hep-1 monoclonal antibody (mAb) was cultivated for 100 days in protein-free medium using a Lambda MINIFOR bioreactor. The CB.Hep-1 mAb showed no modifications on its biochemical properties and target antigen immunopurification capacity. The properties of the cell culture and mAb produced were: 1.1 ± 0.2 × 10^6 cells/mL viable cell density, cell specificity production rate (SPR): 25.7± 9.8 μg/10^6 cells · day, 82.5 ± 6.7 % viability, 122.7 ± 43.6 mg/L mAb concentration, 125.7 ± 43.6 mg/harvest · day, 12.5 g of harvested mAb and 10.1 g of purified mAb. The mAb purity was over 98 %, mAb being obtained with no aggregation or modification of specificity and affinity constant (CKTCTT, 0.9 ± 0.6 × 10^{10} M^{-1}). The bioreactor vessel volume was renovated in up to 19 % per day. Target antigen immunopurification efficiency was similar to that with mAb isolated from other biological sources: 81.4 ± 21.8 μg/mg adsorption capacity, 52.6 ± 14.1 % adsorption efficiency, 60.0 ± 20.8 μg/mg elution capacity, 83.2 ± 11.60 % elution efficiency, 88.5 ± 1.0 % antigen purity, 0.03 ± 0.015 % released-coupled mAb ratio and 0.1-4.8 ng mAb/μg Ag leakage mAb-antigen ratio. These bench-scale results support the scale-up of the CB.Hep-1 hybridoma culture to a 600-L bioreactor under continuous culture without cell retention, or to a 50-L bioreactor with cell retention at 25-30 × 10^6 cell/mL, rendering up to 2025 or 1378 g of CB.Hep-1 mAb per year, respectively. The Lambda MINIFOR bioreactor robustness is adequate for long-term production of mammalian cells at bench-scale.

Keywords: bioreactor, Hepatitis B surface antigen, mammalian cell culture, monoclonal antibody, protein free medium

RESUMEN

Cultivo continuo de células de hibridoma de ratón durante 100 días en el bioreactor Lambda MINIFOR. Se cultivó el hybridoma de ratón productor del anticuerpo monoclonal (mAb) CB.Hep-1 durante 100 días con medio libre de suero en el biorreactor Lambda MINIFOR. El mAb CB.Hep-1 se obtuvo sin cambios en sus propiedades bioquímicas ni en su capacidad para inmunopurificar el antígeno blanco. Las propiedades del mAb y del cultivo celular fueron: densidad de células viables, 1,1 ± 0,2 × 10^6 células/mL; producción específica (SPR), 25,7± 9,8 μg/10^6 células · día; viabilidad 82,5 ± 6,7 %; concentración del mAb, 122,7 ± 43,6 mg/L mAb, 125,7 ± 43,6 mg/cosecha · day; 12,5 g de mAb cosechado y 10,1 g de mAb purificado. La pureza del mAb fue superior al 98 %, y este se obtuvo sin agregaciones, y retuvo su especificidad y su constante de afinidad (CKTCTT, 0,9 ± 0,6 × 10^{10} M^{-1}). El volumen del biorreactor se renovó en hasta un 19 % por día. La eficiencia de inmunopurificación del antígeno blanco fue similar a la del mAb aislado de otras fuentes biológicas: capacidad de adsorción, 81,4 ± 21,8 μg/mg; eficiencia de adsorción, 52,6 ± 14,1 %; capacidad de elusión, 60,0 ± 20,8 μg/mg; eficiencia de elusión, 83,2 ± 11,60 %; pureza del antígeno, 88,5 ± 1,0 %; proporción de mAb acoplado-liberado 0,03 ± 0,015 %), y desacople del mAb (0,1-4,8 ng mAb/μg Ag). Esto permitió estimar el posible escalado del sistema a biorreactores de 600 L o de 50 L, ambos con cultivo continuo, sin o con retención de células a 25-30 × 10^6 células/mL, respectivamente, con rendimientos aproximados de 2025 o 1378 g de CB.Hep-1 mAb por año, respectivamente. El biorreactor Lambda MINIFOR es un sistema robusto para el análisis de cultivos de células de mamíferos a escala de laboratorio por largos periodos de tiempo.

Palabras clave: biorreactor, antígeno de superficie del virus de la Hepatitis B, cultivo de células de mamíferos, anticuerpos monoclonales, medio libre de proteínas

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Introduction

Antibodies are glycoproteins made by B-lymphocytes to react against antigens, these cells are immortalized to produce monoclonal antibodies (mAbs) through the hybridoma technology [1]. Once the given hybridoma is isolated, the mAb is produced by injecting hybridoma cells into the abdominal cavity of histocompatible mice, to generate ascites. Alternatively, mAbs can be obtained by *in vitro* procedures, through cell cultivation in bioreactors operated in batch or fed-batch modes, in continuous mode without cell retention (which could include perfusion), or by the combination of some of these bioreactor operation modes [2].

In vitro production includes cell culture operations, which determine the overall process productivity. Since its discovery, the biopharmaceutical industry has made significant progress in obtaining relative high product titers in fed-batch and continuous (perfusion) operation modes [3]. This included the use of

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smaller bioreactors and minor investments in facilities and equipment. For instance, with contemporary high titers, even blockbuster drugs could be manufactured using bioreactors of up to 2000-L as maximum volume, instead of conventional 15 000-L bioreactors. Furthermore, no additional scale-up steps are required after process validation, therefore, providing the advantage of a simpler operation process with reduced risks due to operation time and scale, and biological processing [4].

Specifically, under the bioreactor continuous operation mode, the constant addition of nutrients and removal of toxic compounds generates cultures reaching high and sustained cell densities for longer periods, while expressing the target mAb consistently [4-6]. Therefore, the target protein can be constantly harvested from cell culture vessels and purified before it can be degraded by the action of cellular proteases, which cleave protein peptide bonds under neutral and slight acid conditions. Unfortunately, the expression of proteases is unavoidable, since they play vital roles in cellular physiological processes [7].

