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Two Approaches for Cell Retention in Perfusion Culture Systems

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TWO APPROACHES FOR CELL RETENTION IN PERFUSSION CULTURE SYSTEMS

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To my parents and my children
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Two Approaches for Cell Retention in Perfusion Culture Systems
Zhaowei Wang

Abstract

A lack of efficient, economical, and reliable cell retention devices has limited the application of perfusion culture systems in the biopharmaceutical industry. Two types of cell retention devices were developed in this work for long-term perfusion culture systems: a modification of an inclined gravity settler and a variation of an ultrasonic filter. Both bench-top and large-scale tests showed that the gravity settler can effectively retain viable cells and preferentially remove nonviable cells in perfusion culture systems. The viable cell retention rate can be maintained well above 90% during long-term perfusion culture period while the nonviable cell retention rate is 20-30% lower than that of viable cells. The design of this settler enables its manufacture as a single-use device to be used in conjunction with disposable bioreactors. An apparatus for measuring the settling velocities of both the viable and nonviable cells, which is both simple and inexpensive to use, was developed in order to predict optimal operation parameters of the gravity settler. The gravity settler was also effective in an algae dewatering process. The ultrasonic filter has a cell retention capacity 21-fold greater than that of the gravity settler for the same working surface area. Due to its unique design, this ultrasonic filter can operate continuously, as opposed to the currently available ultrasonic filters that need on-off intervals for removal of retained cells.
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NOMENCLATURE

A     area of the cross-section of the rectangular channel
b     separation between the two inclined surfaces
β     ratio of particle diameter to vessel diameter
c     volume fraction of particles in fluid
C_f   speed of sound in fluid
d_p   particle diameter
γ_f   compressibility of carrying fluid
γ_p   compressibility of particle
E_{ac} acoustic energy density within suspension
f     frequency of applied ultrasound
F     acoustic contrast factor
F     perfusion flow rate
F_D   hydraulic force
g     gravity acceleration
h     distance
θ     angle between the longitudinal axis of the gravity settler and the vertical
I     electric current
L     length of inclined gravity settler
κ     wave number across acoustic filter
μ     fluid viscosity
\( \mu_{app} \) apparent cell specific growth rate

\( n \) function of Reynolds number (equal to 4.65 when Reynolds number is less than 0.3)

\( P \) power input

\( P_{\text{Initial}} \) antibody concentration in inoculum

\( P_{\text{Max}} \) maximum antibody concentration achieved during batch culture

\( \bar{P} \) average antibody concentration of two consecutive samples

\( \Delta P \) difference in antibody concentration of two consecutive samples

\( q_p \) specific rate of antibody production

\( \rho \) density of carrying fluid

\( \rho_p \) density of solid particle

\( R \) cell retention rate

\( R_v \) viable cell retention rate

\( R_p \) radius of the spherical particle

\( s \) cross section area of settling column

\( S(v) \) volumetric rate of production of fluid clarified of particles

\( t_{\text{Total}} \) total time taken for the batch culture to reach maximum antibody concentration

\( \Delta t \) time interval between two consecutive samples

\( T \) residence time

\( U \) wetted perimeter of cross-section

\( v \) settling velocity

\( V \) volume

\( w \) width of inclined gravity settler

\( x \) distance of cell from the nearest pressure nodal plane
\( X_R \)  
cell concentration in the bioreactor

\( X_O \)  
cell concentration in the overflow stream

\( X_v \)  
viable cell concentration

\( X_T \)  
total cell concentration

\( X_{T(t-\Delta t)} \)  
total cell concentrations at time \( t-\Delta t \)

\( X_{T(t)} \)  
total cell concentrations at time \( t \)

\( \overline{X}_T \)  
average total cell concentration

\( \overline{X}_v \)  
average viable cell concentration
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CHAPTER I

INTRODUCTION

1.1 Overview

Tissue culture technique was first used almost 100 years ago as a way to conduct research work in developmental biology. Carrel succeeded in keeping tissue alive for over two months, which demonstrated the possibility of long term \textit{in vitro} cell culture. The next milestone was that of Katherine Sanford and co-workers cultured single cells successfully in 1948.\textsuperscript{1} Formulated culture medium and the creation of cell lines make cell culture more realistic and quantitative. In 2007, the sales of biopharmaceuticals in the US were over $44 billion, a majority of which were produced in animal cells.\textsuperscript{2}

The main culture modes are batch culture, fed-batch culture, and continuous perfusion culture. Batch culture is the most traditional mode for cell culture. Its major disadvantage is that only low cell concentration can be reached and cells start to die soon after reaching the maximum concentration, resulting in low product titer. And much more time is spent on system shut-down, cleaning, and inoculation compared to continuous cell culture.
In fed-batch culture mode, a fraction of the cell suspension is removed from the system at certain intervals, and an equal amount of fresh medium is added into the system. This kind of culture is better than batch culture because the time used for shutting down and cleaning is less. Indeed, the cell loss still cannot be avoided when a portion of culture is replaced by fresh media. The removal of cells having ability to produce desired product is undisputedly wasteful.

For perfusion culture mode, spent medium is continuously removed from the culture vessel without losing cells, and an equal amount of fresh medium is added into the culture vessel. The most important advantage of perfusion culture mode over batch culture and fed-batch culture is that viable cells are not removed with the spent culture medium. Cells are retained in the culture vessel to continue to grow and express target products. For producing the same amount of desired product, the cost for running a perfusion culture system can be as low as one tenth of fed-batch culture mode.\textsuperscript{3} Perfusion cultures also can better maintain consistent product quality, and allow steady state operation and better cell physiology control.\textsuperscript{4}

Although it is apparent for biopharmaceutical companies to prefer perfusion culture to fed-batch culture mode, the truth is the latter is prevalent for industry. The reason is the lack of a robust, highly efficient and low cost cell retention device. There are many technologies currently available for the retention or recycling of cells grown in
suspension but all of them have drawbacks limiting their broad application in the biopharmaceutical industry.

1.2 Specific Aims

The aims of this work are to develop two cell retention devices for perfusion cell culture systems, which are more robust, efficient and less expensive than currently available systems.

Specific Aim 1: To develop an inclined gravity settler for long-term perfusion cell culture.

Gravity settlers are simple to build, are low-cost and easy to operate. The traditional vertical gravity settler’s volume is too large compared to the working volume of the bioreactor. Several kinds of inclined settlers have been developed, which offer an efficient means to selective cell retention. These settlers have an obvious disadvantage: viable cells are not returned to the bioreactor easily because the cell suspension flow direction is opposed to the direction of the settled down cells returning back to bioreactor. The inclined gravity settler we developed overcomes this shortcoming by allowing the settled cells to move with the fluid in same direction. Meanwhile, the capacity per volume of the gravity settler is improved significantly compared to previously developed devices.
Specific Aim 1A: Characterization of an inclined gravity settler for cell retention.

An inclined gravity settler with flow co-current to particle movement has been designed. The performance of the prototype version was characterized in perfusion cultures with two cell lines, each conducted for over one month. The design was then modified for industrial-scale use, including changing the material of construction to polycarbonate which makes it feasible for both disposable and re-usable applications. Short-term experimental results demonstrate its efficiency for cell retention at the industrial-scale.

Specific Aim 1B: Develop a method for settling velocity measurement.

The cell retention capacity of the settler is proportional to the cell settling velocity. Cell settling velocity varies at different growth phases and for different cell lines, which causes uncertainty of separation efficiency. In order to select operating parameters to maximize the settler’s viable cell retention capacity for each cell line at its specific growth stage, a simple method for measuring viable cell settling velocity is needed. This device was developed and tested with both mammalian cells and standard polystyrene particles.

Specific Aim 1C: Demonstrate feasibility of a perfusion system with inclined gravity settler as inoculum source for large-scale culture.

Since the settler can preferentially remove nonviable over viable cells from the perfusion culture, a perfusion culture using this device is expected to be able to maintain relatively
high viability during the perfusion culture period. The feasibility of using this perfusion culture as an inoculum for a large-scale batch culture was investigated.

**Specific Aim 1D: Application of the gravity settler for algae culture dewatering.**

Algae culture is a promising alternative for producing biofuel. The major limitation to the economic viability of this process is the large expense of the dewatering process, i.e. separation of the cells from the perfusion fluid. Although the algae cells are smaller than mammalian cells, they have many similar characters. The gravity settler developed for perfusion mammalian cell culture was evaluated for use with algae cells.

**Specific Aim 2: To develop an acoustic-based cell retention device.**

Ultrasonic filters utilizing ultrasonic standing waves to retain cell-sized particles have high capacity per volume. The effect of a porous mesh inserted into the ultrasonic filter chamber was determined. The commercially available ultrasonic filters need regular power-off intervals to allow retained cells to leave the filter chamber. An ultrasonic filter which can be operated continually was developed.
Specific Aim 2A: Evaluate performance of cell retention using ultrasonic standing waves with a porous mesh.

It has been found that porous mesh can enhance particle retention in an ultrasonic filter. Cell retention performance of this system was investigated and compared to commercially available acoustic filters.

Specific Aim 2B: Determine the impact of long-term exposure to ultrasonic standing waves on cell growth and antibody production.

The feasibility of using the ultrasonic filter as a cell bioreactor was studied, with specific emphasis on the impact of ultrasound stress on cell growth and antibody production during long term exposure.

Specific Aim 2C: Characterize cell retention using standing ultrasonic waves at an oblique angle with fluid flow direction.

An acoustic filter with unparallel alignment of acoustic transducer and particle suspension flow direction was developed and investigated. The middle chamber of the acoustic filter is unparallel with the transducer and reflector walls. A unique feature of this acoustic filter is that the carrying fluid flows in the same direction as the movement of the captured particles, allowing continuous operation of the filter. The retention efficiency of the device was investigated using mammalian cells and polystyrene.
CHAPTER II
BACKGROUND

2.1 Overview

A variety of cell or particle retention devices have been developed.\textsuperscript{5, 6} Cell retention devices based on filtration process, such as crossflow microfiltration\textsuperscript{7-9} and spin filters\textsuperscript{10-13}, are economical to develop and operate. The disadvantage is that the membranes retain nonviable cells and cell debris, which accumulate in the bioreactor and can foul the sensors within the bioreactor and the retention membrane, resulting in termination of the perfusion culture process.

Continuous centrifuges\textsuperscript{14, 15} were developed for the handling of large-scale cell separations. It can be operated continuously with small residence time of cells in the centrifugal chamber. It is being used nowadays for large scale perfusion culture systems. The main drawback to the system is that it is mechanically complex and might expose cells to harmful shear stress. The cost is relatively high for each unit too.
2.2 Acoustic Filters

The ultrasonic filter has been investigated by many researchers in recent years and has been successfully applied to perfusion cultures.\textsuperscript{16-20} It has been described as the most promising cell retention device for long term perfusion culture.\textsuperscript{21} This kind of device is compact and efficient for cell or particle retention. The cell suspension is first pumped into the lower end of the device. The cells are aggregated by the ultrasonic standing waves. When the ultrasonic field is on the collected particles mostly kept in the working chamber of the filter because the hydraulic force which is upward against the moving down trend of the collected particles. An “off” interval is needed to allow the collected cell clumps to drop down out of the ultrasonic chamber and back into the bioreactor. Then the ultrasonic field is turned on and a new cycle of retention is restarted. This “on-and-off” cycle operation mode not only increases the complexity of the control system but also reduces the retention efficiency since the system is not in a steady status at the beginning of every new cycle. The impact of prolonged exposure to ultrasonic fields on growth and productivity in a wide variety of cell types is unclear.

It is known that ultrasonic waves at high power level can disrupt cell membranes, and this feature is used to disrupt cells in the commercially available lab instrument called sonicator. Other detrimental effects to cells from ultrasonic field exposure are due to shear stresses and microstreaming associated with oscillations of cavitation bubbles.
Conversely, ultrasonic filters for cell retention have been successfully used with no detectable detrimental effect on cell growth or productivity.\textsuperscript{22-28} In fact, it has been found that in some condition, with low power density input, acoustic waves can enhance cell growth.\textsuperscript{29, 30} Zhang et al. also found that both proliferation and matrix production of chondrocytes were enhanced by pulsed low-intensity ultrasound.\textsuperscript{31, 32} In their experiments, the proliferation of chondrocytes was increased by 15\% over control and the secretion of type II collagen was increased by 22\% over control. It needs to be determined if there is significant detrimental impact on cell growth and productivity for prolonged exposure to the ultrasonic standing field at the power lever applied for cell retention using the ultrasonic filters.

