The annual global market for biopharmaceuticals continues to increase from an estimated $12 billion three years ago to $30 billion today. Of the 122 biopharmaceutical products approved in the United States and Europe, 50 are produced using mammalian cells, 39 by prokaryotic cells, 21 using a yeast system, and a further 12 products by undisclosed systems (1). As an increasing number of potential new drugs emerge from the preclinical stage into clinical trials (now estimated at approximately 500 undergoing clinical evaluation), demand for the cGMP manufacture of these products continues to increase. The main methods of making such products are batch, fed-batch, and continuous perfusion. The mode of operation is typically chosen on the basis of product concentration and stability, scalability, simplicity, time, and cost. Perfusion presents a number of advantages over other modes of cultivation, including increased volumetric productivity, reduced vessel requirements, rapid removal of by-products and easily inactivated products from the culture environment (2). This article describes the use, development, and validation of a small-, medium-, and large-scale acoustic perfusion system.

**Figure 1:** Schematic view of a classical perfusion system setup using a 200-L/day acoustic device
The constituents of a biotechnological broth are many and varied, but they generally can be classified by size or function. In a perfusion setup, various components of this broth are separated so that cells are retained, harvest is captured, and medium is refreshed. Such a process maintains consistent nutrient supply and removes products and cellular waste. Perfusion processes, although inherently more sophisticated than batch or fed-batch processes in design and scale-up, have been successfully used to generate large-scale quantities of biopharmaceuticals. Instrumentation, bioreactor design, scale-up, and ease of operation have improved significantly over the past decade (8).

But such improvements also bring new challenges. A combination of the use of serum, protein, or mammalian-source-free medium and achievement of high cell densities leads to poor performance of standard spinfilters in perfusion systems. An alternative option is the use of an acoustic cell retention device. In this case, cells are retained not by a filter material, but by sound waves: a “virtual” filter type avoiding clogging issues that occur with use of standard filter material.

**Acoustic Cell Retention Device:**
Ultrasonic separation of cells (and other particles) is achieved by generating acoustic forces resulting in a standing wave. These forces result from interactions between fluid and cells; their magnitude depends on the differences in compressibility between the cells and the medium (6).

This study investigated three acoustic devices: the 10-, 50-, and 200-L flow/day systems. Figure 1 illustrates the 200-L system setup. A recirculation pump delivers the cell culture broth to the resonator chamber. The resonator chamber comprises two opposed parallel glass surfaces. At least one surface is piezoelectrically activated to act as an ultrasonic source. As a result, specifically selected frequencies generated from the attached controller establish a high frequency acoustic standing field within the cell suspension between the glass walls. This acoustic energy mesh acts as a virtual filter capable of capturing the cells within the antinodes of the acoustic field and thus retaining cells from the fluid. Simultaneously, the harvest pump draws clarified harvest from the resonator. When a weight/level sensor identifies a drop in weight/volume, a feed stream is initiated using the medium pump indicated to maintain a constant working reactor volume.

Trapped cells typically form loose aggregates, which settle out of the acoustic field and are then immediately disaggregated while recirculating to the bioreactor. In essence, the cells are held by ultrasonic forces against the upward flow of the harvest stream. The cells are held within this “virtual” filter for a defined period termed *sonification time* (typically 5–10 minutes) while harvest is withdrawn. After a predefined time, the acoustic field is automatically switched off by the controller for a defined period termed *interrupt time* (typically 3–9 seconds) while the cells are returned to the reactor by gravity. The entire procedure subsequently begins again and continues in this cyclic motion until the end of the culture. In this study, average cell retention efficiency was greater than 90% for the 10- and 50-L systems and greater than 85% for the 200-L system.

**10- and 50-L Acoustic Systems**
The 10-L system was first used according to the manufacturer’s recommendations as detailed in Figure 1, termed *classical setup*. We believe that this setup contributed to cell clumping. Replacing the harvest pump with a regulated harvest/back-flush pump resulted in a noticeable decrease in cell clumping. With this dual-function pump setup, the acoustic chamber was “washed,” and cells were actively dissociated by the backward fluid motion during the interrupt time (7).

Photo 1 shows a photograph of (A) a 7-L fermentor using a regular acoustic retention device setup and (B) a 7-L fermentor using a modified acoustic retention device setup. The clumping of cells is visible by eye in the classical setup (Photo 1a). The adoption of the dual-function pump for this process clearly reduced the clumping (Figure 1b). This procedure was carried forward to the larger scales with no significant change in process operation and with results similar to those of the small-scale device.

**200-L Acoustic System**
A pilot run of the process using the large-scale 200-L acoustic device was conducted to determine the optimal parameter settings at this scale. The influence of five key parameters on separation efficiency (SE) were assessed: temperature, flow rate, power input, frequency selection, and recirculation rate.

**Temperature:** The standing waves of this acoustic system are formed by exciting a ceramic transducer plate. Part of the energy applied to

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**Photo 1:** Photograph of (A) 7-L fermentor growing CHO-K1 cells using a classic acoustic retention system setup and (B) 7-L fermentor growing CHO-K1 cells using a modified acoustic retention system setup.
The transducer is transferred to heat. The acoustic device therefore requires cooling. The 10- and 50-L scales are air cooled, whereas the 200-L system is water cooled (Figure 1). The amount of heat produced is dependent on the power applied to the system. If uncontrolled, this heat can establish convective currents within the chamber, resulting in disturbance of the acoustic field and subsequently a decrease in cellular retention. In addition, temperatures that are too high within the chamber can potentially cause cell damage. Other parameters that influence the temperature in the system are flow rate, the temperature of the environment, and length of the recirculation loop. The correct control of temperature is therefore a critical parameter for the acoustic system at this scale.

