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Advances in Large-Scale In Vitro Culture of Hybridoma Cells: Postprint

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Abstract

Monoclonal antibodies have demonstrated tremendous application value in the fields of biology and medical research, serving as novel reagents in immunoassays and targeted agents for biotherapy. As medical diagnostic reagents, monoclonal antibodies can fully leverage their advantages of excellent specificity, high sensitivity, greater convenience for quality control, and facilitation of standardization and normalization. Traditional methods utilize mouse ascites for monoclonal antibody preparation; however, the technology for large-scale in vitro culture of hybridoma cells to produce monoclonal antibodies has also been continuously developing over recent decades. Monoclonal antibodies produced through in vitro culture of hybridoma cells have been applied in numerous areas, and particularly the demand for monoclonal antibodies in disease diagnosis and treatment has further promoted the development of hybridoma cell in vitro culture production technologies. Due to the semi-adherent nature of hybridoma cells, large-scale in vitro culture of hybridoma cells can be performed using either suspension or adherent culture methods. This review focuses on the preparation of monoclonal antibodies through in vitro culture of hybridoma cells for application in in vitro diagnostic reagents, primarily encompassing hollow fiber cell culture and bioreactor cell culture methods, as well as advances in the optimization of various culture methods.

Full Text

Advancements in Large-Scale In Vitro Culture of Hybridoma Cells

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Abstract

Monoclonal antibodies have demonstrated tremendous application value in biological and medical research, serving as novel reagents in immunoassays and guided weapons for biological therapy. As medical diagnostic reagents, monoclonal antibodies offer distinct advantages including excellent specificity, high sensitivity, and greater convenience for quality control, facilitating standardization and normalization. Traditional methods for monoclonal antibody preparation relied on mouse ascites production. However, over recent decades, technologies for large-scale in vitro culture of hybridoma cells have continuously evolved. Monoclonal antibodies produced through in vitro hybridoma cell culture have been applied in numerous fields, particularly in disease diagnosis and treatment, which has further accelerated the development of hybridoma cell culture production techniques. Due to the semi-adherent nature of hybridoma cells, large-scale in vitro culture can be performed using either suspension or adherent culture methods. This review focuses on in vitro culture techniques for hybridoma cells used in in vitro diagnostic reagents, primarily covering hollow fiber cell culture and bioreactor cell culture methods, along with advancements in the optimization of different culture approaches.

Keywords: Hybridoma; Monoclonal antibody; Bioreactor; Hollow fiber

Since British scientists Milstein and Kohler invented monoclonal antibodies (McAb) in 1975, ushering in a new era transitioning from polyclonal to monoclonal antibodies, monoclonal antibodies have demonstrated incomparable superiority over polyclonal antibodies. Their advantages include high specificity, high titer, high purity, uniform physicochemical properties, strong reproducibility, low cost, and the ability for mass production. The hybridoma technology for monoclonal antibody production represents the most important technological revolution in contemporary immunology, and its advent has greatly advanced the development of medicine and biology.

Monoclonal antibodies have extremely broad applications, with hybridoma cell-produced monoclonal antibodies being utilized in many areas, particularly in in vitro diagnostics and disease treatment [1]. Therapeutic antibody drugs for human use primarily employ genetically engineered recombinant antibodies expressed by CHO, SP20, NS0 and other cells, while monoclonal antibodies produced through hybridoma cell culture are currently mainly applied in diagnostic reagents and veterinary therapeutics.

Currently, most monoclonal antibodies for in vitro diagnostics are prepared using the in vivo method, where hybridoma cells are inoculated into the mouse peritoneal cavity. The hybridoma cells grow in the peritoneal cavity and produce ascites, yielding large quantities of monoclonal antibodies at high concentrations. Mouse ascites production offers high antibody concentration and yield, allows for continuous harvesting, requires no culture media costs, and avoids repeated sterile operations, making it rapid and convenient. However, ascites fluid often contains various mouse 杂 proteins (including IgG) and lipid substances that

frequently require purification before use. There is also a risk of contamination by animal viruses, and significant inter-batch variation due to individual differences among mice. Consequently, large-scale in vitro culture of hybridoma cells under serum-free or low-serum conditions has become a primary research focus [2].