\textbf{2.3 Gravity Settlers}

Gravity settlers are relatively simple to manufacture and are especially suitable for cells sensitive to shear stress. They not only prevent viable cells from being removed with the supernatant, but they also preferentially remove nonviable cells from the culture system.\textsuperscript{33-36} The avoidance of the gradual accumulation of nonviable cells in the bioreactor is important since the proteases and glycosidases released by lysed cells may degrade the secreted antibody.\textsuperscript{27, 37, 38} Removal of nonviable cells and debris also helps to reduce probe fouling resulting in prolonged culture period. Vertical gravity settlers\textsuperscript{39} need a large volume, relative to the bioreactor volume, to separate the cells from the overflow because of the slow settling velocity of animal cells, resulting in difficulty in large-scale
An inclined gravity settler results in a sharply reduced settler volume compared to the vertical gravity settler. When the cell suspension enters the settler from the lower end of an inclined upward-flow settler, the cells settle to the surface of the lower plate and then slide down countercurrently to return to the bioreactor (Figure 2.1). The downward movement of the settled cells on the lower surface is hindered by the upward flowing stream, causing an increase in the residence time of the settled cells in the settler. A long residence time not only has potentially negative impact on cell metabolism but
also allows cells to attach to the lower surface. In order to reduce the chance of cell attachment, pre-chilling and periodic vibration or bubbling has been used to re-suspend the cells stalled on the lower surface.\textsuperscript{42-47}

In a downward-flow gravity settler, cell suspension enters at the upper end of the gravity settler (Figure 2.2). Since the settled cells move downward in the same direction as the fluid, there is no hindrance from the fluid for the settled cells to return to bioreactor. Settled cells have nearly the same speed as the fluid and so the cell residence time is theoretically the same as the fluid residence time. With this design, cells do not accumulate and no action is needed to re-suspend the stalled cells in the settler. This makes it more suitable for continuous operation.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{figure22.png}
\caption{A downward-flow inclined gravity settler, a single cell is also under three forces, but the hydraulic force, $F_h$, facilitates the downward movement of settled cells.}
\end{figure}
With the use of an effective cell retention devices perfusion cultures can attain much higher cell densities than that of batch culture mode, but the cell viability is too low to be used as inoculum for large bioreactors.\textsuperscript{48} The inoculum viability directly affects the cell growth in production bioreactors. Kallel et al. showed that the inoculum viability should be higher than 75%.\textsuperscript{49-51} In practice the cell viability used as inoculum is around 90%. Cell viability during perfusion cultures with total cell retention can be improved above 90\% by increasing cell bleed rate.\textsuperscript{52-54} Using this strategy, Heidemann et. al. developed an innovative method to inoculate large scale cell culture bioreactors in less time. Cells from high density perfusion bioreactors, in which cells were purged to keep the cell viability high, were collected and frozen in liquid nitrogen. Then the frozen cells were thawed and cultured in fed-batch mode to accumulate cells for inoculating a large production bioreactor. The downside of this approach is the use of cryo-preservation equipment as well as the loss of viable cells when using the cell bleed strategy.
CHAPTER III

AN INCLINED GRAVITY CELL SETTLER FOR LONG-TERM HIGH-DENSITY MAMMALIAN CELL PERFUSION CULTURE

3.1 Introduction

In this chapter we present a modified design of the downward-flow inclined gravity settler. This design results in greater operational flexibility and improved efficiency in selective retention of viable compared to nonviable cells. The utility of this design is demonstrated with two different hybridoma cell lines, hybridoma 9E10 and R73, each cultured over one month in perfusion systems with the cell settler device.

Due to the slower growth rate and greater sensitivity to growth factor concentrations compared to bacteria, expansion ratio about 1:10 is required for seeding large-scale bioreactors, with the seed culture normally started from a 1 mL ampoule. Multiple medium-sized bioreactors are involved in the scale-up process as shown in Figure 3.1. Normally it would take 3-4 weeks to prepare a seed culture for large cell culture
Apart from the high cost associated with excessive time and labor required for this process, the risk of contamination is high due to multiple transfers of inoculum. It is obvious that a smaller inoculum volume and fewer steps are desirable for process optimization in the biopharmaceutical industry. A perfusion system with cell recycle not only results in high productivity but can also be used to reduce costs for inoculum preparation for large-scale production. The gravity settler can preferentially remove nonviable cells and cell debris resulting in results in relatively high cell viability with little loss of viable cells. This feature suggests a possible alternative for seed-train expansion strategy. With both high cell concentration and viability, the small-scale perfusion culture can be used to provide inoculum for a large-scale bioreactor. This method can be expected to reduce both time and costs associated with seed expansion. This feasibility of this process is presented in this chapter using the R73 hybridoma cell line.

Figure 3.1 Conventional seed-train expansion process

A perfusion system with cell recycle not only results in high productivity but can also be used to reduce costs for inoculum preparation for large-scale production. The gravity settler can preferentially remove nonviable cells and cell debris resulting in results in relatively high cell viability with little loss of viable cells. This feature suggests a possible alternative for seed-train expansion strategy. With both high cell concentration and viability, the small-scale perfusion culture can be used to provide inoculum for a large-scale bioreactor. This method can be expected to reduce both time and costs associated with seed expansion. This feasibility of this process is presented in this chapter using the R73 hybridoma cell line.
3.2 Materials and Methods

3.2.1 Principles of Settler Design and Operation

The working principle of an inclined gravity settler is described by:  \(^{23, 25}\)

\[
S(v) = vw(L \sin \theta + b \cos \theta) \tag{3.1}
\]

where \(S(v)\) is the volumetric rate of production of fluid clarified of particles with settling velocity \(v\), \(w\) is the width of the settler, \(b\) is the separation distance between the two inclined surfaces, \(L\) is the length of the settler, and \(\theta\) is the angle between the longitudinal axis of the gravity settler and the vertical (Figure 2.1B). The quantity \(w(L \sin \theta + b \cos \theta)\) is the projected area of the inclined gravity settler.

Since normally \(L\gg b\), \(^{23, 25, 28}\) Equation 3.1 can be simplified to:

\[
S(v) = vwL \sin \theta \tag{3.2}
\]

where \(wL \sin \theta\) denotes the projected area of the inclined gravity settler.

Equation 3.2 shows that the processing capacity of an inclined settler is determined by the product of the projected area of the settler and the cell settling velocity. By knowing the settling velocity, the projected area needed for processing a given volumetric rate of cell suspension can be determined.
With the traditional inclined gravity settler, the only means for changing the projected area during the culture period, and thus allow changes in the perfusion rate, is to change the inclination angle. In previously published work, the upward-flow settlers were operated with inclination angles of 25 or 30 degrees\textsuperscript{23, 25, 28, 40}. The upper limit of the inclination angle is limited by the need to allow the settled cells on the lower plate to slide down to the outlet port and thus prevent stalling on the lower surface.

### 3.2.2 Design Details of Downward-Flow Inclined Gravity Settler

In order to increase the flexibility of the inclined gravity settler, we designed a downward-flow inclined settler with multiple inlets as shown in Figure 3.2. By changing the position of the inlet the length $L$ is changed and thus the projected area is also changed proportionally (Equation 3.2), to meet the need of a wide range of processing flow rates. The settler volume is 45 mL at the farthest inlet position, which is less than 5% of a bioreactor with 1 L working volume. The working volume for this settler is changeable and is determined by the inlet position. The closer the inlet position is to the outlet (i.e. the smaller the distance $L$), the lower the settler working volume, and thus, a lower corresponding perfusion rate. The upper inlets are connected to the bioreactor via a fluid distributor. Cell suspension is pumped into the settler via one of the inlets while valves to the other inlets are closed. The concentrated cell suspension is returned to the bioreactor via port 1 and the supernatant is pumped to the harvest tank via port 2.
The material of construction is borosilicate glass. The width ($w$) is 2 cm and the thickness ($b$) is 1 cm. There are 12 inlets on the upper surface and two outlets from the lower surface. The maximum distance between the inlets and port 1 is 20.5 cm. This length was calculated using Equation 3.2 in order to accommodate a flow rate of 2.4 L/day through the settler, based on a viable cell settling velocity of 2.9 cm/hour (hybridoma cell line AB2-143.2) at an inclination angle of 60°. From our experience, this settler can be operated at a 60° inclination angle with an occasional shake to loosen stalled cells. Cell accumulation was virtually eliminated at 55°, which is the angle used in this work.

### 3.2.3 Cell Lines and Media

As shown in Table 3.1, two hybridoma cell lines were cultured in the perfusion culture system. The 9E10 and R73 cells were cultured in BD Cell™ Mab serum free medium
Both media were supplemented with 0.1% Pluronic F68 (Sigma, St. Louis, MO) for bioreactor culture. No other components were added or adjustments made to the media during the culture process.

Table 3.1 Cell line and culture media

<table>
<thead>
<tr>
<th>Cell line</th>
<th>9E10 (CRL-1729)</th>
<th>R73</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell Source</td>
<td>ATCC</td>
<td>Cleveland Clinic Foundation</td>
</tr>
<tr>
<td>Cell Type</td>
<td>Mouse-Mouse hybridoma</td>
<td>Mouse-Mouse hybridoma</td>
</tr>
<tr>
<td>Antibody Isotype</td>
<td>IgG1</td>
<td>IgG1</td>
</tr>
<tr>
<td>Culture Medium</td>
<td>BD Cell Mab Medium Serum Free</td>
<td>BD Cell Mab Medium Serum Free</td>
</tr>
<tr>
<td>Cellular Products:</td>
<td>Monoclonal antibody against human myc (c-myc) protein</td>
<td>Monoclonal antibody against rat TcR</td>
</tr>
<tr>
<td>Bioreactor Used</td>
<td>2 L B. Braun stirred bioreactor</td>
<td>2 L B. Braun stirred bioreactor</td>
</tr>
</tbody>
</table>

3.2.4 Analytical Methods

Cell density and viability were evaluated using trypan blue exclusion method with a hemocytometer. Concentrations of antibody IgG1 in culture supernatants were measured using a standard ELISA kit (Alpha Diagnostic International, San Antonio, TX) see appendix A.

3.2.5 Bioreactor System and Culture Protocol

The perfusion culture system is shown in Figure 3.3. A 2-L B.Braun stirred bioreactor (B. Braun biotech, Allentown, PA) with 1 L working volume was used for culturing 9E10
and R73 cells. A four-gas control module was applied to maintain the DO at 50%. The set point of pH was 7.2 but it fluctuated between 6.8 and 7.2 since no base or acid was supplied during the culture. A 1-L B.Braun stirred bioreactor with 1 L working volume was used for the inoculation test with R73 cells.

Cells were cultured in T-flasks in a humidified 5% carbon dioxide incubator at 37°C.

**Figure 3.3** Schematic of the perfusion culture bioreactor system

Cells in exponential growth phase were inoculated in the stirred bioreactor. Perfusion culture was started at the stationary growth phase of the bioreactor batch culture. Samples were taken from the bioreactor and the line to the harvest vessel (port 2). The recirculation rate was maintained constant at 0.8 L/day, while the perfusion flow rate was varied from 0.8 to 1.6 L/day.
3.2.6 Inoculation Test Protocol

The viable cell concentration reached $49 \times 10^6$ cells/mL after the perfusion rate was doubled gradually over a period of 6.4 days (see Figure 3.4). At this point, a 4 mL cell suspension was withdrawn from the perfusion bioreactor and used to inoculate the 1-L bioreactor for a batch culture as shown in Figure 3.5, resulting in a $2 \times 10^5$ viable cells/mL initial cell concentration at a 1:250 expansion rate. A control batch culture was conducted with inoculum from T-flasks with a 1:5 expansion ratio, resulting in an initial cell concentration of $2 \times 10^5$ viable cells/mL.

3.2.7 Calculations

The cell retention rate, $R$, is defined as:

$$R = \frac{X_R - X_O}{X_R} \times 100 \%$$  \hspace{1cm} 3.3

where $X_R$ is the cell concentration in the bioreactor; $X_O$ is the cell concentration in the overflow stream that exits the gravity settler via port 2 to the harvest tank.

Antibody volumetric productivity $p_v$ is the amount of the antibody produced per day per reactor working volume, given by:
Figure 3.4 R73 cell culture results. A. cell growth curve B. cell retention rate C. viable cell concentration vs. antibody concentration D. specific cell growth rates vs. specific antibody production rates
$p_v = \frac{(P_{\text{Max}} - P_{\text{Initial}})}{t_{\text{Total}}}$  \hspace{1cm} (batch culture)  \hspace{1cm} 3.4

and

$p_v = \bar{P} + \frac{\Delta P}{\Delta t}$  \hspace{1cm} (perfusion culture)  \hspace{1cm} 3.5

where $P_{\text{Max}}$ is the maximum antibody concentration achieved during the batch culture; $P_{\text{Initial}}$ is antibody concentration in the inoculum; $t_{\text{Total}}$ is the total time taken for the batch culture to reach the maximum antibody concentration (assuming that the batch culture is conducted ideally, i.e. the batch culture terminated as soon as the maximum antibody titer is achieved.); $\bar{P}$ is the average antibody concentration of two consecutive samples; $\Delta t$ is the time interval between two consecutive samples; $\Delta P$ is the difference in antibody concentration of two consecutive samples.
Specific rates of cell growth and antibody production are calculated using the following equations, which were described previously:  

\[
\mu = \frac{X_T}{X_v} \mu_{app} = \frac{X_T}{X_v} \left[ \frac{1}{\Delta t} ln \frac{X_{T(t)}}{X_{T(t-1)}} + (1 - R_v) \frac{F}{V} \right]
\]  

3.6

\[
q_p = \left( \frac{F}{V} \bar{P} + \frac{\Delta P}{\Delta t} \right) \frac{1}{X_v}
\]  

3.7

where \( \mu \) is the specific rate of cell growth (h\(^{-1}\)); \( q_p \) is the specific rate of antibody production (pg/viable cell/h); \( \mu_{app} \) is apparent cell specific growth rate (h\(^{-1}\)); \( F \) is perfusion flow rate (L h\(^{-1}\)); \( V \) is the working volume of bioreactor; The perfusion rate in day\(^{-1}\) is defined as \( F/V \); \( X_v \) and \( X_t \) are viable and total cell concentrations (cells/mL), respectively; \( X_{T(t-\Delta t)} \) and \( X_{T(t)} \) are total cell concentrations at time \( t-\Delta t \) and time \( t \); \( R_v \) is viable cell retention rate; and \( \bar{X}_t \) and \( \bar{X}_v \) are the average cell concentration of two consecutive samples; \( (1-R_v)F/V \) is the viable cell bleed rate. Equation 3.6 shows that in steady-state during perfusion culture, the apparent specific growth rate is equal to the viable cell bleed rate.