Furthermore, the main mAb capture step is affinity chromatography, using Protein A- or Protein G-based resins [8, 9], which can recognize mAb fragments and use low pH values to elute mAb, thereby activating acid proteases. Hence, these aspects are highly relevant for regulatory approval of proteins intended for pharmaceutical use, which are produced, among others, in bioreactors operated under continuous mode. There are other concerns, including the challenges in operating cell cultures for long periods, the increased contamination probability and potential inconsistency during harvests, the purification of multiple subbatches of a given bulk material, and the production of non-homogenous products (with altered glycosylation pattern). All these could affect the binding and the biological activity of the target mAb molecule.

In this regard, the CB.Hep-1 mAb was generated by hybridoma technology three decades ago, for the large-scale immunoaffinity chromatography purification of the hepatitis B surface antigen (HBsAg) used as active pharmaceutical ingredient of the Cuban hepatitis B vaccine [10-12]. Since CB.Hep-1 mAb does not require any modification of genes encoding the mAb aminoacid sequence to be applied as immunoreagent, all the processes were developed by using the hybridoma cells to produce ascites [13]. Due to the drastic reduction in vaccine price in the last years, ethics and biosafety issues, and the remarkable advances in non-animal use technologies, the CB.Hep-1 mAb production was implemented in other processing platforms. They included *in vitro* production using bioreactors operated under continuous mode, to reduce the capital investment and running costs. For such purposes, protein free medium (PFM) and culture age effects on mAb productivity were analyzed for further scale-up of the production processes, in bioreactors operated under continuous mode.

However, hybridoma technology can be affected under these conditions since cells could show certain degree of instability. Some reports have demonstrated a sporadic loss of chromosomes during long-term cultivation of hybridoma cells, specifically those involved in expression of mAb heavy chain [14, 15]. This phenomenon has been better evidenced in inter-species hybridomas, which gradually lose chromosomes, preferentially, from one of the parent cell lines. Besides, as myeloma cell lines are often tetraploid and B-lymphocytes are diploid cells, cell fusion results in a mixed population of multikaryons, not purely hexaploid. Then, some of them tend to lose chromosomes for gaining stability, in response to cell culture stressing conditions, even for cells of the same species [16]. This provokes that non-producing cells could eliminate producing cells via competition in energy consumption.

Therefore, this work was aimed to demonstrate the stability of CB.Hep-1 hybridoma cells cultivated in the Lambda MINIFOR bioreactor operated under continuous mode and supplemented with PFM, for 100 days instead of the initial period of 15 days [17]. This process is intended to produce CB.Hep-1 mAb without modifications in the purity, specificity, affinity constant, and target antigen immunopurification capacity. Such a prolonged time study was necessary to evidence the cell line stability and process economy, and to define the culture end-point needed to perform specific virus determination tests. Finally, this study also demonstrated the robustness of the Lambda MINIFOR bioreactor technology to perform such prolonged bench-scale studies, decisive for the analysis of large-scale experiments of mammalian cell culture.

Materials and methods

Hybridoma cell generation

The SP2/0-Ag14-derived hybridoma (CB.Hep-1) was obtained using a Balb/c mouse immunized with the HBsAg obtained from a hepatitis B chronically-infected patient. Hybridoma cells were isolated and cultivated in RPMI-1640 (GIBCO-BRL, Gaithersburg, Maryland, USA), supplemented with 8 % fetal bovine serum (GIBCO-BRL, Gaithersburg, Maryland, USA), 2 mM L-glutamine, 1 mM sodium pyruvate, 17 mM sodium bicarbonate and 25 mg/L gentamicin [10]. The cell cultures were maintained at 37 °C under a 5 % CO_2 atmosphere [10].

PFM

The animal component free (PFHM-II) cell culture medium (Life Technologies, Paisley, UK) was used, supplemented with 1 g/L Kolliphor® P-188 (SIGMA life Science, St Louis, USA), 2 mM L-glutamine, 1 mM pyruvate, 2.5 g/L HEPES, and phenol red. The PFM was used during the experiment, with osmolality ranging 285-300 mOs/kg H_2O and pH 7.2.

Hybridoma cell count and viability determination

The hybridoma cell count and viability were measured by the trypan blue exclusion method [18] using the following formula:

$$X_{(v,\,d)} = \frac{N \times D \times 10^4}{Nq}$$

Where:

X_v, X_d: Concentration of living or dead cells, respectively,

N: Cell count,

D: Dilution factor,

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10^4: Factor of the Neubawer chamber,
Nq: Number of quadrants

Characterization of CB.Hep-1 hybridoma cell culture in Spinner flasks

The CB.Hep-1 hybridoma cells were seeded into a 25 cm^2 T-flask at a concentration of 3×10^5 cells/mL. Then, the culture was expanded to a 75 cm^2 T-flask, to produce the cell amount needed to inoculate a 250-mL Spinner-flask. Cell cultures were always kept at 37 °C under 5-7 % CO_2 atmosphere and PFM was replaced every 48 h to reach maximum viable cell density. Next, supernatant samples were filtered through a Sartorius 0.22 µm filter for antibody quantification. The maximum cell exponential growth rate (µ) was calculated using the following formula:

$$\mu\ (h) = \frac{Ln\ X_2 - Ln\ X_1}{t_2 - t_1}$$

where: X_1 and X_2 are the concentrations of viable cells at times 1 and 2, respectively, and EGR, considering all Xv and two timepoints of the exponential growth phase.

The cell population doubling time (PDT) was estimated as:

$$PDT\ (h) = \frac{Ln\ 2}{\mu}$$

The CB.Hep-1 mAb specific production rate (SPR) per day was estimated, by calculating the ratio of antibody concentration and cell concentration as follows:

$$SPR\ (pg\ IgG/cell \cdot h) = \frac{(C_2 - C_1) \times (LnX_2 - LnX_1)}{(t_2 - t_1) \times (X_2 - X_1)}$$

where:

C_1 and C_2: mAb concentrations expressed as pg/mL at t_1 and t_2, respectively,

X_1 and X_2: the number of living cells/mL at t_1 and t_2, respectively,

t_1 and t_2: timepoints (h) of samplings 1 and 2, respectively.