For those reasons, flowrate, power input, frequency selection, and recirculation rate were controlled at the same settings, but not the temperature. A range of temperatures was assessed from 1 °C higher (+1 °C) than the cultivation temperature to 4 °C lower (−4 °C). Figure 2 depicts the separation efficiency (%) of a 200-L acoustic retention device versus temperature difference (°C) between the cultivation temperature in the reactor and the cooling water jacket surrounding the acoustic chamber. A temperature difference of 1.5 °C lower than the cultivation temperature showed the best separation efficiency. This finding is in broad agreement with what other groups have observed (8).

Flow Rate: Flowrate is a critical parameter that significantly influences the separation efficiency (SE) of the acoustic device. The current large-scale device has a recommended vendor operating range of 50–200 L/day. Actual data demonstrating this within a mammalian culture are limited. This study assessed the effect of 50–485-L flowrates/day. Figure 3 shows the flowrate (L/day) versus separation efficiency (%) using a 200-L acoustic retention device with 5–6 × 10^6 cells/mL in the culture. A flowrate of 100-L/day was shown to be optimal for this process. A rate of 485-L/day, an almost 2.5-fold increase to the upper recommended operating range, still provided a separation efficiency of 63%.

Power Input to the Chamber: The power applied to the resonator chamber bears a direct correlation with the resulting separation efficiency (Figure 4). The impact of the power setting is best described as influencing the intensity of the acoustic field and hence the resulting acoustic mesh. When the power input was increased from 20W to 40W, the separation efficiency also increased. However, this increase in power input will influence the temperature of the chamber as previously described. A balance of these parameters is critical for stable and robust separation efficiency.

Frequency Selection for Controller Device: Within the resonator chamber, at specific frequencies generated from the controller, a highly frequent acoustic standing field is established that traps the cells within the antinodes of the waves. During normal operation, an automatic frequency selection scans a defined frequency range to obtain a level at which cells are trapped. This frequency will change during the time course of a culture as the matrix (cell culture fluid) changes in which the field is generated. The speed of sound changes in relation to the matrix through which it travels. The frequency used at that time reflects responses in changes to cell density, nutrient, metabolite, and by-product concentrations.

Therefore, the impact of
automatic and manual frequency selection on separation efficiency was assessed. Automatic selection resulted in good separation efficiency of 86% at this cell density, but an improvement to 93% resulted in frequency selection of 2.14 MHz (Figure 5). This result demonstrates that SE performance can be improved by selecting the optimal frequency at specific conditions. This can be best done during the stationary phase of a continuous perfusion culture when no significant changes in matrix composition occur.

Recirculation Rate: The rate of recirculation using the pump depicted in Figure 1 influences the cell culture performance over long runs. One key parameter influenced by the recirculation rate is the dissolved oxygen (DO) concentration within the resonator chamber known to have an impact on cell culture performance.

In this study, three varying rates of recirculation were assessed on the basis of their impact on SE and DO concentration directly under the resonator chamber (within the resonator bowl). The best performance on both parameters was attained by a recirculation rate of 3 (Figure 6). At that rate of recirculation, an SE of 93% and a DO value of 9% was attained.

Balance: This initial study did not address synergistic effects of each of the above discussed parameters with the large-scale acoustic device. An increase in flowrate is known to influence the performance of a defined power input; therefore, a concurrent adjustment of the power input may be necessary. It is important to “balance” all parameters to obtain a consistent and robust high separation efficiency.

VALIDATION

Subsequent to the initial pilot run, which defined the optimal settings for this process, the system and associated equipment were validated. In addition, the system setup was validated with regards to sterilization and operation. After completion of the validation study, the first cGMP-manufacturing run using the large-scale acoustic system was performed in quarter four of 2002. This is believed to be the first cGMP run using this device at this scale (9).

NEW AND IMPROVING

The use of the large-scale acoustic system as a perfusion device remains relatively novel. This is reflected in sales figures supplied by the vendor (Applisens, The Netherlands) with approximately 150 of the 10-L/day, 50 of the 50-L/day, and eight of the 200-L/day systems having been sold. The work presented in this study demonstrates that improved cellular retention can be obtained with the large-scale acoustic device using variations in temperature, flowrate, power input, frequency selection, and recirculation rate.

A larger scale acoustic device — the 1000-L/day — is now being tested. This device consists of five of the 200-L/day devices configured together. Photo 2 shows a prototype model. The device is likely to help propel the acoustic system as a serious perfusion method in biopharmaceutical production. A commercial launch is planned for 2004.

REFERENCES

8 Keijzer, T; et al. Integrating Acoustic Perfusion in Mammalian Cell Culture — Temperature Control. 18th ESACT meeting, Granada, Spain (2003).
9 Crowley, J; et al. cGMP Manufacturing of a Fusion Protein in Mammalian Cells Using a Large-Scale Acoustic Perfusion System. 18th ESACT meeting, Spain (2003).