\section*{3.3 Results and Discussions}

\subsection*{3.3.1 9E10 Perfusion Culture}

Figure 3.6 shows the perfusion culture results with 9E10 cells. The maximum viable cell concentration reached during the perfusion culture period is 10-fold that achieved at day
3.5 in batch culture period (Figure 3.6A). The difference in retention rates between viable and nonviable cells is 20% on average (Figure 3.6B). When the perfusion rate was doubled from 0.8 day\(^{-1}\) to 1.6 day\(^{-1}\) the cell concentration oscillated with no sustained change and did not increase accordingly (Figure 3.6A). At the 1.6 day\(^{-1}\) perfusion rate and measured retention rate, the cell bleed rate is 0.16 day\(^{-1}\); at these conditions the perfusion culture is unlikely to be limited by nutrient availability.\(^{61}\) However, at 1.6 day\(^{-1}\) the average viable cell retention rate decreased to 90% (Figure 3.6 B). Therefore the expected increase in cell numbers due to increased perfusion rate was partially offset by the lower cell retention rate of the 9E10 cells. This phenomenon is in good agreement with results reported by Dalm et. al.\(^{49}\) The perfusion culture was terminated earlier than planned due to malfunction of the pH probe.

The gravity settler was designed for a maximum flow rate of 2.4 L/day based on a 2.9 cm/hour settling velocity of viable cells. However, the settling velocity of viable 9E10 cells is less than 2.9 cm/hour (Table 3.2). According to Equation 2, the length of the gravity settler needed for achieving 100% cell retention of the 9E10 cells at these conditions is 22.9 cm, which is longer than the 20.5 cm device used here. This finding supports the necessity of using a long device with flexible inlet positions for maximizing retention of different cell lines with different settling velocities.
Figure 3.6 9E10 cell culture results. A. cell growth curve B. cell retention rate C. viable cell concentration vs. antibody concentration D. specific cell growth rates vs. specific antibody production rates
Table 3.2. Cell settling velocity comparison

<table>
<thead>
<tr>
<th>Cell Line</th>
<th>Viability</th>
<th>Viability</th>
<th>Settling Velocity (cm/h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>9E10</td>
<td>97% Viable</td>
<td>Viable</td>
<td>2.6</td>
</tr>
<tr>
<td></td>
<td>65%</td>
<td>Viable</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nonviable</td>
<td>0.9</td>
</tr>
<tr>
<td>R73</td>
<td>95% Viable</td>
<td>Viable</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>65%</td>
<td>Viable</td>
<td>0.9</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nonviable</td>
<td>0.6</td>
</tr>
</tbody>
</table>

During most of the batch culture and perfusion culture with 0.8 day⁻¹ perfusion rate, the antibody concentration is almost linearly proportional to viable cell concentration (Figure 3.6C). When the perfusion rate was doubled the antibody concentration decreased by 50%. However, the specific antibody productivity is similar for the two periods (Figure 3.6D). The specific cell growth rate fluctuates dramatically during the culture. Although it has been broadly reported that specific antibody production rate drops when the specific growth rate increases, this relationship is not clearly demonstrated here. In general, the antibody production for this cell line is growth associated, i.e., the higher the cell concentration, the higher the antibody titer (Figure 3.6C). Therefore, with this cell line, the same result as that concluded by Hiller et. al. is true, i.e., a higher antibody volumetric productivity can be reached at higher perfusion rate as long as cell retention is maximized.

3.3.2 R73 Perfusion Culture

The maximum viable R73 cell concentration during perfusion culture at 0.8 day⁻¹ is about 8-fold of that achieved during the batch culture (Figure 3.4A). Compared to results with
9E10 cell line, the viable cell retention rate is much lower with the R73 cells, with an average of 88% throughout the perfusion culture period (Table 3.4, Figure 3.4B). The lower retention rate is a direct result of the significantly lower settling velocity of the R73 cells, measured at 1.8 cm/hour (Table 3.2). The settler length needed for 1.6 day\(^{-1}\) perfusion rate with this settling velocity is 33.3 cm (from Equation 3.2), which is larger than the maximum length of device used here. Notwithstanding this calculation, at many points the cell retention rate over 95% was achieved even with 1.6 day\(^{-1}\) perfusion rate. This result is attributed to the observation that the initial speed of the fluid as it enters the device is perpendicular to the settling surface, which results in a higher actual cell settling velocity. As a result, this inclined gravity can handle perfusion rates somewhat higher than the theoretical calculation based on settling velocity.

**Table 3.3.** Average cell retention rate comparison between the two cell lines throughout the perfusion culture period

<table>
<thead>
<tr>
<th></th>
<th>Hybridoma 9E10</th>
<th>Hybridoma R73</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Average viable cell</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>retention rate (%)</td>
<td>94±4</td>
<td>88±9</td>
</tr>
<tr>
<td><strong>Average nonviable cell</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>retention rate (%)</td>
<td>72±7</td>
<td>75±9</td>
</tr>
</tbody>
</table>

As with the 9E10 cell line, when the perfusion rate was doubled in one step from 0.8 day\(^{-1}\) to 1.6 day\(^{-1}\) the viable cell concentration did not increase accordingly. But when the perfusion rate was increased in seven steps from 0.8 day\(^{-1}\) to 1.6 day\(^{-1}\), the viable cell concentration almost doubled. At the first three steps of perfusion rate increase, the cell retention rates are higher than most of those achieved during perfusion culture with 0.8 day\(^{-1}\) perfusion rate. As the perfusion rate gradually increases, the cell viability also increases as shown in Figure 3.4A, which most likely increases the settling velocity, as
shown in Table 3.2. The combination of these two effects results in lower equivalent bleed rate and thus higher viable cell concentration. This result suggests that a gradual increase in perfusion rate is more effective in achieving high cell density than one step increase. Both the antibody titer and the antibody volumetric productivity were significantly improved, 7-fold (Figure 3.6C) and 40-fold (Figure 3.7), respectively, during the steady state of culture period with 0.8 day\(^{-1}\) perfusion rate (first phase) compared to the batch culture.

As shown in Figure 3.4C, the antibody concentration is roughly proportional to the viable cell concentration during batch culture and perfusion culture with 0.8 day\(^{-1}\) perfusion rate (first phase), similar to the results with the 9E10 cells. However, in contrast with the 9E10 cells, both the R73 antibody concentration and specific antibody
production rate decreased sharply right after the perfusion rate doubled from 0.8 day\(^{-1}\) to 1.6 day\(^{-1}\). Furthermore, the specific antibody production rate did not recover even after the perfusion rate was changed from 1.6 day\(^{-1}\) to 0.8 day\(^{-1}\). There are several possible explanations for this loss in productivity. Ozturk et al. reported the antibody productivity significantly decreased for a hybridoma cell line cultured in 1.25\% serum media compared to that cultured in 20\% serum media. It took four weeks of culture in 20\% serum media to recover the antibody productivity.\(^{65}\) In our experiments, the higher perfusion rate may have diluted some factors associated with antibody production which could not be accumulated quickly enough during the short periods of low perfusion rate. Secondly, it has been shown that an increase in perfusion rate, and thus the bleed rate, increases the accumulation rate of a nonproducing subpopulation of cells in the culture.\(^{66}\) In fact, it has been suggested that the ideal perfusion rate should be around 1 day\(^{-1}\).\(^{67,68}\) Higher perfusion rates more than 1 day\(^{-1}\) were used here to investigate the capacity of the settler with a volume-limited bioreactor.

According to Equation 3.2, a downward-flow settler at 55\(^{\circ}\) can process 64 - 94\% more cell suspension compared to an upward-flow settler with the same dimensions at 30 - 25\(^{\circ}\), respectively. With co-current movement of the settled cells and the supernatant, there is insignificant cell accumulation in the gravity settler, and thus there is no need for methods to facilitate removal of the accumulated cells. This results in a much simplified operation. The adjustable inlet position permits operation of the device over a wide range of perfusion rates and with cell lines with significant variation in settling time. A
minimized settler working volume is advantageous for reducing the time of exposure of the cells to the unfavorable environment of the settler.

### 3.3.3 Residence Time Comparison

In a traditional upward-flow gravity settler, the hindrance of hydraulic force and friction force from the settler surface (Figure 2.1 A) makes the settled cells prone to stall on the lower surface. The average residence time of settled cells is 60% longer than that of the fluid, and over 10% of the cells were shown to have stalled in the settler even with the assistance of fluid pre-chilling and vibration.²⁸

In the downward-flow gravity settler presented here, settled cells move in the same direction as the cell supernatant stream. Therefore the movement of settled cells are facilitated rather than hindered by the fluid movement (Figure 2.1B). During the steady states of the perfusion cultures, for all cell lines tested, no cell accumulation on the lower surface was observed. The settled cells formed a thin layer and moved downward along with the fluid in the settler.

At day 44.3 (Figure 3.4A) of the perfusion culture of R73 cell line, cell concentration in the bioreactor was 3.7x10⁷ cells/mL. The flows into the gravity settler and out via port 1 were temporarily shut off and the cell suspension was completely collected via port 2 after vigorous shaking of the gravity settler. The cell concentration in the gravity settler was measured at 3.9x10⁷ cells/mL, which is negligible difference from the stream
entering the gravity settler. This indicates that cell stalling in the downward-flow gravity settler was negligible and that the settled cells move at almost the same rate as the cell suspension. No pre-chilling, periodic vibration, or bubbling was needed to facilitate the cell removal. Only an occasional shake of the settler was performed once every one to two days to help remove cells retained in some dead corners.

### 3.3.4 Inoculation Test

Cell growth curves using the inoculum from the perfusion systems and T-flasks as control are shown in Figure 3.8. The maximum cell concentration is similar for the two cultures but the lag phase of the culture with inoculum from perfusion culture is longer than that of culture with inoculum from T-flask culture. This can be explained by the initially lower growth factor concentrations that occurred in the batch reactor when using the smaller inoculum volume (4 mL from perfusion, compared to 200 mL from T-flask). With a smaller inoculum volume, more time is needed to accumulate enough growth factors, leading to elongation of the lag phase. The influence of conditioned medium on lag time was demonstrated by Ozturk et al., who reported that the maximum cell concentration was reached about 24 hours earlier for culture with addition of conditioned media to 1.25% serum media compared to the unconditioned media with same serum concentration\(^6\). They also showed that the maximum viable cell concentrations reached in the two cultures were similar.
The duration of the stationary phase of the culture with the perfusion inoculum is shorter than that of the culture with the T-flask inoculum. This will not be a problem for semi-continuous or continuous production culture since medium replacement begins before cell viability drops below 90%.

The cell seed from the T-flask was obtained when the viable cell concentrations reached about $1 \times 10^6$ cells/mL, resulting in a 1:5 expansion, as is commonly used. However, the viable cell concentration in bioreactor batch culture can reach as high as $3.5 \times 10^6$ cells/mL as shown in Figure 3.8, suggesting that the expansion ratio with inoculum from a batch culture reactor can be increased up to 1:18. This improvement in maximum viable cell concentration, compared to values traditionally obtained, can be attributed to

Figure 3.8 Cell growth curve for batch culture with inoculum both from T-flasks and perfusion culture bioreactor
the optimized medium formulations and improvements in cell lines. A potential 1:18 expansion ratio from batch culture is still significantly surpassed by the 1:250 ratio achieved with inoculum from the perfusion culture bioreactor, while the latter has potential to be further improved with additional optimization studies.

As shown in Figure 3.9 the maximum antibody concentration in the batch culture with the perfusion inoculum (18.7 mg/mL at 144 hour) was similar to that obtained with the T-flask inoculum (17.8 mg/mL at 117 hour). It has been shown that the antibody production linearly relates to the integral of viable cell concentration regardless of initial inoculum size and serum concentration. So it is not surprising to see comparable antibody production based on the similar maximum viable cell concentration as shown in Figure 3.8.

![Figure 3.9 Antibody production comparison between batch cultures with inoculum both from T-flasks and perfusion culture bioreactor](image)
The time difference for reaching the maximum antibody concentration (27 hours) is similar to the time difference (29 hours) of reaching maximum cell concentration between these two cultures. When batch culture is used for production, the cell suspension should be collected around the time when maximum antibody concentration is reached. We can define a collection window as shown in the Figure 3.9, in which the antibody concentration is above 16 mg/mL. The length of the collection window is about 20 hours with the T-flask inoculum and about 10 hours with the perfusion inoculum. This difference in the collection window should not be a problem with frequent sampling near the end of the culture.