Culture of CB.Hep-1 hybridoma cells in Spinner flasks for bioreactor inoculum preparation

CB.Hep-1 hybridoma cells were seeded into a 25 cm^2 T-flask at a concentration of 3×10^5 cells/mL. Then the culture was expanded using PFM in a 75 cm^2 T-flask to produce the number of cells needed to inoculate a 1-L Spinner flask. The cell cultures were always kept at 37 °C under a 5-7 % CO_2 atmosphere, and the PFM was replaced every 48 h until reaching the number of cells required to inoculate the Lambda MINIFOR bioreactor, which were harvested in the advanced exponential phase.

CB.Hep-1 hybridoma cell culture in the Lambda MINIFOR bioreactor

A Lambda MINIFOR bioreactor (Lambda Laboratory Instruments, Zürich, Switzerland) with a 6-L vessel was inoculated with the inoculum of CB.Hep-1 hybridoma cells prepared in the 1-L Spinner-flask, at 0.3 $\times 10^6$ cells/mL using PFM as described above. Cells were harvested from the Spinner-flask at the middle of the logarithmic phase with 90 % viability. Bioreactor operating parameters were settled as follows: 2 L, initial volume; 6 L maximum volume, pH 6.8-7.2, 36-37 °C, 4 Hz agitation, 0.01-0.02 L/min aeration, and 5.5-1.1 mg/L dissolved oxygen (DO_2). The fed-batch operation mode was employed in the first five days of culture (1-5 L), and continuous bioreactor volume renovation started on day 6, at 0.144 vvd (720 mL/day), to guarantee a constant culture EGR. Samples of bioreactor supernatant were taken every 24 h of culture to monitor the viable cells concentration (X_v), cell viability and mAb production. The CO_2 supply was not required. The SPR, defined as the concentration of IgG, was calculated following the formula:

$$SPR = q\ IgG \int Xv dt$$

ELISA for CB.Hep-1 mAb quantification

MaxiSorp microtiter plates (Nunc, Roskilde, Denmark) were coated with 10 µg/mL of HBsAg in 100 mM Na_2CO_3/$NaHCO_3$, pH 9.6. After incubation, plates were washed with 150 mM phosphate buffered solution (PBS), pH 7.6/0.05 % Tween-20. All samples were diluted in 150 mM PBS, pH 7.6/0.2 % bovine serum albumin/0.005 % Tween-20, and incubated again for 20 min at 50 °C. Subsequently, wells were washed five times with the same washing solution and incubated with 100 µL/well of a goat anti-mouse IgG horseradish peroxidase conjugate for 20 min at 50 °C [11].

CB.Hep-1 mAb purification

Bioreactor supernatants were harvested, centrifuged at 4800 × *g* at 4 °C for 20 min, and filtered through a 0.22 µm membrane. Filtered supernatants were directly applied onto a 117-mL Prosep va Ultra affinity chromatography column (Millipore, Bangalore, India), using 150 mM PBS, pH 8.0, as adsorption buffer and 100 mM citric acid, pH 3.0, as elution buffer. The whole chromatographic step was done at a linear flow rate of 100 cm/h. Extensive washings (10 column volumes) were done with 150 mM PBS, pH 8.0, before antibody elution, to remove contaminants derived from the secreted material or broken cells. Subsequently, the eluted CB.Hep-1 mAb was incubated in 100 mM citric acid, pH 3.0, at 4 °C, to inactivate viruses if present. Then, the sample buffer was changed to 20 mM Tris/150 mM NaCl, pH 7.6, by a size-exclusion chromatography using Sephadex G-25 coarse in an XK-16 column (GE Healthcare Life Sciences). The column was operated at 130 cm/h, and the collected fractions were filtered through a 0.22-µm membrane under sterile conditions. The purified CB.Hep-1 mAb was store at 4 °C until the coupling to Zetarose CL4B (Emp-BIOTECH, Berlin, Germany).

Zetarose CL4B activation by BrCN procedure

Zetarose CL4B (Emp-BIOTECH, Berlin, Germany) was activated with BrCN (neoFroxx, Darmstadt, Germany) according to the modified procedure reported by Axen, Porath and Ernback [19]. The original protocol was modified, with no drying of the matrix with acetone, and no suction in any activation step to drain the matrix. First, several washings with water were applied to remove matrix preservation solution (ethanol 20 %). Next, BrCN was dissolved in nitrile acetate, at a ratio of 1 g of BrCN per mL of nitrile acetate, in a glass flask. The solution was poured in a 5-L reaction bowl, which contained the Zetarose CL4B suspension. The pH of the reaction was controlled in the range 10.5-11.0 using 4 M NaOH, at 18-21 °C and completed in

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just 4 min. Subsequently, continuous washings were performed, with 4 mL of water, 2 mL of 100 mM acetic acid, and 5 mL of water, per mL of Zetarose CL-4B each. The reaction was agitated using an anchor impellent at 400 rpm. A 231 g/2.2 L BrCN-Zetarose CL4B ratio was used to obtain 40-60 µmoL of cyanate esters groups/mL of matrix.

CB.Hep-1 mAb immobilization procedure

The CB.Hep-1 mAb immobilization was developed according to the procedure described by Valdés *et al*., [11]. The coupling efficiency (ξ) was determined by an indirect method, according to the formula:

$$\xi\,(\%) = \frac{\varphi}{\alpha} \times 100$$

where:

φ: the coupled protein amount measured as the difference between initial amount of ligand, and

α: the amount detected in the filtered and washing fractions after the coupling reaction. The excess of reactive groups was blocked adding 100 mM glycine, pH 8.0. Immunosorbents were washed five times, alternately with 100 mM sodium acetate/500 mM NaCl, pH 4.0, and 100 mM $NaHCO_3$/500 mM NaCl, pH 8.3. Finally, immunosorbents were stored in 150 mM PBS, pH 7.2, at 4 °C.