The establishment of monoclonal antibody preparation processes through hybridoma cell in vitro culture typically requires four stages: (1) selection of mouse myeloma cells, cell fusion, and monoclonal screening; (2) selection of in vitro culture methods for mouse hybridoma cells; (3) process optimization for hybridoma cell in vitro culture; and (4) establishment and optimization of purification processes. Two main approaches exist for producing monoclonal antibodies through in vitro culture of mouse hybridoma cells: hollow fiber system culture and bioreactor culture. The advantages of hollow fiber systems include: (1) high cell density (>10^8 cells/mL), (2) efficient nutrient distribution and timely removal of metabolic waste, (3) long maintenance duration (up to several months), particularly suitable for long-term continuous culture, and (4) small space occupation. The disadvantages include: (1) difficulty in scale-up, suitable for products with annual demand below 10g, and (2) relatively expensive disposable consumables—once contamination occurs, the consumables must be discarded, increasing costs. The advantages of bioreactor culture include: (1) easy scale-up (from several liters to hundreds or even thousands of liters), (2) mature and stable processes, and (3) flexibility to select different culture processes such as batch, fed-batch, or perfusion culture according to requirements. The disadvantages include: (1) relatively low antibody concentration in the culture medium, and (2) large downstream purification volumes. This review focuses on monoclonal antibodies for in vitro diagnostics, summarizing the characteristics, selection, process optimization, and current research progress and application results of these two hybridoma cell culture systems.

1. Characteristics and Culture Methods of Hybridoma Cells

Mouse hybridoma cell lines are formed by fusion of mouse myeloma cells with spleen cells from immunized mice. Generally, mouse hybridoma cells are not strictly anchorage-dependent cells (ADC), but rather semi-adherent cells that can be cultured both adherently and in suspension, though the degree of anchorage dependence varies among different cell lines. Hollow fiber systems primarily employ adherent culture, while bioreactor culture can be performed either in suspension or with microcarrier-assisted adherent culture.

The demand for monoclonal antibodies differs between in vitro diagnostics and therapeutic applications. In vitro diagnostic antibodies typically require smaller annual quantities, generally 10-100g/year, though some special-purpose antibodies like blockers may require up to 1-2kg/year. In contrast, biopharmaceutical antibodies generally require 100-1000kg/year [3-4].

Bioreactors are the most commonly used method for monoclonal antibody preparation, though roller bottles and hollow fiber systems are also frequently employed in the diagnostic reagent industry. Culture methods include batch culture, fed-batch culture, and perfusion culture. Perfusion culture is the most effective method for small-scale monoclonal antibody production. Hollow fiber systems operate as perfusion cultures, while bioreactor systems can perform batch, fed-batch, or perfusion culture. Perfusion culture continuously adds fresh medium and removes metabolic waste, enabling rapid achievement of high cell density, which in turn enables high antibody secretion and productivity. Studies have shown that perfusion culture productivity can be more than 10 times higher than batch or fed-batch culture [5].

Raisa V et al. [6] statistically compared the differences between bioreactor and hollow fiber systems for monoclonal antibody preparation, including cost differences (shown in [Figure 1: see original paper] and [Figure 2: see original paper]) and productivity differences (shown in). Costs include direct and indirect costs, with direct costs comprising core raw materials, consumables, miscellaneous items, labor, and QA/QC, while indirect costs include general equipment, maintenance, and depreciation. The primary difference is that equipment depreciation accounts for 39% of costs in bioreactor systems versus 25% in hollow fiber systems, with hollow fiber systems incurring 10% higher consumable costs than bioreactor systems. In terms of productivity, a 10L bioreactor perfusion culture achieves annual production of 425g with antibody concentration of 0.17mg/mL. A single 70mL hollow fiber column produces 10.6g per batch, with 5 batches per year. By simultaneously culturing 8 hollow fiber columns per batch, annual production of 425g can be achieved. The cost per gram of antibody is similar between the two systems, with bioreactor systems being only 7% higher than hollow fiber systems.