Although the specific antibody production rate decreased significantly for cells grown in the perfusion culture with 1.6 day$^{-1}$ perfusion rate compared to those in the batch culture as shown in Figure 3.4D, apparently the specific antibody production rate fully recovered when the cells from the perfusion culture were inoculated into the batch culture. This is in good agreement with the finding of Morrill, $^{71}$ who demonstrated that the decrease of antibody productivity in high density culture can be reversed when they are transferred into low cell density culture.
CHAPTER IV
A SIMPLE APPARATUS FOR MEASURING CELL SETTLING VELOCITY

4.1 Introduction

Gravity settlers have been successfully applied as cell retention devices in perfusion cell cultures from the bench-top to large-scale industrial applications.\textsuperscript{23, 25, 26, 39, 40, 72-76} The capacity of an inclined gravity settler to clarify cell suspension is described in equation:

\[ S(v) = v \cdot w(L \sin \theta + b \cos \theta) \] \hspace{1cm} (4.1)

where \( S(v) \) is the volumetric flow rate of fluid clarified of particles with sedimentation velocity \( v \); \( w(L \sin \theta + b \cos \theta) \) is the projection area of an inclined gravity settler; \( w \) is the settler width, \( b \) is the separation between the two inclined surfaces, \( L \) is the length of the settler, and \( \theta \) is the angle of inclination of the settler from the vertical. Batt et al. and
Davis et al. have successfully predicted the cell retention efficiency of gravity settlers based on theoretically calculated cell settling velocities. The accurate determination of the viable cell sedimentation velocity is critical for controlling the operation of the gravity settler to maximize viable cell recycling and thus viable cell concentration in the bioreactor. During long-term perfusion culture, the cell suspension is a mixture of viable and nonviable cells, and the nonviable cells have settling velocities that are less than that of the viable cells.

Viable cell settling velocity can vary significantly among mammalian cell lines; for instance, the settling velocity of hybridoma cell line AB2-143.2 and CHO cell line M1-59 are 2.9 cm/h and 1.45 cm/h, respectively. This two-fold difference demonstrates the necessity of measuring this parameter for every new cell line to be used in a gravity settler/perfusion system in order to properly select the gravity settler with appropriate capacity.

Moreover, the settling velocity of viable cells may change substantially during the course of a long-term perfusion culture due to changes in cell size. It is thus important to measure the distinct settling velocity of the viable and nonviable cell populations periodically during a long-term perfusion culture in order to optimize the operation of the gravity settler in real-time.
The measurement of erythrocyte sedimentation rate (ESR) has been widely used for over 50 years as a simple, standardized medical screening test.\textsuperscript{79-84} Although many modifications have been made to speed-up the procedure,\textsuperscript{85-87} the basic operational principle is the same. A sample of blood is placed in a narrow tube (Westergren Tube) and after a period of time a visible interface forms between the clarified plasma and the red blood cells. By reading the scale at the interface after a defined period of time the sedimentation can be determined. This method assumes the red blood cells have uniform size and settling velocity; therefore the movement of the red blood cell population is taken as the distance that the cells at the top of the tube can move in certain time. This method is not directly applicable to mammalian cell culture, since there is not a clear color difference between the cells and the clarified supernatant. For the same reason, the method used to determine plant cell settling velocity is not practical for animal cell culture.\textsuperscript{88} Even if there is a clearly identifiable interface, only the settling velocity of the smallest nonviable cells can be determined in this manner. This measurement is much less important than that of the viable cells for optimizing the gravity settler operation.

Particle image velocimetry (PIV) has been used primarily for directly measuring the settling velocity of individual particles.\textsuperscript{89} Despite the complexity of this process, it cannot distinguish between viable and nonviable cell settling velocity. Another method, the “Owen Tube”, is a 1-L column used for determining the settling velocity of suspended particulate matter in natural body water.\textsuperscript{90-92} Periodic samples are removed from the bottom of the Owen Tube and the dry weight measurement is used to determine the settling velocity. This method is not accurate for small sample amounts, the presence
of cell debris would contribute to measurement error, and the process can not distinguish the viability of the cells.

Stokes’ Law can be used to theoretically calculate the settling velocity of particles in fluid when the Reynold’s number is less than 0.2, given by:

\[
v = \frac{gd_p^2(\rho_p - \rho)}{18\mu}
\]

where \(d_p\) = particle diameter; \(\mu\)=fluid viscosity; \(\rho_p\)= density of solid particle; \(\rho\)= density of carrying fluid; \(g\) is acceleration due to gravity. The particle diameter is normally determined by means of a Particle Size Analyzer (Particle Data Inc.) or Coulter Multisizer (Beckman Coulter, Fullerton, CA). The particle density is measured using neutral buoyancy measurement or density gradient partitioning methods. A glass capillary viscometer can be used to determine the fluid viscosity. The fluid density is easily determined from weight and volume measurements. Using this procedure, the settling velocity of viable and nonviable hybridoma and CHO cells have been determined.\(^{23,74}\) This method is not practical for routine measurements during long-term perfusion culture since multiple measurements are needed for a single settling velocity determination, which is time-consuming and increases the potential for measurement error.

We have developed a simple, inexpensive, and rapid method for measuring settling velocity of both viable and nonviable cells in a mixed population, based on a modification of the Westergren Tube. The accuracy of this method is demonstrated using
polystyrene particles with known physical properties. The method is then used to measure the settling velocity of three different hybridoma cell lines.

4.2 Materials and Methods

4.2.1 Settling Column

A schematic of the settling device is shown in Figure 4.1. The device consists of a rectangular settling column made of 2.4 mm glass plate. The column has an internal width of 1.4 cm an internal length of 2.0 cm, and a height of 11.5 cm. There is a 0.6 mm wide slot in the narrow side of the column, at a distance of 4 cm from the bottom. At the same height as the slot is a 0.6 mm wide and 0.5 mm deep groove on the other three sides of glasses. The groove is filled with silicone glue (General Electric). The edge of the plate glass at the slot is also coated with the silicone glue. A shutter is made of 0.5 mm thick and 4.5 cm long stainless steel plate which is slightly wider than the width of the slot. The groove in the glass works as a track to guide the shutter through the slot. The function of the cured silicone glue is to help seal the contact between the shutter and glass surface. When conducting the settling velocity measurement, high vacuum grease was also applied to the interface between the glass plate and the shutter to help seal the contacts. When the shutter is pushed into the column, the lower part of the column can be totally closed. The settling column is exactly perpendicular to the supporting 7 cm x 7 cm glass plate, to which it is glued. The device should be located on a leveled horizontal surface so the settling column is strictly vertical.
Figure 4.1 Schematic of the settling column. (A) Side view. (B) 3-D view.

### 4.2.2 Standard Particles

Monodisperse standard polystyrene particles (Sigma, St. Louis, MO) with $15 \pm 0.2 \ \mu$m diameter (mean ± standard deviation) and $1.05 \ \text{g/cm}^3$ density (both values reported by the manufacturer) were used to verify the reliability of the device. The particles were suspended in DI water supplemented with 0.1% Triton X-100 (Sigma, St. Louis, MO), which helps prevent the particles from aggregating. The viscosity of the solution (without the particles) at $28 \ ^\circ\text{C}$ is 0.0084 poise, as measured using a size 25 glass capillary viscometer (Cannon Instrument Co. State College, PA). The density of the fluid is $0.996 \ \text{g/cm}^3$. The concentration of the particle suspension is $1.8 \times 10^5 \ \text{particles/mL}$, resulting in $0.03\%$ volume fraction. All the particle settling velocity measurements were conducted in a $28\ ^\circ\text{C}$ incubator.
4.2.3 Cell Lines and Cell Culture

Three hybridoma cell lines, HB-159 (ATCC), 9E10 (ATCC) and R73 (Cleveland Clinic Foundation, Cleveland, OH), were tested with the settling column. All cells were cultured with BD Cell™ Mab serum free medium (BD Diagnostic Systems, Sparks, MD) and maintained in 250mL T-flasks in a 37°C incubator with 5% carbon dioxide. Cell settling velocity measurements were conducted with cells in the exponential growth phase, in the second day after inoculation; and with cells in death phase, in the fifth day after inoculation. The nonviable cell settling velocity was measured only when the population viability was lower than 70% in order to obtain enough nonviable cells to be counted accurately using a hemacytometer. The cell suspension in the T-flask was pipeted several times to disassociate any cell clusters. The cell suspension was not diluted before being introduced into the column, at concentrations of 1 – 2 x10^6 cells per mL. All cell settling velocity measurements were conducted in the 37 °C incubator. Cell size distribution measurements of HB-159 were conducted with cells cultured 2, 6, 9, 13 and 17 days after inoculation.

4.2.4 Particles and Cell Counting

The polystyrene particles were counted using a Z2 Coulter Counter (Beckman Coulter, Fullerton, CA) equipped with a 70 um ampoule aperture tube. The lower threshold was set at 14 um and higher threshold was 16 um. The Coulter Counter was also used to determine the cell size distribution of the HB-159 cells. The cell concentration and
viability were measured using a hemacytometer and trypan blue exclusion method. More than 1000 viable cells were counted for each sample.

4.2.5 Settling velocity Measurement Procedure and Analysis

A sample containing 28 mL of the well-mixed monodisperse particle suspension is added to the settling column (Figure 4.2A) with the shutter open. Assuming uniform particle density and diameter, the particles settle down at the same rate, traveling a distance $h'$ over the settling time period $t$ (Figure 4.2B). The particle settling velocity is given by:

$$v = \frac{h'}{t}$$  \hspace{1cm} (4.3)

The volume of the column vacated by the particles is given by:

![Figure 4.2 Protocol of particle settling velocity measurement using the cell settling column. A. Fill the column with well mixed monodisperse particle suspension with known concentration. B. Particles moved downwardly $h'$ distance in one hour. C. Separate the column in two parts by pushing in the shutter to stop particles from moving into the lower part of the column. D. Remove the particle suspension above the shutter. E. Re-suspend the settled particles from the bottom of the column and well mix, then sample and measure particle concentration.](image)
\[ V' = h' \cdot s \]

where \( s \) is the cross section area of the settling column. Similarly, the volume below the shutter, \( V \), and the volume between the shutter and the interface, \( V'' \), are given by:

\[ V = h \cdot s \]

\[ V'' = h'' \cdot s \]

At \( time=t \), the shutter is closed (Figure 4.2C). The particle suspension above the shutter is first removed (Figure 4.2D). The settled particles on the bottom of the column are then resuspended and thoroughly mixed with the remaining particle suspension below the shutter. Mass is conserved between \( time=0 \) and \( time = t \), yielding:

\[ X_1(V' + V'' + V) = X_1V'\prime + X_2V \]

Where \( X_i \) is the particle concentration in the initial suspension added to the column; \( V \) is the volume of the column below the shutter; and \( X_2 \) is the particle concentration after re-suspending and mixing the settled particles in the space below the shutter (Figure 4.2E).

Combining Equations 3, 4, 5(a), and 6 yields:

\[ v = \frac{h(X_2 - X_1)}{tX_1} \]
The distance $h$ is fixed by the device design and $X_l$ is known from the sample preparation. The experiment is conducted for a known amount of time $t$, such that the particle interface remains above the shutter position during the time $t$. Therefore the only measurement needed is that of the particle concentration in the lower volume.

The same experimental and analytical procedures were used for the calculation of the settling velocities of the viable and nonviable cells. Samples were counted for both viable and nonviable cell numbers and Equation 7 applied to each population.

### 4.3 Results and Discussion

#### 4.3.1 Theoretical Calculation of the Particle Settling Velocity

The settling velocity of 15 um polystyrene particles at 28°C was calculated using Equation 1 and the data in the Methods section to be 2.81 cm/h. To confirm the applicability of Stokes’ law to this system, the Reynolds number was calculated using:

$$\text{Re} = \frac{\rho_p d_p v}{\mu} \quad 4.8$$

The calculated Re number is 0.0014, indicating that the settling velocity is indeed governed by Stokes’ law.
The corrected settling velocity can be calculated taking into account particle concentration and wall effects by means of:

\[ v_{ts} = v (1 - c)^n/(1 + 2.1\beta) \]  

where \( v_{ts} \) is the corrected settling velocity, \( v \) is the settling velocity of a single particle calculated from Stokes’ law; \( c \) is the volume fraction of the particles in the fluid; \( n \) is a function of Reynolds number, equal to 4.65 when Reynolds number is less than 0.3; and \( \beta \) is the ratio of particle diameter to vessel diameter. The hydraulic diameter is commonly used to calculate the equivalent diameter when handling flow in noncircular channels, defined as:

\[ d_h = \frac{4A}{U} \]

where \( A \) is the area of the cross-section of the rectangular channel and \( U \) is the wetted perimeter of the cross-section. The hydraulic diameter of the settling column used here is 1.47 cm.