Source of purified HBsAg

HBsAg particles were produced by fermentation of *Pichia pastoris* yeast cells. Cells were harvested by centrifugation from a 3000-L bioreactor and disrupted on a Dyno®Mill KDL disintegrator (Willy A. Bachofen AG, Muttenz, Switzerland). The homogenate was subjected to acid precipitation and centrifugation at 10 000 × *g* for 30 min. Next, the supernatant was placed into contact with the Celite® Hyflo Supercell (Merck KGaA, Darmstadt, Germany) under continuous stirring. The centrifuged matrix was washed with 2 M potassium thiocyanate solution, and the HBsAg particles were eluted with 20 mM Tris-HCI/3 mM EDTA/100 g/L sucrose, pH 8.2. Then, HBsAg particles were purified by established purification steps including Zetarose CL4B-based immunoaffinity chromatography, ion-exchange chromatography and size-exclusion chromatography [20, 21].

Immunoaffinity chromatography

Immunosorbents containing 3.7 mg of the CB.Hep-1 mAb ligand per milliliter of matrix were used. The applied material was a semi-purified material, diluted in a non-purified material obtained from a fermentation process done with no genetically modified *P. pastoris* yeast. A 155 µg HBsAg/mg mAb antigen-coupled antibody ratio applied to the column was used. Next, columns were washed with ten column volumes of 20 mM Tris/3 mM EDTA/0.5 M NaCl, pH 7.4, and the antigen elution was carried out using 20 mM Tris/3 mM EDTA/0.5 M NaCl/3 M KSCN, pH 7.4. The immunosorbents were regenerated after each purification cycle by applying 60 mL of 0.1 mol/L Tris/0.5 mol/L NaCl solution, pH 8.5, 100 mL of purified water, 60 mL of a 0.1 mol/L sodium acetate/0.5 mol/L NaCl solution, pH 4.5, and 100 mL purified water. All chromatography steps were done at a 1 mL/min flow rate. Sample absorbance was measured at 280 nm and data were acquired and processed with the aid of the BIOCHROM software, developed by the Center for Genetic Engineering of Havana, Cuba.

Protein quantification

Protein was quantified following procedure described by Lowry *et al*. [22]. The calibration curve ranged 100-500 µg/mL, and absorbance was measured at 730 nm in an Ultrospec UV/Visible spectrophotometer (Pharmacia Biotech Ultrospec 2000, Cambridge, England). Purified samples were also quantified by measuring absorbance at 280 nm and using molar extinction coefficients 1.37 for CB.Hep-1 mAb and 5.00 for the HBsAg particle.

Determination of CB.Hep-1 mAb and HBsAg purity and molecular homogeneity by SDS-PAGE and SEC-HPLC

The identity pattern and purity of the CB.Hep-1 mAb and HBsAg particles were analyzed by electrophoresis on a 12.5 % (w/v) SDS-PAGE gel, followed by staining with Coomassie® brilliant blue R-250 (Bio-Rad Laboratories, California, USA), as described by Laemmli [23]. The molecular homogeneity of CB.Hep-1 mAb was estimated by SEC-HPLC using a TSKgel G3000PW column (3000 mm/7.5 mm ID, Tosoh Bioscience, Japan). The chromatographic mobile phase was 150 mM PBS, pH 7.0, and 100 µg of the sample were dissolved in 150 mM PBS, pH 7.0, and applied directly into the system. A 0.2 mL/min volumetric flow rate was employed and absorbance was measured at 226 nm.

The molecular homogeneity of the HBsAg particles was estimated using a SEC-HPLC TSK gel G6000PW (6000 mm/7.5 mm ID, Tosoh Bioscience, Japan). The chromatographic mobile phase was 150 mM PBS, pH 7.0, and 100 µg of sample were dissolved in 150 mM PBS, pH 7.0 and applied into the system. A 0.6 mL/min volumetric flow rate was used and absorbance was measured at 226 nm.

Determination of the CB.Hep-1 mAb released from immunosorbents by ELISA

Costar® high-binding plates (Costar, Cambridge, Mass., USA) were coated with a sheep anti-mouse polyclonal IgG in 100 mM Na_2CO_3/$NaHCO_3$, pH 9.6, at 4 °C, overnight. Plates were blocked for 30 min at 37 °C. Next, wells were washed and samples from immunosorbents were added and incubated at 37 °C for 3 h, with 1 % non-fat milk/150 mM PBS, pH 8.0. After washing, plates were incubated with 100 µL/well of a goat anti-mouse polyclonal IgG-horseradish peroxidase conjugate (Merck KGaA, Darmstadt, Germany, USA). The reaction was then revealed using 100 µL/well of 0.05 % O-phenylenediamine and 0.015 % H_2O_2 in citrate buffer, pH 5.0, and stopped with 50 µL/well of 1.25 M H_2SO_4. The absorbance was measured in a Multiskan ELISA reader (Labsystems, Helsinki, Finland) using a 492 nm filter [11].

Statistical analysis

Experimental results of five harvested supernatant pools were statistically compared by a one-way ANOVA test, with a confidence level (α) of 0.05. The test was applied using the software Statgraphics®Plus

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v. 5.0 (Statpoint Technologies, Virginia, USA), and the remaining mathematical analyses were done using Excel 2003 software (Microsoft, Washington, USA).

Results and discussion

In a continuous culture, part of the cell population is continuously removed together with the spent medium. At the same time, cell growth is controlled by adding fresh medium, expressed as dilution rate. When cell growth rate equals the dilution rate, the steady state of equilibrium is reached, in which cell concentration, nutrient availability, and product and waste formation are theoretically constant into the bioreactor. Hence, the continuous culture mode is aimed to prolong cell cultivation time, eliminate multiple inoculum preparations and inoculation moments, reduce bioreactor size and increase productivity, as improved operational parameters in comparison with batch and fed-batch operation modes [4, 24].