2. Hollow Fiber System Culture of Hybridoma Cells

Hollow fiber cell culture technology emerged in the early 1970s. In 1972, Knazek et al. reported their design and fabrication of a small hollow fiber cell culture device along with experimental results, demonstrating that cells could form multi-layered tissue-like structures on hollow fibers [7]. Currently, several U.S. companies are engaged in the research, development, and production of hollow fiber systems, including FiberCell Systems and Cell Culture Company. Hollow fiber systems can provide extremely large surface areas within small volumes. FiberCell Systems offers medium-sized hollow fiber bioreactors (C2011 and C2008) with column volumes of 15mL providing 2200cm^2 surface area, and larger reactors (C2018 and C2003) with 60mL column volumes providing 1.2m^2 surface area. Cell Culture Company can provide reactors with 150mL column volume offering 2.1m^2 surface area.

A key feature of hollow fiber bioreactors is that cell density can exceed 10^8 cells/mL, enabling high antibody concentrations. Another fundamental difference from other cell culture technologies is that hollow fibers form porous per-

meable supports that facilitate cell attachment, most closely resembling in vivo cell growth patterns. Due to bottom-to-top nutrient delivery, cells easily stack upon each other, forming multi-layered cell structures. A third characteristic is the ability to selectively control the passage of substances with different molecular weights through the hollow fiber membrane pore size (or molecular weight cut-off), enabling exchange of nutrients and metabolic waste while retaining and accumulating the target product within the system. The most common application is the production of monoclonal antibodies by hybridoma cell lines, representing the first large-scale application of hollow fiber bioreactors. Functional hollow fiber membranes have pore sizes of 20-30kD, with serum-containing medium circulating inside the fibers to continuously provide nutrients, while the extracapillary space serves as the cell growth compartment. Amino acids, inorganic salts, vitamins, and small molecules from serum in the intracapillary medium can permeate through the hollow fiber membrane for cell utilization, while metabolic waste products such as lactate and glucose from cells in the extracapillary space can be removed through the membrane. By replacing the intracapillary medium daily and harvesting antibody from the extracapillary space, continuous hybridoma culture and long-term antibody production can be achieved [8]. Due to the large surface area and small culture volume, with cell numbers reaching over 10^9, the harvested antibody concentration is relatively high. Hollow fiber culture systems typically have long culture cycles, generally ranging from 1-6 months depending on requirements [9].

Legazpi et al. [10] cultured HB-8852 hybridoma cells using a hollow fiber column with 30kD molecular weight cut-off and 2050cm^2 surface area. Throughout the culture period, glucose concentration gradually decreased from 4.5g/L to 3g/L, and glutamine concentration decreased from 0.4g/L to 0.2g/L. After 16 days of continuous culture, antibody secretion reached 0.27mg/mL, revealing the metabolic kinetics of hybridoma cell culture in hollow fiber systems, though this cell line showed relatively low antibody secretion capability compared to 0.03mg/mL in shake flask culture. R van Erp et al. [11] applied a hollow fiber system to culture mouse hybridoma cells for preparing two hCG monoclonal antibodies. Roller bottle culture was used to prepare cell seeds in DMEM/F12 medium with 10% serum. Log-phase cells were inoculated into the hollow fiber system extracapillary space at a density of 5×10^6 cells/mL. Two weeks post-inoculation, extracapillary fluid was harvested at a frequency of twice per week, enabling continuous culture for 80 days with average cell density reaching 7×10^7 cells/mL and average antibody secretion of 4mg/mL. Jackson et al. [12] evaluated the feasibility of using in vitro hollow fiber systems to replace in vivo ascites methods for monoclonal antibody preparation. Three hybridoma cell lines were cultured using both methods over a 65-day production period. Results showed: for cell line 2B11, in vivo yield was 455mg versus 211mg in vitro; for cell line 3C9, in vivo yield was 446mg versus 565mg in vitro; for cell line RMK, in vivo yield was 997mg versus 1023mg in vitro. Average antibody concentrations were 4.07-8.37mg/mL for in vivo ascites methods and 0.71-11.1mg/mL for in vitro hollow fiber culture, demonstrating that hollow

fiber systems can be used for in vitro preparation of antibodies with annual demand below 5g.