The corrected theoretical settling velocity for the polystyrene particles, calculated using Equation 9 with concentration of \( 1.8 \times 10^5 \) particles/mL (volume fraction of 0.03%), is 2.80 cm/h. The difference between the calculated value using Stokes’ law and the corrected one is less than 0.4%, indicating that the effects of concentration and the wall
are negligible. The concentration effect on settling velocity increases sharply at concentrations greater than $1 \times 10^7$ particles/mL (Figure 4.3A). Neglecting the concentration effect at the expected cell concentration during perfusion culture, $5 \times 10^7$ cells/mL, causes a 30% error from the actual settling velocity. The large effect of cell concentration on settling velocity demonstrates the advantage of a direct measurement of this property using the settling column, rather than a theoretical calculation using Stokes’ law. As shown in Figure 4.3B, the wall effect is still negligible even at half the hydraulic diameter of the current settling column prototype. This result indicates that the minimum cell suspension volume needed for use in the settling column can be reduced to less than 10 mL with no material impact on the accuracy of the measurement.

### 4.3.2 Standard Polystyrene Particle Settling Velocity Measurement

The settling velocity of the polystyrene particles, measured using the settling column, is $2.70 \pm 0.08$ cm/h (mean ± standard deviation; Figure 4.4). This value is 3.6% smaller than the theoretical value, corrected for wall and concentration effects. This small deviation is most likely caused by particle inertial effects, which are difficult to predict.\textsuperscript{89, 94-98} It is almost impossible to totally avoid swirling or convective motion of the particle suspension. Particle inertia influences the settling velocity at both the micro-scale and macro-scale. In order to minimize the inertial effect by avoiding convection caused by a temperature difference, the settling column and particle suspension should be at the same temperature before the suspension is added to the column. Convection can also be reduced by the slow addition of the suspension to the column.
Figure 4.3 Calculation of concentration effect and wall effect based on polystyrene particles with 15 um diameter. (A) Concentration effect. (B) Wall effect.
4.3.3 Hybridoma Cell Settling Velocity Measurements

The size distributions of the HB-159 hybridoma cell line at different lengths of time in culture are shown in Figure 4.5. Two peaks are evident, at diameters of 10 µm for cultures aged 13 and 17 days and at diameters of 14.5 µm for cultures aged 2 days. The percentage of cells with the lower diameter increased with decreasing viability while the percentage of cells at the higher diameter decreased with decreasing viability. The first peak at 10 µm most likely denotes nonviable cell population and the second peak at 14.5

Figure 4.4 Comparison of the settling velocities of 15 um polystyrene particles, calculated using Stokes’ law (corrected for wall and concentration effect) and the measurements described in the Methods section (left) and measured using the settling column (right). The error bar indicates the standard deviation and n is the number of repetitions. P=0.09
µm denotes the viable cell population. These results are in agreement with results reported by Searles et al. in which the mean size of nonviable cells is significantly smaller than that of the viable cells. Not only are nonviable cells smaller than viable cells, but also the nonviable cells decrease in diameter as the population viability decreases. It has also been reported that viable cell diameter increases over 20% when the cells progress from lag phase to exponential phase.

Table 4.1 shows the settling velocities of three hybridoma cell lines measured using the settling column along with settling velocities of two cell lines reported in the literature. The variation in settling velocities of the three hybridoma cell lines is significant, with a two-fold variation between the HB-159 and the R73. These measurements are similar to values reported for a hybridoma cell line and a CHO cell line, obtained using Stokes’ law. The two-fold variation in settling velocities indicates that the cell line with the lower velocity will need a gravity settler that is double in size to achieve the same cell
retention capacity as that of the faster-settling cell line. This confirms the necessity of measuring the settling velocity before selecting the gravity settler and starting the perfusion culture.

The settling velocities of the nonviable cells are 30-50% lower than the corresponding viable settling velocity (Table 4.1). This difference is the basis of preferential removal of nonviable cells using gravity settler in perfusion culture bioreactors.

Table 4.1 also shows that even for the same cell line, the viable cell settling velocity decreases significantly, up to 50%, when viability of the cell suspension decreases from...
97% to 65%. Since a lowered viability is a normal outcome during long-term perfusion culture, this result indicates that the minimum capacity of the gravity settler would need to be doubled to maintain the cell retention efficiency throughout the culture period. Otherwise, loss of viable cells would likely occur. Therefore it is necessary to measure the cell settling velocity periodically during the perfusion culture especially when the cell viability drops significantly.\textsuperscript{101}

Since each cell population has a size distribution as indicated in Figure 4.5, it is expected that each population would also have a distribution of settling velocities. The settling velocity reported here represents a number-average value for each population, since it is determined from a count of cell number. One could determine an average settling velocity for each subpopulation defined by a size criterion using a particle counter to quantify both total cell number and size distribution. However, in practical application, a population-average settling velocity is sufficient for optimizing operation of an inclined gravity settler.\textsuperscript{23, 25}

### 4.3.3.1 Analysis of the Settling Column Method for a Heterogeneous Population of Particles

An actual cell sample contains a distribution of cell sizes, as shown in Figure 4.5, and thus is expected to have a distribution of settling velocities. The interpretation of the settling velocity, as measured with the method described above, for a non-uniform population of particles, is presented here.

Suppose the particle mixture has distribution of diameters, where each diameter is represented by $d_i$, with $i=1, \ldots, n$ where $n$ is the number of different discrete particle sizes. The sample concentration, $X_o$, can be represented by:

$$X_o = \sum_{i=1}^{n} x_{i,o}$$  \hspace{1cm} 4.11
where \( x_{i,o} \) is the concentration of particles in the mixture with diameter \( d_i \). The analysis presented in equations 4.3-4.7 for a homogeneous population of particles can be written for each particle of diameter \( d_i \), i.e.:

\[
v_i = \frac{h(x_{i,f} - x_{i,o})}{x_{i,o}t}
\]

4.12

Let \( \bar{v} \) be the number-average settling velocity of the population, defined by:

\[
\bar{v} = \frac{1}{X_o} \sum_{i=1}^{n} v_i x_{i,o}
\]

4.13

Substitution of Eqn. 12 into Eqn. 4.13, and rearranging, yields:

\[
\bar{v} = \frac{h}{X_o t} \left( \sum_{i=1}^{n} x_{i,f} - \sum_{i=1}^{n} x_{i,o} \right)
\]

4.14

Substitution of Eq. 11 for the initial concentration, \( X_o \), and the equivalent expression for the final concentration, \( X_f \), yields:

\[
\bar{v} = \frac{h}{X_o t} \left( X_f - X_o \right)
\]

4.15

The right hand sides of Eqns. 4.15 and 4.7 are identical; thus the measured population settling velocity, \( v \), is identical to the number-average velocity, \( \bar{v} \).
CHAPTER V

SCALEUP OF INCLINED GRAVITY SETTLER FOR RETENTION
OF ANIMAL CELLS AND ALGAE DEWATERING

5. 1 Introduction

A small-volume inclined gravity settler made of borosilicate glass for cell retention used in bench-top perfusion cell culture bioreactor was described in Chapter III. The capacity of the settler tested was 1.6L/day. This flow rate is high enough for conducting bench-scale bioreactor perfusion culture but not for large scale industrial perfusion culture, which is up to 3600 L/ day. This chapter focuses on the scale-up of the design.

Besides the cell retention application for mammalian cell perfusion culture systems, the inclined gravity settler is also tested with algae dewatering purpose. Since some algae cells have similar properties to mammalian cells. The two kind of cells both have diameters around 10um and density close to water. For perfusion cell culture systems the
inclined gravity settler is used for to remove spent media and return the retained cells back to bioreactor. For algae culture dewatering purpose, the gravity settler is used to remove water and concentrate the algae culture.

Whether the application is large-scale biopharmaceuticals for mammalian cells, or algae dewatering, several modifications should be made from the small-scale device. The first is the material of construction. Because of the brittleness of glass, the material used for constructing the small-scale settler, glass, is not practical for large-scale use. An incidental impact or pressure shift might break the glass wall of the settler and terminate the culture. It is also more difficult to manufacture using glass.

Polycarbonate is light-weight compared to glass or steel. It is tough (virtually unbreakable), glass-like transparent and autoclavable. It can be extruded into desired form like many other thermoplastics. Polycarbonate sheet can be easily machined with standard metal tooling machines and is dimensionally stable. FDA compliant grade is available, which is critical for cell culture processes producing therapeutical pharmaceuticals for human uses. It is a better material than glass for making the gravity settler provided that the cell retention efficiency is not adversely impacted.

The second modification concerns is about the geometry and outlet arrangement on the settler. The dimension of the settler will be scaled up by increasing the length and width. The thickness will be kept similar as the small volume inclined gravity settler. As shown
in Figure 5.1, the outlet of the concentrated stream will be moved to the lower end the settler.

5.2 Materials and Methods

5.2.1 Device Description

![Diagram of the 10L/day inclined gravity settler](image)

**Figure 5.1** Schematic of the 10L/day inclined gravity settler.
Two settlers were made and tested, which are made of 9.5mm thick polycarbonate plate (McMaster, Aurora, OH). The first one as shown in Figure 5.1 was built based on the design shown in Figure 3.2. The fundamental difference between them lies in the outlet design. For the previous one as shown in Figure 3.2, the outlet for cell returning to bioreactor is located on the lower plate of the settler next to the lower end of the inclined gravity settler as shown in Figure 3.2. For the new one, both outlets are located at the end of the settler and the outlet to bioreactor is underneath the outlet to harvest tank. The change was made because cells tended to accumulate at the joint area between the outlet and lower plate (Figure 3.2) due to the slow flow rate at that area. Another reason for the change is to facilitate stacking the individual settlers to a group for supporting large scale culture. The cell separation capacity is adjusted by selecting different inlets along the

![Diagram of inclined gravity settler](image)

**Figure 5.2** Side view of inclined gravity settler for algae dewatering.
longitude axis.

The second design shown in Figure 5.2 has inlets only at one position near the upper end of the settler. The inlet position is located on the side end of the settler to make it easier to stack. One more difference is that there is no separator between the two outlets like that shown in Figure 5.1 since it was found that the separator was not necessary during the test with the first prototype.

5.2.2 Cell Line and Media

Hybridoma HB159 cells were cultured in BD Cell™ Mab serum free medium (BD Diagnostic Systems, Sparks, MD). The media was supplemented with 0.1% Pluronic F68 (Sigma, St. Louis, MO) for bioreactor culture. No other components were added or adjustments made to the media during the culture process. Algae, Scenedesmus dimorphus, is used for the algae dewatering test. Both hybridoma and algae cell numbers were determined using hemocytometer.

5.2.3 Hybridoma Perfusion System and Culture Protocol

The perfusion culture system is shown in Figure 5.3. A 2 L B.Braun stirred bioreactor (B. Braun Biotech) with 2 L working volume was used. A four-gas control module was applied to maintain the DO at 50%. The set point of pH was 7.2 but it fluctuated between 6.8 and 7.2 since no base or acid was supplied during the culture.
Cells were at first cultured in T-flasks in a humidified 5% carbon dioxide incubator at 37°C. Cells in exponential growth phase were inoculated in the stirred bioreactor. The culture was started as batch culture then transitioned to perfusion culture in order to
achieve high cell concentration for the short-term recycle cell retention test. The actual perfusion flow rate was 1L/day. Viable cell density was maintained over $1 \times 10^7$ cells/mL in the bioreactor. The flow rate from the outlet port I is taken as virtual perfusion flow rate. It is called virtual perfusion flow rate because the system is not really perfused with that amount of fresh media but it can show the real capacity that the retention device can process. In order to simplify the discussion, we use the term “perfusion” to replace the “virtual perfusion”

5.2.4 Algae Perfusion System

At first the algae were in cultured in 150 mL Erlenmeyer flasks and then transferred to 2 L Pyrex bottles. Finally 5 L of algae suspension was collected and maintained in a 10 L Pyrex bottle sitting on a stirring plate for the algae dewatering test with the scaled up inclined gravity settler. The tested algae culture was maintained in room temperature and aerated with air supplemented with less than 5% CO$_2$. The agitation speed of the stirring bar was adjusted to keep all algae in suspension. The experimental set up is shown in Figure 5.4 and Figure 5.5A.

5.2.5 Calculations

The cell retention rate, $R$, is defined as:

$$R = \frac{X_a - X_o}{X_o} \times 100\%$$

5.1
where $X_R$ is the cell concentration in the bioreactor; $X_O$ is the cell concentration in the overflow stream that exits the gravity settler via port I to the harvest tank.

If the two outlets of the settler are combined, then cell concentration in the combined outlet stream will be equal to the cell concentration in the bioreactor, $X_R$, during steady state. Let the average cell concentration in the settler be $X_{a}$; then the total cell number in the settler is $X_{a} V$, where $V$ is the working volume of the inclined gravity settler. The speed at which the cells leave (and enter) the settler is $F \cdot X_R$ where $F$ is flow rate of cell suspension entering the settler. The average time needed to remove these retained cells is defined as the cell residence time in the settler, given by:

$$T = \frac{V \cdot X_{o}}{F \cdot X_{R}}$$

**Figure 5.4** Setup of algal dewatering with the gravity settler.
Figure 5.5. A. Algae dewatering setup with the inclined gravity settler. B. Settled down algae formed a thin layer sliding downwardly during steady status. C. Contrast view between concentrated stream and clarified stream at outlets.
The algae concentration ratio, $C$, is defined as:

$$C = \frac{X_u}{X_R}$$

where $X_R$ is the cell concentration in the bioreactor; $X_u$ is the algae cell concentration in the harvest stream.