Meanwhile, the *in vitro* cell culture of mammalian cells replaced *in vivo* methods for mAb production, under conditions enabling a relative high level of mAb expression. The initial simple batch cultures subsequently derived into culture systems with controlled feeding (fed-batch), successfully increasing the maximum cell concentration, culture longevity, and with relative higher mAb titers. This made the fed-batch the first-choice bioreactor operation mode for biopharmaceutical production of natural and recombinant mAbs [5]. Nevertheless, continuous manufacturing is also recognized as one of the best methods to increase bioprocess productivity and to reduce weight of capital investment in large scale manufacturing processes.

Regarding host cells for mAb production, mouse hybridomas are the main host cell lines used to produce commercial mAb in bioreactors. This happens because some mAbs do not need modification as to improve their biochemical properties, even when mouse hybridomas are difficult to engineer for protein expression at high levels and they usually grow to only moderate densities in bioreactors.

In bench-scale bioreactors used for mAb production, systems supporting low cell densities (1-2 × 10^6 cells/mL) and antibody concentrations (approximately, 100 µg/mL) can be implemented [25, 26]. Therefore, these systems cannot be used to produce significant mAb amounts, but they can be used for a deep characterization of the cell culture and mAb molecule, and to design the scaled-up processes. In this sense, one of the most attractive examples of bench-scale bioreactors is the Lambda MINIFOR bioreactor (Lambda Instruments), designed for measuring and control important parameters of biological cultures (Figure 1) [27]. Within the attractive modifications of this bioreactor are the versatility of vessel volume, the vertical fish-tail agitation system and the infrared radiator, with a gilded parabolic reflector used to warm the culture medium without overheating the system.

The initial culture characterization done in Spinner-flasks showed a typical hybridoma growth curve, that corroborated the results reported by Valdés *et al*. in 2012 [17], when culturing the CB.Hep-1 hybridoma in Spinner-flasks using PFM (PFHM-II). The static hybridoma batch culture showed a lag-phase of about 18 h, whereas the culture highest cell density was reached at 90 h. The cell density increased from 4.0 × 10^5 cells/mL at 18 h to a maximum of 2.1 × 10^6 cells/mL at 90 h. In this sense, Merten *et al*. [28, 29] described three main SPR patterns to classify hybridoma cell lines. Cells showing the highest immunoglobulin-production during lag-phase and the onset of the log-phase can be classified as either group I and II. Hybridoma cells belonging to group II may manifest another high SPR at the onset of the cell culture growth steady and death phases. Group III hybridomas comprises cells with a quite constant production of mAb during the growth phase; but it is completely stopped after the beginning of stationary phase. Considering this, the maximum SPR measured for the CB.Hep-1 hybridoma in the defined culture conditions was detected in the stationary phase, indicating the system performs as group II and supporting the bioreactor operation under continuous mode. This operation mode has demonstrated to be quite efficient, despite concerns on the cell line stability and the properties of the produced mAb molecules. Taking this into account, the continuous culture of the CB.Hep-1 hybridoma was studied during 100 days in the Lambda MINIFOR bioreactor, to characterize the cell line stability, the properties of the mAb molecule and to scale up the production based on real data.

Figure 1. Illustration of Lambda-MINIFOR fish-tail bioreactor (Lambda Laboratory Instruments, Zürich, Switzerland)

The first five days of culture consisted on the batch and fed-batch operation modes of the bioreactor. This provided a 2.1 × 10^6 cells/mL maximum cell concentration and a high cell viability (96.9 %) on day six, ready to start the continuous operation mode. The parameters of the continuous cell culture stage are shown in table 1. As shown, viable cells concentration ranged 1.0 ± 0.1 × 10^6-1.3 ± 0.1 × 10^6 cells/mL (1.1 ± 0.2 × 10^6 cells/mL average) and cell viability ranged 76.3 ± 6.2-87.5 ± 4.4 % (82.5 ± 6.5 % average). Normally, the cell culture densities obtained in a bioreactor operated under continuous mode are up to 80 % of that reached in fed-batch mode, but here 54 % was obtained (Figure 2). This can be explained by the low medium volume renovation per day (up to 19 %). But this was advantageous, since the highest expression of CB.Hep-1 mAb is detected when almost the entire cell population is not dividing. An alternative explanation may be related with the PFM composition, since this formulation was not developed specifically for this hybridoma cell line. Both aspects have a re-

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Table 1. Results of production and characterization of the CB.Hep-1 mAb obtained using a Lambda-MINIFOR bioreactor for hybridoma cells culture for 100 days

Harvest pools (days)	Parameters											
	Cell concentration (× 10^6/mL)	Cell viability (%)	mAb concentration (mg/L)	Cell specific production rate (µg/10^6 cells · day)	mAb production (mg/ harvest · day)	mAb production (g/harvest)	mAb recovery (%) IAC and incubation at pH 3.0	mAb recovery (%) size exclusion-HPLC	mAb purity (%) SDS-PAGE	mAb purity (%) size exclusion-HPLC	mAb aggregate level (%)	mAb affinity constant (× 10^{10} M^{-1})
6-33	1.16 ± 0.4	87.5 ± 4.4	147.7 ± 21.6	34.1 ± 9.1	138.8 ± 48.0	3.7 ± 0.4	75.5	96.5	99.0	99.5	3.7 ± 0.4	1.1
34-50	1.30 ± 0.1	82.7 ± 5.3	129.7 ± 1.3	24.3 ± 3.3	125.4 ± 1.2	2.0 ± 1.2	80.1	98.5	100	99.8	2.0 ± 1.2	1.8
51-75	1.11 ± 0.1	79.6 ± 4.5	136.6 ± 13.5	22.5 ± 5.2	118.6 ± 12.3	3.1 ± 0.1	90.0	95.0	98.5	99.7	3.1 ± 0.1	0.1
76-86	1.02 ± 0.1	76.3 ± 6.2	151.5 ± 13.0	26.8 ± 3.2	173.3 ± 28.4	1.6 ± 0.2	89.5	97.2	99.2	99.9	1.6 ± 0.2	0.4
87-100	1.13 ± 0.1	76.8 ± 3.6	157.8 ± 22.7	25.6 ± 3.9	162.0 ± 20.5	2.1 ± 0.2	90.0	95.2	98.9	100	2.1 ± 0.2	1.0
Total (average, 100 d)	1.13 ± 0.2	82.5 ± 6.7	122.7 ± 43.6	25.7 ± 9.8	125.7 ± 43.6	3.1 ± 0.5	85.0 ± 6.7	96.4 ± 1.4	99.1 ± 0.5	99.7 ± 0.1	3.1 ± 0.5	0.9 ± 0.6
CV (%)	25.6	8.1	35.5	38.1	35.7	26.4	7.9	1.5	0.5	0.1	26.4	–