3. Bioreactor Culture of Hybridoma Cells

Hybridoma cell culture systems encompass various types and scales, including roller bottle culture systems, stirred-tank bioreactors, air-lift bioreactors, and single-use bioreactors, each with distinct characteristics and applications. Culture scales range from several liters to tens, hundreds, or even thousands of liters, with stirred-tank bioreactors being the most widely used in the industry. However, different hybridoma cell lines exhibit varying antibody secretion capabilities and may have different nutritional requirements, with the most suitable culture media also differing. Monoclonal antibody production by mouse hybridoma cells in vitro is influenced by numerous factors including promoters, inhibitors, and additives. Many researchers have employed various approaches to enhance monoclonal antibody production capacity in vitro, such as genetic engineering modification, re-screening of monoclonal cells, and nutrient optimization [13]. Bioreactor culture of hybridoma cells requires dual regulation of bioreactor control parameters and nutrients [14].

3.1 Bioreactor Parameter Control

The most economical and effective monoclonal antibody production process requires thorough understanding of how bioreactor process parameters affect cellular physiology. Therefore, kinetic parameters for cell growth, nutrient consumption, and antibody secretion must be evaluated, as these parameters are also useful for process scale-up [11].

Ayyildiz TD et al. [15] compared roller bottle culture systems (Modular Cell Production Roller Culture Apparatus), stirred-tank bioreactors (Biostat B plus cc, Sartorius, 5L), and single-use static cell culture systems (CELLine CL350, Sartorius) for culturing A5A8 hybridoma cells to prepare anti-Salmonella O-antigen-specific monoclonal antibodies, aiming to understand how different bioreactor process parameters affect monoclonal antibody production, including cell density, cell viability, glucose and glutamine consumption, lactate and ammonia production, and antibody secretion. Results showed that when cells were cultured in DMEM/F12 medium with 10% serum, bioreactor culture outperformed single-use static culture systems, which in turn outperformed roller bottle culture systems, with specific antibody production rates of 20mg/L/d, 16mg/L/d, and 5mg/L/d, respectively.

The most commonly used stirred-tank bioreactors have multiple factors affecting cell growth and antibody productivity during culture, including bioreactor process parameters such as temperature [16], dissolved oxygen [17], pH [18], and agitation [19]; nutrients and metabolic waste such as glucose [20], amino acids [21], and hydrocortisone [22]; and chemical inducers of antibody secretion such as sodium butyrate [23]. Of course, changes in these factors may also affect an-

tibody charge distribution [24], glycosylation patterns [25], and may even cause crystallization phenomena [26], as shown in .

Agarabi CD et al. [29] utilized Plackett-Burman design from DOE experimental design methodology to conduct 12 experiments validating the effects of 11 parameters at two levels on monoclonal antibody production in bioreactor-cultured hybridoma cells. The 11 parameters included dissolved oxygen, temperature, agitation, aeration, inoculation density, pH, temperature shift, feeding strategy, non-essential amino acids, fatty acids, and cortisol. Results showed that antibody secretion across the 12 experiments ranged from 20mg/L to 120mg/L. Bioreactor process parameters did not produce sustained significant effects on cell density, but agitation speed and temperature had more significant effects on cell density than fatty acid addition. Appropriate levels of glucose and glutamine were beneficial for cell growth and antibody secretion. Different feeding strategies had minimal impact on cell growth and antibody secretion. Antibody yield decreased when temperature was reduced to 34°C, though interestingly, some studies have shown increased antibody yield at lower temperatures.

3.2 Nutrient Optimization

Batch culture of hybridoma cells in vitro yields low cell density and antibody secretion [30]. Jo EC et al. [31] cultured anti-HBsAg monoclonal antibody-secreting hybridoma cells primarily in Erlenmeyer flasks using a repeated intensive feeding strategy with specific basal and feed media. The culture medium contained 50 times the nutrients of RPMI-1640 basal medium. Feeding was based on maintaining glucose concentration at 1g/L, with daily addition of 10% initial volume of feed while removing 10% volume to maintain stable culture volume. Maximum cell density of 1×10^7 cells/mL was reached at 70 hours, after which cell density was maintained at 0.5×10^7 cells/mL for 2500 hours, with antibody secretion maintained at 1g/L for 2500 hours.