### 5.3 Results and Discussion

#### 5.3.1 Hybridoma Cell Retention Test

![Graph](image)

**Figure 5.6** Cell retention at different entry point vs. perfusion rate for HB 159 Hybridoma
Table 5.1 Viable cell retention rate vs. L and perfusion amount.

<table>
<thead>
<tr>
<th></th>
<th>Inlet I</th>
<th>Inlet II</th>
<th>Inlet III</th>
</tr>
</thead>
<tbody>
<tr>
<td>L (cm)</td>
<td>16</td>
<td>33.5</td>
<td>57</td>
</tr>
<tr>
<td>Perfusion amount (L/day)</td>
<td>5.8</td>
<td>10.8</td>
<td>15.8</td>
</tr>
<tr>
<td>Viable cell retention rate (%)</td>
<td>99</td>
<td>98</td>
<td>96</td>
</tr>
</tbody>
</table>

Figure 5.6 shows the hybridoma cell retention at different perfusion amount using the settler shown in Figure 5.1. The maximum perfusion rate was achieved is 17.28 L/day with 90% viable cell retention efficiency. This is about a 10-fold improvement in capacity compared to the bench top device described in Chapter 3. This result verified that this inclined gravity settler is scalable.

![Cell Retention Capacity Graph](image)

Figure 5.7 Actual cell retention capacity and predicted cell retention capacity based on settler length (cell retention \( \geq 96\% \)).
Surprisingly, the cell retention capability (maximum perfusion account with cell retention over 95%) is not linearly proportional to the length, L, as predicted by Equation 3.2. Hereof L is the distance between the entry point and upper end of the separator. As shown in Table 5.1 and Figure 5.7, the amplitude of cell retention capacity increase is less than that of the length. It is contradicted to the prediction: cell retention capacity is linearly proportional to the length.

The observed discrepancy with the theoretical prediction is mostly likely caused by the different cell residence times in the gravity settler as shown in Figure 5.8. While the supernatant residence time is almost unchanged, the cell residence time is approximately proportional to the length. This means the sliding speed of the settled cells almost stays

![Figure 5.8 Average residence time of cells vs. supernatant](image)

**Figure 5.8** Average residence time of cells vs. supernatant
constant while the linear velocity of cell suspension or supernatant increases along with the perfusion rate. The longer residence time of settled cells can cause cell accumulation in the settler. Upper layer of cells of the settled cells will be dragged by the hydraulic force due to the difference of movement speed between settled cells and the fluid. From direct visual observation through the transparent upper surface of the gravity settler, the settled cells slide down like traveling dunes. The uneven distribution would induce turbulence in the laminar flow, and then reduce the cell retention efficiency. This result indicates that the inclined gravity settler can not be linearly scaled up by simply increasing the length, which would lower the cell retention efficiency per unit area.

Another important issue that should be taken into account is the residence time issue of viable cells. The environment in the gravity settler is not suitable for cell growth since there is no control over DO and pH. The residence time of viable cells in the settler should not be too long. It was found cell growth and antibody productivity were not impacted significantly when the residence time in the settler was less than 1.5 hours.\textsuperscript{23, 28} When the third inlet was used, the residence time of viable cells in the settler we tested was 1.2 hours as shown in Figure 5.8, which is still acceptable for supporting a perfusion culture system as a cell retention device.

Further increase of the length is not practical, which will increase viable cell residence time. Therefore the length of the downward flow inclined gravity settler should not be over 60 cm.
For retention over 95%, the maximum perfusion amount of cell suspension the settler can handle is 15.8L/day. If the perfusion rate is 1/day, then the maximum working volume of the bioreactor this settler can support is 15.8L. The working volume of the settler used here is 361 mL (b=0.66cm, w=9.6 cm and L=57) which is about 2.3% of the bioreactor working volume, 15.8 L, it can handle. The maximum thickness of the settler should not be increased freely since As shown in equations 3.1 and 3.2, the increase of thickness, $b$, of the settler is negligible to the cell retention capacity and the volume of gravity settler working volume should be less than 5% of the bioreactor working volume. Therefore the maximum thickness of the downward-flow inclined gravity settler we recommend here is 1.3 cm.

Although the length and thickness can not be increased at will, the width, $w$, can be increased with no limitation as long as enough inlets are arranged for even distribution of flow. If taking the convenience of operation into consideration, the maximum of width can be set as 150 cm, which turns out the maximum capacity a single unit of the settler is 246 L/day, while the working length is 57 cm and the thickness is 0.66 cm. For further scale up, multiple single settler as shown in Figure 5.2 can be stacked up, with which there is no limitation virtually for the maximum capacity.

The retail price of 0.5 cm thick polycarbonate sheet, which was used in building the settler, is $51/m^2$. To build a settler with 80 L/day capacity, the total size of polycarbonate sheet needed is 0.6 m$^2$ with cost $31. The material cost will be $40 by
adding 30% cost for the outlet part. We estimate the manufacturing cost is $100 for large quantities. Then the cost for producing a unit with 80L/day is $140.

5.3.2 Algae Dewatering Tests

Results with algae are shown in Figure 5.5 and 5.9. As shown in Figure 5.5 C using the settler shown in Figure 5.2, it is visually apparent that the algae concentration in harvest stream is much higher than that in the clarified steam. Figure 5.5 B shows steady status of the algae dewatering process. Settled algae formed a thin film and move downwardly along the inclined settler smoothly. The process appeared to operate smoothly over a
period of 3 weeks at which point it was terminated due to a leak in the settler. The quantitative results are reported in Figure 5.9. 4-fold increases in concentration was achieved when the flow rate is 12 + 2 mL/min which denotes that the clarified stream flow rate was 12mL/min and the harvest stream flow rate was 2 mL/min. This implies, for achieving a given amount of dry algae mass, the volume is about 25% concentrated algae suspension needed to be processed compared to regular unprocessed algae suspension. When the total flow rate increased from 14ml/min to 18+2 mL/min (clarified stream flow rate was 18mL/min and the harvest stream flow rate was 2 mL/min), the concentration ratio decreased to 2.7-fold. To determine which flow rate will be used for practical application it depends on the algae dry mass recovery rate as shown in Figure 5.10, in which we assume the algae concentration is 1x10^8 algae/mL concentration in the algae suspension to be processed, which is transferred to 2 g/L dry mass (data from lab

![Figure 5.10](image.png)

**Figure 5.10** Recovery of dry algae mass per square meter of the inclined gravity settler from the harvest stream. Error bar shows standard deviation.
communication). The recovery rates are almost same for two flow rates. So increasing the total flow rate from 14 to 20 mL/min did not increase the amount of algae recovery. At same level of recovery rate, higher flow rate means higher energy consumption in pumping system. More tests need to be conducted to determine the optimal flow rate for maximum algae recovery rate.

When the flow rate is 12+2 mL/min, the expected recovery rate of algae mass is 424 g dry wt/day/m². If we assume the dry algae oil content is 50%, the area of inclined gravity settler needed to collect enough algae for producing 1 ton oil per day is 4761 m² which is 69m x 69m area. It is more than 1 acre. To reduce the land usage, a multiple layer of design should be considered. Meanwhile the manufacturing cost can be reduced significantly. For any middle plate in a multi-plate settler will function as upper piece and lower piece, which is almost 50% building material saving and the materials for the middle pieces can be much thinner than that of the one in a single layer settler, which acts as frame as well to support its own weight.
CHAPTER VI

CELL RETENTION WITH ACOUSTIC FILTERS

6.1 Introduction

In Chapter III, IV and V, the first approach of cell retention using an inclined gravity settler was presented. In this chapter, two modifications of currently available ultrasonic filters for performance improvement will be described.

The range of ultrasound frequency for applications in cell retention is 0.5--3 MHz. Particles mainly undergo two forces in the ultrasonic standing field, “primary acoustic force” and “secondary acoustic force”. When particles in suspension are exposed to a resonant acoustic wave field, the particles experience a time-averaged force known as the primary acoustic force. It moves the particles to the pressure nodal planes or antinodal planes determined by physical properties of the particle and carrying fluid. This force is related to compressibility and density of the particles and the carrying fluid and can be calculated using the following equation.103, 104
\[ F_{ac} = 4\pi R^3 \kappa E_{ac} \sin(2\kappa x) F \]

where \( \kappa \) is the wave number across the acoustic filter, \( E_{ac} \) is energy density within the suspension, \( x \) is the distance of the cell from the nearest pressure nodal plane, \( R \) is the particle radius, and \( F \) is the acoustic contrast factor. This factor \( F \) is characteristic of the suspension and is given by: \(^{103,104}\)

\[
F = \frac{1}{3} \left( \frac{5\rho_p - 2\rho_f}{\rho_f + 2\rho_p} \frac{\gamma_f}{\gamma_p} \right)
\]

Where \( \rho_p \) is the density of the particle, \( \rho_f \) is the density of carrying fluid, \( \gamma_f \) is the compressibility of carrying fluid, \( \gamma_p \) is the compressibility of the particle. Movement of particles to either the pressure nodal planes or antinodal plane is determined by the sign of \( F \).

Particles also experience forces due to local scattering of the acoustic field by nearby particles, known as the secondary acoustic force. This force between particles exists because of the reflection of the acoustic field by one particle to the other particles and is given by:

\[
F_s = \rho_f c_f^2 f^2 E_{ac} \gamma_f^2 (1 - \frac{\gamma_{p1}}{\gamma_f})(1 - \frac{\gamma_{p2}}{\gamma_f})(\frac{VV_s}{d^2})
\]

Where \( c_f \) is the speed of sound in the fluid, \( f \) is the frequency of the applied ultrasound, \( V \) is the particle volume, \( d \) is distance between two particles. This equation is valid if both particles are less compressible or both particles are more compressible than the carrying
fluid.\textsuperscript{18} Obviously it is valid for suspensions of particles made of the same kind of material.

This force causes the nearby particles to aggregate to form clusters and drive the concentrated cells to the local minima of pressure amplitude within the pressure nodal planes.\textsuperscript{105} The particle cluster can either stay in the ultrasonic field, or drop out of the field when the gravity force is equal to or bigger than the hydraulic drag force. Various types of acoustic filters using this approach have been used to retain cells in microorganism fermentation or animal cell culture.\textsuperscript{16, 17, 19-21, 106-110} Commercially available acoustic filters utilizing standing ultrasonic waves to retain mammalian cells have been successfully used in large scale perfusion culture with capacity up to 1000 L/day.\textsuperscript{108, 109, 111}

A porous media placed within the acoustic filter has been shown to increase the particle retention efficiency.\textsuperscript{18, 112, 113} The interaction of the incident plane waves with waves reflected from the fibers of the porous mesh enhances the capture of particles in the acoustic filter. A single fiber model has been used to explain the mechanism of the particle retention improvement effect of porous mesh in the acoustic standing field.\textsuperscript{114-116} A single particle can be captured by a single fiber of the porous mesh due to the secondary force between the fiber and particle. When a particle cluster formed under the primary and secondary force is larger enough, it can be blocked by the limited opening spaces of the porous mesh.\textsuperscript{18}
The enhancement of mammalian cell retention by filling acoustic filter with porous mesh is studied in this chapter. In contrast to a mechanical filtration process, the porous mesh has a much larger pore size compared to cell size.

The acoustic transducers are glued directly to the glass wall or frame of the commercially available acoustic filters for mammalian cell culture.\textsuperscript{17, 105, 107, 108, 110, 117-119} Compared to this rigid attachment of transducer, Dr. Feke’s group at Case Western Reserve University, used a flexible approach to attach the transducer to the acoustic filter.\textsuperscript{18, 112-116, 120, 121} The acoustic transducer attachment methods, rigid and flexible, are also compared in this chapter. The objective is to increase the cell retention capacity per unit of power input compared to available acoustic filters via this approach.

\section*{6.2 Materials and Methods}

\subsection*{6.2.1 Cell Line and Medium}

T10B9 Hybridoma cells (ATCC) were used for the cell retention tests conducted within the acoustic filter. A serum free medium, BD Cell Mab Medium Serum Free, (BD Biosciences - Advanced Bioprocessing) supplemented with 0.1\% Pluronic F68 (Sigma, St. Louis, MO) was used for cell culture. Cells were cultured in T-flasks first, then transferred to a 2-L B.Braun stirred bioreactor and the working volume was 1.5 L.
6.2.2 Experimental Setup

Four acoustic filters were tested in this study. As shown in Figure 6.1\textsuperscript{121} and 6.2, the first design using flexible attachment method is essentially the same as that made by David Rusinko for retention of polystyrene particle, with the exceptions that the polycarbonate was used for making the frames instead of acrylic or polyethylene, and that silicon sheets replaced latex sheets.\textsuperscript{121} The 10mm thick transducer (PZT, Navy Type I, Model EC-64, EDO Electro Ceramics, New York, NY) and the glass reflector shown in top left of the photo, are attached to the central frame by two fixing frames. Resonant frequency around 1.1 MHz was used for the excitation to maintain standing wave field in the acoustic filter. The acoustic filter inner volume is 13 mL. Figure 6.3 shows the experiment setup using

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Figure6.1.png}
\caption{Schematic of the first acoustic filter tested for cell retention. (Modified form of model by David Rusinko).}
\end{figure}

this device called “single chamber acoustic filter”.