The mAb recovery in the centrifugation and filtration supernatants were not accurately determined, but they were estimated as 95 %, according to the volume lost. In all the harvests, the mAb successfully recognized the HBsAg aminoacid peptide (CKTCTT). mAb aggregation was not detected for all the harvest.

markable influence on the cell density and viability of the cell culture (1.13 ± 0.2 cells/mL and 82.5 ± 6.7 % average). Noteworthy, both parameters showed a relatively small coefficient of variation (25.6 and 8.1, respectively), demonstrating the cell culture consistency for 100 days.

The relation between cell density and SPR of CB.Hep-1 hybridoma was also analyzed (Figure 2). SPR values ranged 22.5 ± 5.2-34.1 ± 9.1 µg mAb/10^6 cells · day (25.7 ± 9.8 average, 38.1 CV; Table 1). These SPR values higher than 10 µg mAb/10^6 cells · day were coincident with other reports [29], and supported the further scale up of CB.Hep-1 mAb production in a bioreactor. Regarding mAb concentration (Figure 3), it ranged from 129.7 ± 1.3-157.8 ± 22.7 µg/mL (122.7 ± 43.6 µg/mL average), the mAb production harvested per day 118.6 ± 12.3-173.3 ± 28.4 µg/mL (125.7 ± 43.6 µg/mL average). The amount of mAb produced per harvest was 1.6 ± 0.2-3.7 ± 0.4 g (3.1 ± 0.5 g), accounting for 12.5 g of mAb harvested in 100 days in PFM under the operational conditions described.

Traditionally, affinity chromatography has been used as the initial step for mAb purification using conventional packed bed adsorbents based on the streptococcal Protein G [8, 9, 30]. This technology requires the clarification of the crude feed before loading, to avoid interactions of cells with the resins. In our procedure, cells were eliminated and the supernatant clarified by combining steps of centrifugation and filtration through 0.22-µm membranes, for a 95 % volume recovery. It was followed by a step of affinity chromatography using Protein A-Prosep va Ultra [31] and incubation of the eluted CB.Hep-1 mAb at low pH, as to inactivate the potential viral contaminants. This purification stage provided a very high mAb recovery, 85.02 ± 6.79 % average, CV as low as 7.9 %, derived from the use of PFM for cell culture. Usually, the use of PFM renders mAb in bioreactor supernatants with purity above 80 % [32, 33]. This is remarkably higher than that obtained when producing mAbs by the ascites *in vivo* procedure (< 30 %, recovery around 60 %) or by cell culture in fetal bovine serum-supplemented media. Next, the buffer of affinity chromatography elution fractions was exchanged to 20 mM Tris/150 mM NaCl, pH 7.6, by size- exclusion chromatography. The recovery of this step was 96.4 ± 1.4 %, as recommended by the manufacturer (> 95 %), with a very low CV among harvest groups (1.5 %; Table 1), and 10.1 g of mAb were obtained.

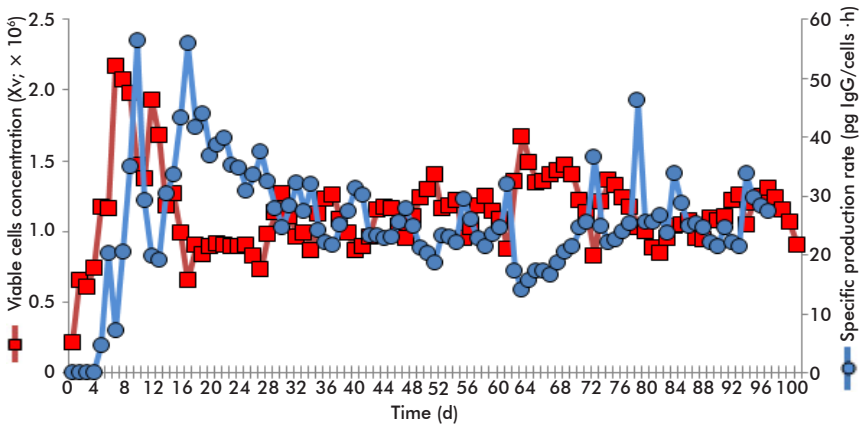

Figure 2. CB.Hep-1 hybridoma concentration and specific production rate for 100 days of culture in a Lambda-MINIFOR fish-tail bioreactor.

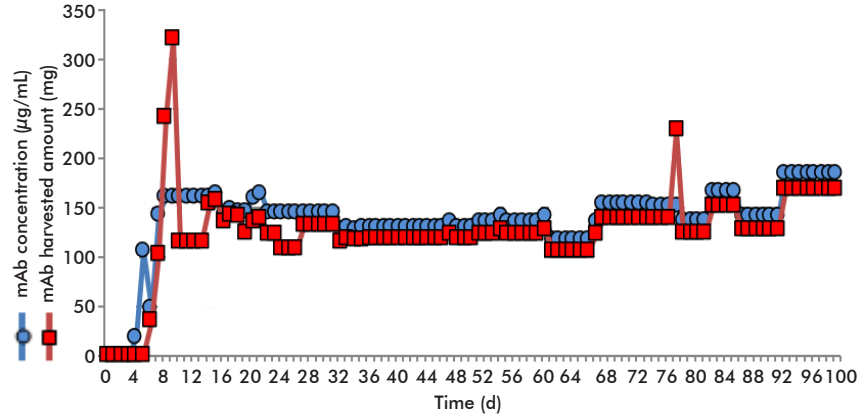

Figure 3. CB.Hep-1 mAb concentration and harvested amount in the supernatant of a CB.Hep-1 hybridoma cell culture in a Lambda-MINIFOR fish-tail bioreactor for 100 days.