It is well known that cell death during culture is caused not only by glucose or amino acid deficiency but also by accumulation of metabolic toxins such as ammonia from glutamine metabolism and lactate from glycolysis. Appropriately reducing glucose and glutamine concentrations can decrease accumulation of metabolic waste such as ammonia and lactate, but excessively low concentrations with inadequate feeding may lead to rapid cell death. Therefore, nutrient concentrations must be reasonably controlled to prevent cell death from either nutrient deficiency or metabolic waste accumulation [32-34]. In the 1990s, Liangzhi Xie et al. [35-37] conducted extensive work on medium and feeding studies through investigation of hybridoma cell metabolic kinetics, analyzing nutrients and energy required for cell growth and metabolism. Fed-batch culture increased maximum viable cell density from 6.3×10^6 cells/mL to 1.7×10^7 cells/mL, extended culture duration from 340h to 550h, and achieved final antibody secretion of 2400mg/L, while reducing the glucose-to-lactate conversion ratio from 67% to 3.4%.

Conventional hybridoma cell culture uses serum-containing basal media such as DMEM or DMEM/F12. However, with the development of serum-free media technology, various serum-free and low-serum media for hybridoma cells have emerged. Keisuke S et al. [38] developed a serum substitute for in vitro culture of mouse hybridoma cell line CRL-1606 (from ATCC) secreting human fibronectin monoclonal antibody. This serum substitute contained 12 components: insulin (0.017mM), transferrin (0.0013mM), p-aminobenzoic acid (0.015mM), pyridoxine hydrochloride (0.11mM), sodium selenite (0.00038mM), sodium pyruvate (7.5mM), hypoxanthine disodium (0.074mM), linoleic acid (0.00073mM), lipoic acid (0.0025mM), putrescine (0.0025mM), thymidine (0.0075mM), and glutathione (0.0081mM). Bioreactor culture (1L, Able Co, Japan) was performed at 800mL working volume with feeding every 12 hours based on nutrient consumption. The entire culture lasted 25 days, achieving maximum viable cell density of 3×10ˆ6 cells/mL and antibody secretion of 400mg/L, representing a 5-fold increase compared to pre-feeding levels. Chua GK et al. [39] developed a low-serum medium consisting of DMEM basal medium supplemented with 0.4% fetal bovine serum, 311.8mM ferric citrate, 17.3nM sodium selenite, and 4.5mM zinc sulfate. Compared to the control group using 2% fetal bovine serum, this low-serum medium reduced costs by 64.6% while increasing single-cell antibody production capacity by 3-fold.

Marc Cherlet et al. [40] used a 2L bioreactor to culture hybridoma cells secreting anti-human T lymphocyte CD3 monoclonal antibody to validate the effects of butyrate on hybridoma cells. Butyrate can promote antibody secretion but inhibit cell growth, necessitating selection of appropriate timing and concentration for addition. Adding 1mM butyrate during the logarithmic growth phase doubled antibody secretion. Rokni M et al. [41] investigated the effects of the trace element all-trans retinoic acid (ATRA) and the unsaturated fatty acid docosahexaenoic acid (DHA) on antibody secretion by mouse hybridoma cells both in vivo and in vitro. Three experimental groups and one control group were established: ATRA alone, DHA alone, and combined ATRA and DHA. Results showed that 单独添加 1 M ATRA or 10 M DHA enhanced antibody secretion, with ATRA showing better promotion than DHA. Combined addition of 0.5 M ATRA and 5 M DHA also promoted antibody secretion, with effects similar to 单独添加 10 M DHA, doubling antibody secretion. Konno Y et al. [42] demonstrated that coenzyme Q10 addition could also promote antibody secretion.

With the development of monoclonal antibody technology, thousands of hybridoma cell lines secreting monoclonal antibodies have been established, and methods and equipment for large-scale in vitro hybridoma cell culture continue to evolve. For the rapidly developing in vitro diagnostics field, demand for monoclonal antibodies continues to increase. Limitations in mouse breeding scale and environmental assessment regulations will further promote rapid development of hybridoma cell in vitro culture technology. Currently, multiple in vitro diagnostics companies are developing hybridoma cell in vitro culture technologies, while also actively developing recombinant antibodies for some diagnostic

antibodies to improve antibody quality.

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