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The second acoustic filter tested as shown in Figure 6.4 is made of 2 mm thick borosilicate glass. The acoustic filter has two chambers (called “double chamber” device) with equal dimension, which is similar design by Dobhoff-Dier, et. al.\(^\text{16}\) Dimension of each chamber of the double chamber acoustic filter is 150 x 50 x 22 mm (height x width x thickness). The inner volume of each chamber is 152 mL. The separator is 10 micron thick polyethylene film. The acoustic impedance of polyethylene (1.79 MRayls) is close to that of water (1.48 MRayls at 20 °C) and it is thin so the sound energy loss across the
separator is minimal. Four pieces of 25 x 25 x 1 mm piezoelectric lead zirconate titanate transducers (APC 880, American Piezo Ceramics, Inc., Mackeyville, PA) with frequency around 2.1 MHz were used for the excitation to maintain resonance in the acoustic filter. The transducers were directly attached to the glass carrier. The side wall, transducer carrier and reflector were all made of 2 mm borosilicate glass. The chamber next to reflector is the active chamber. The flow distributor is made of 1 mm thick phenolic sheet.

Figure 6.3 Cell retention test with the first acoustic filter with single chamber.

Figure 6.4 Acoustic chamber coupled with water cooling chamber (Second, third and fourth filter.)
The diameter of holes on it is 1.2 mm and the distance between each hole is 3 mm.

The third acoustic filter is also a double chamber with the same design as the second acoustic filter but with smaller dimensions called the “small double chamber acoustic filter”. Dimension of each chamber of this filter is 150 x 22 x 22 mm (height x width x thickness). One piece of transducer was used with dimension 50 x 18 x 1 mm (height x width x thickness). Inner volume for each chamber is 59 mL. The separator material is 127 um thick TPX (polymethylpentene) film which is not only autoclavable and FDA compliant but also stronger than polyethylene. Like polyethylene, TPX material also has acoustic impedance (1.84 MRayls) close to water so minimal ultrasonic energy loss occurs across the TPX film.

For the second and third acoustic filters, the piezoelectric transducer was attached on a piece of 2 mm thick glass carrier using low viscosity glue, Loctite Super Bonder 420 (Loctite, Avon, OH) with viscosity of 2 cp. The low viscosity glue can minimize the thickness of the gap between the piezoelectric transducer and glass carrier, in order to minimize the power input needed for cell retention capacity.

The fourth acoustic filter has same dimension as the third acoustic filter. As mentioned in Rusinko’s work a flexible piezoelectric transducer attachment approach can reduce power loss due to transverse vibration of the transducer and the absorption due the carrier layer. The fourth acoustic filter with double chambers was constructed with the transducer flexibly attached on the cooling chamber side (“flexible double chamber”). In
this experiment the piezoelectric transducer edge was glued on a piece of 0.5 mm silicone membrane. All other features are identical to the third acoustic filter. The reason to build the third and forth acoustic filter is that it is easier to keep the suspended transducer parallel to the reflector plane in a device with smaller dimensions compared to the original large double-chamber device. Experiments with same parameters were conducted with the third and forth acoustic filters for evaluating the effect of the rigid vs flexible transducer attachment method.

Figure 6.5 shows experimental setup for tests conducted with the second, third and forth acoustic filters. Temperature of water bath was 35°C and the cooling water was degassed.

Figure 6.5 Experiment setup of cell retention test with double chamber acoustic filters.
Temperature in bioreactor was maintained at 37 °C. The flow rate of cooling water is double that through the active chamber. Cell density in the bioreactor is 1-1.2 million cells/mL with over 90% viability.

The transducers were driven by an ENI 240L RF Power Amplifier (ENI Co., Rochester, NY) with signal input from a HP 33120A signal generator (HP, Loveland, CO). The voltage and current crossing the transducer were measured by a TDS 420 oscilloscope (Tektronix, Beaverton, OR). The resonance status was evaluated by the power factor, the cosine value of phase angle between voltage and current. For all experiments conducted, the power factor was over 90% on average. The power input is calculated from the following equation:

\[ P = VI \cos \theta \]  

Where \( P \) is the power input, \( V \) is the voltage and \( I \) is the electric current. \( \theta \) is the phase angle between voltage and current and \( \cos \theta \) is the power factor. The power input is the average value over the time period of the measurement.

The porous mesh used to fill the acoustic filters is reticulated polyester with pore size 1250 microns and the porous mesh was made of polyurethane (Foamex International Inc., Media, PA). The wires conducting power to transducer were attached to the transducer with rosin core solder (Alpha Metals Inc, Jersey City, NJ). The melting point of the solder is 190.6 °C. The transducer’s Curie Point is 310 °C. The maximum operating temperature for the transducer is Curie point/2, which is 165 °C.
6.2.3 Analytical Methods

Cell density and viability were determined using hemocytometer count and trypan blue exclusion method. Samples from the collection flasks were gathered during the quasi-steady period assumed to be reached after four residence times after the changes in operating condition. The cell retention rate, $R$, is defined as:

$$ R = \frac{X_R - X_O}{X_R} \times 100 \% $$

where $X_R$ is the viable cell density in the bioreactor; $X_O$ is the viable cell density in the effluent from the acoustic filter at the point of sampling.

6.3 Results and Discussion

Figure 6.6 shows the cell retention results using the single chamber filter. Significant improvement of cell retention capacity for the acoustic filter filled with porous mesh compared to the empty one was noticed at the highest tested linear flow rate of cell suspension, 1.6 mm/s. The cell retention rate of chamber filled with porous mesh is about 3-fold that of the one without mesh. The effect of mesh is not significant when flow rate is at the lowest tested, 0.25mm/s. This is because the power input is high enough at the low flow range to trap the cells at the nodal planes. With higher flow rate, the difference becomes more apparent since the power input is not enough to hold most the cells at the nodal planes in the absence of the porous mesh. At these two flow rates (0.75mm/s and 1.6mm/s), the higher retention rates confirmed the retention enhancement capacity of the
In Doblhoff-Dier et al.’s tests with a double chamber acoustic filter, with power input density of 0.4 W/mL, nearly 100% of cell retention was achieved at 1 mm/s flow rate. The power input density with our single-chamber acoustic filter the power input density is much higher, about 1.1 W/mL but the cell retention rate was lower than 90% at 0.25 mm/s flow rate. For higher power input and lower flow rate with the single chamber acoustic filter, lower cell retention efficiency was achieved compared to the double chamber acoustic filter. The major barrier to achieve high retention efficiency with our single-chamber acoustic filter probably is natural convective flow. Filling the acoustic filter with porous mesh could not offset this effect even at the lowest flow rates tested.

Figure 6.6 Cell retention for the two designs at three flow rates. The power input for the chamber with mesh is 1.12 W/mL, and 1.15 W/mL in the absence of mesh (the first chamber).

fine particles, like mammalian cells, with acoustically energized porous mesh fibers.18,112,114-116
The convective flow is caused by temperature gradient induced by heat generated from the acoustic transducer, which disrupts the lineup of retained cells.\textsuperscript{118,122}

Figure 6.7 shows the cell retention results with the second acoustic filter (large double chamber). Each curve denotes the cell retention under a range of power inputs with a given flow rate. As expected, retention increased with power input for each linear velocity. With the aid of the cooling chamber this device achieved a 95% cell retention, at 1 mm/s flow rate with 0.3 W/mL, without porous mesh, which is in the same range as the result reported by Doblhoff-Dier et al., which was 95% retention with 0.28 W/mL.\textsuperscript{16} For perfusion cultures, more than 95% of viable cell retention is desired, so we select the first point above 95% retention on the curves as the points to compare power.
consumption but the two points to be compared must be as equal as possible. Compared to the results achieved with acoustic filter without porous mesh filled, the cell retention achieved in acoustic filter with porous mesh filled, for reaching the similar level of cell retention, power input was reduced 36%, 39% and 14% respectively for flow rates 0.5mm/s, 0.75mm/s and 1mm/s respectively. These results confirm that for reaching same level of cell retention efficiency less power input can be reduced by filling the acoustic filter with porous mesh. Likewise at the same level of power input and linear velocity, the presence of the porous mesh increased cell retention.

Visual observation indicates that the fibers of the porous mesh can help stabilize the clustered cells in the acoustic filter by two mechanisms. First, the porous mesh acts like a matrix to which the cells loosely attach in the ultrasonic standing wave field. Second, although the pore size of the porous mesh is about two orders of the magnitude larger than that of the single cell, the size of some cell flocs formed in the resonating sound field is bigger than the mesh pore size, and thus the flocs may be trapped in the mesh.

Figure 6.8 shows cell retention for the fourth filter. For the same retention efficiency (95%), the fourth filter with the suspended transducer used 46% less energy than the third filter with the rigidly attached transducer. Since there is no direct rigid contact between the piezoelectric transducer edges and the acoustic filter frame, the transverse mode vibration of the piezoelectric transducer is minimized. And the energy loss due to attenuation and reflection caused by the glass carrier layer is also eliminated.
When the voltage across the transducer was increased from 600mVpp (corresponding to 0.38W/mL for the fourth acoustic filter with rigid transducer attachment) to 650mVpp, the solder for fixing the conducting wire to the transducer surface started to melt. This means the transducer is depolarized since the ½ Curie Point (155 °C, above which the transducer depolarizes) of the transducer is lower than the solder melting point (190.5 °C). This implies the power input cannot be increased freely otherwise the transducer is under risk of damage. A portion of the power input through the transducer was transferred into heating within the transducer. It is not only an issue of energy waste but also seriously limited the operating range of the acoustic filter. Here the operating range is defined as the minimum power input needed to achieve 95% cell retention to the maximum power input can be tolerated by the transducer.

**Figure 6.8** Cell retention test for no porous mesh filled acoustic filters with different transducer affixing modes. Flow rate of cell suspension was 0.5mm/s (Fourth filter).
The solder melting point is 35.5 °C higher than the \( \frac{1}{2} \) Curie Point so before the solder on the transducer melted the temperature at the point might have already surpassed the maximum allowed operating temperature of the transducer. Suppose the highest power input points we tested are the highest power input can be reached without damaging the transducer. Then the operating range of power input for the flexible transducer mode is 1-340% (point A to D in Figure 6.8), while the power operating range of acoustic filter with transducer rigidly affixed is 1-120% (point B to C in Figure 6.8). It is almost three times than the latter. When using the flexibly attached transducer compared to the one with rigid attachment, the maximum power input was increased at least 50% (Point C to D in Figure 6.8) before depolarization occurred.
CHAPTER VII
IMPACT OF EXPOSURE OF ULTRASONIC STANDING WAVES ON CELL
GROWTH AND ANTIBODY PRODUCTION

7.1 Introduction

In chapter VI, cell retention in acoustic filters was shown to be enhanced in the presence of porous mesh. It was found that there is no detectable negative impact of ultrasonic field on cell viability and antibody productivity in short-term exposure time, i.e. less than an hour.\textsuperscript{19, 123, 16} Ultrasonic filters have been successfully applied in perfusion culture systems as cell retention devices.\textsuperscript{20, 21, 106-108, 118, 119} When used as a cell retention device, cell residence time within the ultrasonic filter chamber is less than an hour.

The supposed cell retention device can be eliminated in a perfusion system if the ultrasound is applied to the bioreactor to retain cells within the bioreactor. The impact of ultrasonic standing waving on cell growth and productivity during long-term exposure was investigated.
7.2 Materials and Methods

7.2.1 Cell line and Medium

The cell line used is a hybridoma (mouse, ATCC HB-159), which produces immunoglobulin against H-2 Kd. The cell culture medium is RPMI 1640 supplemented either with fetal bovine serum or without serum (serum free medium). Cells were maintained in 250 mL T-flasks in 5% carbon dioxide incubator.

7.2.2 Analytical methods

Viable and non-viable cell concentrations are determined using a hemocytometer with trypan blue exclusion method. A mouse IgG ELISA kit (Alpha Diagnostic International, San Antonio, TX) was used to measure the antibody concentration.

7.2.3 Experimental Setup

The same acoustic filter shown in Figure 6.1 and 6.2 was used. The experimental setup is same as shown in Figure 6.3 was used for short term impact test. Cell suspension was kept in a stirred bottle and pumped through the acoustic filter. There was no control of
temperature, DO and pH in the test. As shown in Figure 7.1, a 1 L B.Braun stirred bioreactor (B. Braun biotech, Allentown, PA) with 1 L working volume coupled with the acoustic filter was used to test the long term impact of ultrasonic standing waves on cell growth and productivity. Using the bioreactor system the recycled media can be maintained with stable pH, Temperature and DO. The left side of Figure 7.1 shows the ultrasonic signal generating system. The ultrasonic signal is generated by the signal generator and is amplified by the power amplifier. The amplified high frequency signal drives the PZT transducer to produce ultrasonic waves. A power meter is used to check whether the resonant waves are occurring inside the chamber. On the right side of the figure is a traditional bioreactor system, which was used to control DO, temperature, and pH for the
cell suspension that flows through the ultrasonic filter. The cells were first inoculated in the traditional bioreactor, and after the lag phase, the cell suspension was pumped through the acoustic chamber. The cells were then retained by the ultrasonic field in the chamber.

7.3 Results and Discussion

7.3.1 Effect of short-term exposure to ultrasonic fields in controlled environment

Here “short-term” means time range less than a day, in contrast to several days for regular cell culture. Figure 7.2 shows the results of experiment done by Paul Grabenstetter, who did preliminary research work on cell retention using the acoustic filter filled with porous mesh.