Subsequently, the mAb molecule purified from the different harvests of bioreactor supernatants was highly pure (SDS-PAGE, 99.1 ± 0.5 % (0.5 CV); SEC-HPLC, 99.7 ± 0.1 % (0.1 CV), with no mAb aggregation detected in SEC-HPLC samples, an 0.1-1.8 × 10^{10} M^{-1} affinity constant, and the mAb specific for

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the HBsAg epitope CKTCTT (Table 1). All these data were coincident with those previously reported [11].

Then, the HBsAg was immunopurified with the CB.Hep-1 mAb immunosorbents. The previously purified HBsAg particles were added to a non-purified material. This last was obtained from a fermentation process done with no genetically modified *P. pastoris* yeast. Eight parameters were evaluated to assess immunosorbent efficiency: antigen adsorption capacity, antigen adsorption efficiency, antigen elution capacity, antigen elution efficiency; antigen SDS-PAGE purity, SEC-HPLC purity, released-coupled mAb ratio, and mAb leakage (Table 2). Results demonstrated an average HBsAg adsorption capacity and efficiency equal to 81.4 ± 21.8 µg HBsAg/mg mAb and 52.6 ± 14.1 %, respectively. Both parameters were statistically similar over eight purification cycles. Results of adsorption capacity are coincident with those previously reported [34], but not for the antigen adsorption efficiency [34]. It could be elucubrated that ligand density has to be carefully controlled, due to its influence on immunosorbent adsorption and the elution efficiency, for a better adsorption efficiency. The HBsAg elution capacity ranged 64.0 ± 3.5-87.7 ± 4.8 µg HBsAg/mg mAb, with a 60.0 ± 20.8 µg HBsAg/mg mAb average, coincident with our prevoius reports [34]. A 83.2 ± 11.6 % elution efficiency was achieved, its high value corroborating the hypothesis that the real ligand density was lower than expected. Higher ligand densities did not have any statistical influence on antigen binding efficiency, but decrease elution efficiency values.

Meanwhile, SDS-PAGE of HBsAg eluted from the CB.Hep-1 immunosorbents assessed in this study was done under moderate reducing conditions (Table 2), its purity ranging 87.66 ± 1.15-89.60 ± 1.00 %, above the 80 % threshold of acceptance for this chromatographic step [19, 34].

Regarding the antigen aggregation, it was analyzed by SEC-HPLC (Figure 4), providing the chromatographic profiles of HBsAg purified with immunosorbents using CB.Hep-1 mAb as ligand. It was characterized in different pooled harvests of bioreactor supernatants in separate day ranges: 6-33, 34-50, 51-75, 76-86, 87-100 days, respectively. As shown, all profiles were similar, showing only one peak with 13 min of retention time (> 98 % purity), coincident with previous reports and the approved profile for the HBsAg used as active pharmaceutical ingredient of a hepatitis B vaccine for human use [11, 19].

Another essential aspect to strictly monitor in all mAb immobilization and target antigen elution procedures is the leakage of coupled mAb, which can disadvantageously contaminate the target antigen preparation. Hence, the HBsAg purification process is not an exception, in order to avoid contamination of the hepatitis B vaccine with significant amount of mouse immunoglobulins. In this regard, the approved values for this contaminant are < 1 % for released-coupled mAb ratio and 3 ng mAb/µg HBsAg for mAb leakage [11, 35]. These results demonstrated that the process complied with the thresholds established for both parameters in eight purification cycles, 0.03 ± 0.01 % and 2.1 ± 2.3 ng IgG/µg HBsAg, respectively, corroborating the very low level of co-eluted ligand. A relative high value of mAb leakage was only detected in the first purification cycle, which can be explained by the lower adsorption capacity of antigen and not by a high level of mAb leakage by itself. This indicated that the production of CB.Hep-1 mAb in a bioreactor operated under continuous mode does not

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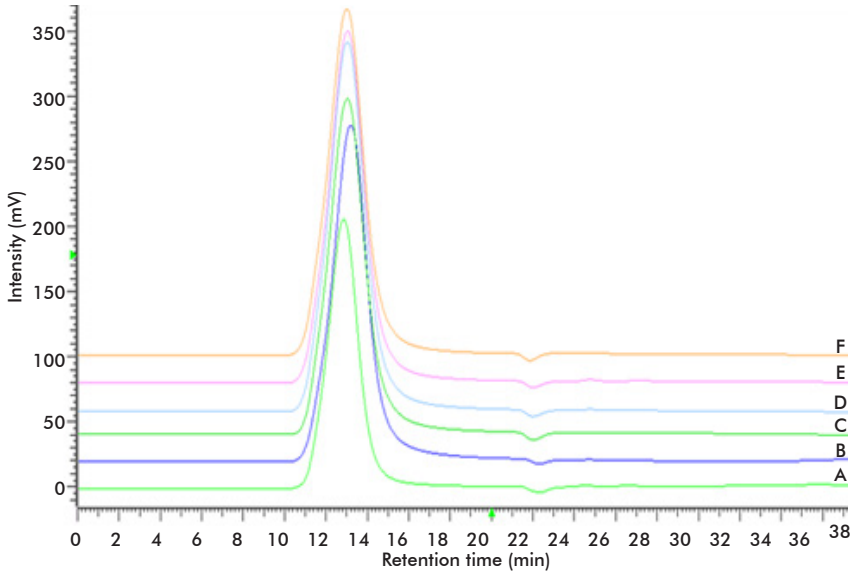

Figure 4. HPLC-SEC purity profiles of HBsAg purified with CB.Hep-1 mAb obtained from the CB.Hep-1 hybridoma cell culture in the Lambda-MINIFOR fish-tail bioreactor for 100 days. Eight profiles are shown, corresponding to five harvest pools and the average: A) 6-33 days. B) 34-50 days. C) 51-75 days. D) 76-86 days. E) 87-100 days. F) 100 days.

Table 2. HBsAg immunopurification parameters using immunosorbents with the CB.Hep-1 mAb obtained using a Lambda-MINIFOR bioreactor for hybridoma cells culture for 100 days, with data of three independent experiments and eight purification cycles

Purification cycles	Adsorption		Elution		SDS-PAGE purity (%)	Released-coupled mAb ratio (%)	mAb leakage (ng mAb/µg HBsAg)
	Capacity (µg HBsAg/ mg mAb)	Efficiency (%)	Capacity (µg HBsAg/ mg mAb)	Efficiency (%)			
1	76.2 ± 6.8	49.7 ± 4.2	64.0 ± 3.5	84.0 ± 3.0	88.3 ± 0.5	0.03 ± 0	4.8 ± 0.4
2	107.4 ± 7.0	69.2 ± 4.5	87.7 ± 4.8	81.6 ± 1.0	–	–	–
3	99.0 ± 17.8	64.1 ± 11.5	86.7 ± 17.7	87.3 ± 3.6	–	–	–
4	83.8 ± 29.7	54.2 ± 19.2	80.6 ± 23.5	98.1 ± 8.3	–	–	–
5	62.8 ± 22.7	39.9 ± 14.4	40.8 ± 14.8	65.0 ± 2.4	89.6 ± 1.0	0.009 ± 0	1.4 ± 0.1
6	82.7 ± 20.7	53.6 ± 13.4	59.6 ± 23.4	70.0 ± 12.5	–	–	–
7	70.3 ± 19.4	45.3 ± 12.5	65.9 ± 18.8	93.5 ± 1.1	–	–	–
8	69.3 ± 19.9	44.6 ± 12.8	59.1 ± 16.0	85.7 ± 2.0	87.6 ± 1.1	0.001 ± 0	0.1 ± 0
Average ± SD	81.4 ± 21.8	52.6 ± 14.1	60.0 ± 20.8	83.2 ± 11.6	88.5 ± 1.0	0.03 ± 0.01	2.1 ± 2.3

affect the efficiency of the HBsAg immunopurification by CB.Hep-1 immunosorbents.

Finally, data of production and characterization of CB.Hep-1 mAb produced in the Lambda MINIFOR bioreactor, operated by continuous mode in 100 days, were used to make a theoretical scale up of CB.Hep-1 mAb production (Table 3). The analysis allowed to estimate that a 600-L bioreactor with a 500-L working volume, operated under continuous mode without cell retention, can be used to produce up to 2025 g of CB.Hep-1 using 27 immunosorbents 16 L-CB.Hep-1. Alternatively, operating a 50-L bioreactor with the same working volume under the same operational mode, but with cell retention, can generate 1378 g using 20 immunosorbents of the same type. Ultimately, this saves thousands of mice per year, avoid biological risks associated to potential contamination with murine and bovine viruses, and a significantly reduces the costs associated to the production of CB.Hep-1 mAb.

Table 3. Scale up modeling data of CB.Hep-1 mAb production in bioreactors operated in continuous mode with and without cell retention

Parameters	Bioreactors			
	Lambda MINIFOR	Stirred bioreactor	Stirred bioreactor	
Volume (L)	6	600	50	
Operation mode	Continuous without cell retention	Continuous without cell retention	Continuous with cell retention	
Working volume (L)	5	500	40	
Culture medium (PFM)	PFHM-II	PFHM-II	PFHM-II	
Total operation time (days)	100	300	300	
Number of fermentation batches	1	3	3	
Harvest number	76	234	246	
Medium volume replacement per day (vvd)	0.20	0.20	1	2
Total medium consumption (L)	80	22 000	11 000	22 000
Harvested supernatant (L/day)	1	86	40	80
Average cell viability (%)	82.5 ± 6.7	80-85	80-85	75-80
Average cell density (× 10^6 cells/mL)	1.1 ± 0.2	1.1-1.3	10	25-30
Average mAb concentration (mg/L)	122.7 ± 43.6	≥ 122.7	150	100
Harvested mAb (mg/harvest/day)	125.7	~9000	6000	8000
IgG purity in supernatant (%)	> 70	> 70	> 70	> 70
Total harvested mAb in supernatant (g)	12.5	~2500	1476	1968
Medium consumption by mAb produced (L/g)	7.7	8-9	7.45	16.2
Cell culture system required for inoculum preparation	1L-Spinner flask (1)	1-L Spinner flask (6) 50-L Wave bioreactor (1)	1-L Spinner flask (4)	1-L Spinner flask (4)
Perfusion system (alternating flow filtration system)	No	No	Yes	Yes
Purification process recovery (%)	81.9	70	70	70
Total purified mAb (g)	10.1	2025	1033	1378
Immunosorbent process recovery (%)	89.7	89.7	89.7	89.7
Total mAb for immobilization (g)	8.9	1802	927	1236
Immunosorbent mAb density (g/L)	3.8	3.8	3.8	3.8
Total immunosorbent volume (L)	2.3	450	244	325
Number of 16-L immunosorbents	–	27	15	20
Animals required for ascites production	380	> 18 000	> 110 000	> 147 600

Conclusions

The CB.Hep-1 hybridoma is able to stably produce the CB.Hep-1 mAb without modifications on its biochemical properties and its target antigen immunopurification capacity during one-hundred days. The Lambda MINIFOR bioreactor demonstrated to be a robust culture system as to proof the long-term cultivation and characterization of mammalian cells at lab-scale. Based on the Lambda MINIFOR bioreactor results, it was estimated that the CB.Hep-1 mouse hybridoma culture could be lineally scale-up to a 600-L or a 50-L bioreactors, both operated under continuous mode, but without or with cell retention, respectively to remarkably scale up the yearly production of purified mAb using 16-L CB.Hep-1 immunosorbents, with reduced operational costs.

Conflicts of interest statement

The authors declare that there are no conflicts of interest.

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