From Figure 7.2 we can find that, after a short period of exposure to the ultrasonic fields (5 hours), the impact of exposure to the ultrasonic standing waves on cell viability is negligible. This result provided the basis for proposing a long-term “ultrasonic bioreactor”.

Figure 7.3 shows the results of another set of short-term exposure experiments. The flow rate was 30 ml/min and the acoustic filter contained the mesh. In this short term experiment Vsg was set to 150 mVpp at which level cells can be effectively retained in the ultrasonic filter.
The first two bars show cells cultured in serum-free medium. 4-hour exposure to ultrasonic field significantly reduced cell viability, by 70%. Meanwhile the viability of the control experiment with voltage applied did not decrease at all after 24 hours. This implies that the ultrasonic field at the power density needed to retain cells could stress the cells and cause cell death.

For the second group of bars, the cells were cultured in a 10% serum medium. After 4 hours exposure to ultrasonic field, the cell viability was not influenced. This is consistent with result presented in Figure 7.2. It is apparent that the serum in the medium provides some protection to the cells exposed to the ultrasonic standing waves. However, after 16 hours, the cell viability decreased more than 80%. This means the serum can only delay but not eliminate all the negative impact of ultrasonic field on cells.

![Figure 7.2](image)

**Figure 7.2** Effects of exposure of 5 hours to ultrasonic fields on viable cell grown in PRMI 1640 media supplemented with 10% serum when the acoustic transducer is powered with three variation of voltage.
Both experiments about short time exposure to ultrasonic field were conducted without temperature, DO and pH control. It is possible that the uncontrolled culture conditions contributed to the quick cell death. In order to identify the impact of these factors’ potential negative influences on cell growth, experiments for investigating the influence of temperature, DO and pH control were conducted.

### 7.3.2 Long term exposure to the ultrasonic field in controlled environment

In this set of experiments, the ultrasonic filter was combined with a 1 L B.Braun bioreactor with which DO, pH, and temperature was well maintained around the set point. Although the DO and pH number in the ultrasonic filter were not able to be controlled, they were indirectly maintained by circulating the culture medium from the bioreactor.
where the two parameters were well controlled. In this way, we can figure out if the two parameters at uncontrolled situation are the cell death cause when the cells are exposed to ultrasonic field.

Figure 7.4 (a) shows the cell growth curve for control experiment. The cells were cultured with a serum-free medium in 1 L B.Braun bioreactor. 1.4 million cells/mL concentration was achieved. This result is typical for this type of system. A culture was conducted in the system coupled with the ultrasonic filter (Vsg=600mVpp).

Figure 7.4 (a) shows the cell growth curve for control experiment. The cells were cultured with a serum-free medium in 1 L B.Braun bioreactor. 1.4 million cells/mL concentration was achieved. This result is typical for this type of system. A culture was conducted in the system coupled with the ultrasonic filter (Figure 7.4(b)). At first cells were inoculated in the bioreactor. Thirty six hours after inoculation, flow of the cell suspension through the acoustic filter was initiated. Every 12 hours, one sample was taken from the traditional bioreactor with the ultrasonic field on. The cells were killed quickly within 12 hours, as shown in Figure 7.4(b). In Figure 7.4(a), at about the 76th hour during the culture, the viable cell density is above $1 \times 10^6$ cells/mL, but in Figure 12(b), at about 76 hours, the viable cell density almost was zero. All cells retained in the ultrasonic filter were flushed back to the bioreactor before sampling. It is noticed that the
sum of viable cell number and nonviable in the last sample is about equal to the sum of viable and nonviable cells in the sample right before the ultrasonic filter was powered on. This means that almost no cell growth happened. Viable cells just were killed in the process.

From this comparison and the comparison shown in Figure 7.3, we can conclude the ultrasonic standing waves at the energy levels we used in the experiments negatively affect cell growth.

**7.3.2.1 Temperature effects**

To investigate whether cell death was caused by heat generation in the filter, we measured temperatures in the system. Phosphate buffered saline (PBS) buffer was...
Figure 7.6 Viability and cell concentration after 24 hour incubation.

circulated through the ultrasonic filter shown in Figure 7.1 and the fluid temperature at various points in the chamber was recorded. PBS flow rate in through the filter is 4.7mL/min, which is the minimum flow rate used for the cell retention tests. The temperature was set to 37 °C in the traditional bioreactor. When the fluid reached the lower inlet of the ultrasonic filter, the temperature of the fluid had been cooled down to 32 °C by the room environment. As shown in Figure 7.5, the temperature at the chamber center was 33.5 °C and the temperature at the transducer surface was 34.5 °C, both during flow conditions. When the flow fluid is stopped, the temperature at the surface rises to 39 °C, which would not kill cells quickly. Actually the fluid is always moving in experiment as shown in Figure 7.4, in which the temperature was not over 37 °C. Based on the results we believe that excessive heat generation would not be a cause of cell death.
Next, it was verified that the low temperature (33.5 °C) did not significantly affect cell growth. Cells were cultured in 33 °C and 37 °C incubators with same inoculation for 24 hours. The result is shown in Figure 7.6. The difference in cell density and cell viability between cells grown in different temperatures is negligible. Therefore, the slightly lower than normal temperature in the ultrasonic filter should not have a significantly negative impact on cell survival.

### 7.3.2.2 Effect of serum

In previous experiments (Figure 7.3) we have found the serum has some protective effects on cells influenced by ultrasonic fields. Therefore, subsequent experiments were done in the serum medium. Figure 7.7 shows the culture results with 10% serum medium at different levels of power. At time=24 hours, the transducer was energized (except the control experiment). Every 12 hours the bioreactor culture vessel for the control culture experiment and the experiments with Vsg=40 mVpp were sampled and the concentration reported in Figure 7.7. Because there is almost no cell retention in the ultrasonic filter when Vsg=40 mVpp, there is no need to turn off the power and flush the system. However, at Vsg=300 mVpp, sampling was done by the following procedure: the transducer was turned off for 5 minutes, and the ultrasonic filter was flushed in reverse to return the retained cells in the ultrasonic filter back to the bioreactor. Then a sample was taken from the bioreactor and counted. After the sampling procedure was complete, the transducer was turned on again.
From Figure 7.7, we can see that the maximum viable cell densities and viabilities decreased as applied voltage $V$ increased. No threshold level of acceptable power was observed; even at $V_{sg}=40 \text{ mVpp}$ where no cell retention occurs and exposure time to the ultrasonic field is shortened, a detrimental effect on cell growth was observed. The maximum signal generator voltage used in this set of experiment was 300 mVpp, at which level of power input, cell concentration was more than tripled at 96 hours, while the cells died out at 80 hours for culture shown in Figure 7.4. This difference is caused by two reasons. The first is that culture shown in Figure 7.4 used 600 mVpp voltage with the signal generator which is double of the latter. It is shown in Figure 7.7 that the higher voltage of signal generator, the more negative impact on cell growth. The second is that
the culture shown in Figure 7.4 was conducted using serum free medium while the culture shown in Figure 7.7 used 10% serum medium. The presence of serum in the medium provided the cells some protection from the sound stress.

Serum concentration was increased from 10% to 15% to see whether the higher concentration serum would protect the cells from sound-induced damage. The ultrasonic filter was combined with the 1 L bioreactor. The sampling procedure is similar as the above experiment. The results shown in Figure 7.8 illustrate that cell growth and viability are basically same for cultures with 10% and 15% serum supplement. The maximum cell density achieved and growth period in both two culture runs are very close when the signal generator voltage was 300 mVpp. This implies that further increase of serum concentration in the culture medium cannot provide extra protection to the cells from negative impact induced by exposing to ultrasonic field.

The antibody production at different levels voltage input with signal generator is shown in Figure 7.9. The data come from the culture experiments shown in Figure 7.7 and Figure 7.8. As shown in Figure 7.9 antibody production decreased drastically, over 50%, when the voltage at signal generator is 40 mVpp. If take into account of the amount of antibody existed in inoculum which volume was counted for 20% of final culture volume the antibody production reduction is even higher compared to control culture. At this level of voltage input at signal generator, cells can not be retained in ultrasonic filter, cells just flow through the ultrasonic filter and were briefly exposed to the ultrasonic field.
When voltage at signal generator was increased from 40 mVpp to 300 mVpp to able to retain cells in the ultrasonic filter, though the serum concentration was increased from 10% to 15%, antibody production was still sharply reduced over 70% compared to the control experiment. Similarly, if the antibody that existed in the inoculum is taken into account, less than 10% of antibody was produced compared to the control test. This should be caused by higher stress from the ultrasonic field and longer exposure time for the cells grown in 15% serum free medium.

### 7.3.2.3 Effect of oxygen and residence time

The following experiment was done to rule out oxygen limitation as a cause of cell death when almost all the cells were retained in the ultrasonic filter. A 140 mL spinner flask
was used to couple with a 13 mL ultrasonic filter for the test. Oxygen for supporting the retained cells in the ultrasonic filter is brought by the continuous influent culture medium pumped from the spinner flask, in which the DO is 75%. 24 hours after inoculation, the ultrasonic filter was powered on and circulation was started. Every 24 hours the transducer was powered off, the retained cells were flushed back to spinner flask for sampling to check the cell concentration and viability.

Signal generator voltages used in this set of experiments were 150 mVpp and 200 mVpp. The results shown in Figure 7.10 indicate that the cells in the system with the ultrasonic filter coupled to the 140 spinner flask died even more quickly than those cultured in the ultrasonic filter coupled to the 1 L bioreactor when the voltage input in signal generator was 300 mVpp, which is higher than the both tests shown in Figure 7.10. As shown in

**Figure 7.9** Antibody production at the end of cultures
Figure 7.7, cell concentration increased indeed at 48 hours for the test with 300 mVpp voltage input at signal. With same serum concentration, supposedly, cells should grown better in the tests shown in Figure 4.10 with lower power level. In the contrast, cell concentration dropped at 48 hours for both tests in Figure 7.10.

![Graph showing cell concentration](image)

**Figure 7.10.** Long-term exposure to ultrasonic field for cells cultured in 10% serum medium (the ultrasonic filter is combined with 140 mL spinner flask as shown in Figure 6.3).

The oxygen concentration in the spinner flask is 75% (measured with an oxygen probe) when viable cell concentration is $5 \times 10^5$ cells/mL and the oxygen concentration in the cell suspension that leaves the ultrasonic filter is estimated to be about 60% (See Appendix B for calculation procedure). At this level cell growth and metabolism are not limited by oxygen supply at all so oxygen limitation is not a cause for the fast cell death.

The volume of cell suspension in the spinner flask is smaller than that in the 1 L traditional bioreactor, so the residence time of cells in this system was much longer than
that in the 1 L bioreactor system. As shown in Appendix C, the exposure time to ultrasonic field in the bioreactor system is about 5 hours (in 24 hours) while in the spinner flask the exposure time is 21.7 hours (in 24 hours) when the Vsg and flow rate are same. Therefore the negative effects largely relate to the exposure time of cells to the ultrasonic field. Cell rupture was visually observed under the microscope after 24 hours exposure. The sound field appears to be the cause of decrease in cell viability. This result is in agreement with the results shown in Figure 7.3, in which Vsg is also 150 mVpp, for cells cultured in 10% serum culture medium, about 90% cells were killed in 16 hours.

It was reported that the damage to yeast cells which are exposed to standing ultrasonic waves is directly correlated to the exposure duration, especially when the cells are in the displacement of pressure nodal planes. Both intracellular material leakage and prolonged cell growth lag time were observed after the yeast cells exposing to ultrasonic standing waves for one hour. Plant cell viability can be markedly influenced by exposing to ultrasonic standing waves. In a period of 40 min exposing to standing ultrasonic filed which able to retain the cells, the viability of plant cells dropped 80%. Cavitation effect is unlikely the cause of death since the energy intensities used in that study was relatively low. The damage mechanism is still not clear but mechanical stress induced by acoustic pressure is very likely a major reason.
CHAPTER VIII

AN ULTRASONIC FILTER FOR PARTICLE RETENTION USING STANDING ULTRASONIC WAVES AT AN OBLIQUE ANGLE WITH FLUID FLOW DIRECTION

8.1 Introduction

Ultrasonic filters currently are being used in long term mammalian cell perfusion systems.\textsuperscript{16, 17, 20, 108} To retain the cells in the filter, the acoustic force must dominate the hydraulic drag force of the fluid carrying the cells. The hindrance of hydraulic drag force will prolong the residence time of collected cells in the ultrasonic filter, which is an unfavorable environment for cell growth since there is no control of pH and DO. In order to shorten the residence time, high recirculation flow rate was applied to facilitate the removal of collected cells but in this way the average residence time of cell suspension time is increased unavoidably.
A novel ultrasonic filter is presented in this chapter. It was developed for separating particles, like animal cells from an aqueous suspension. As with the inclined gravity settler, it is expected to be used as a cell retention device for long-term perfusion cultures. Unlike currently available ultrasonic filters, in which the particles concentrated by standing ultrasonic waves leave the ultrasonic chamber in the opposite direction of the carrying fluid, the collected particles in this device move in the same direction as the carrying fluid. In this way the hydraulic drag force facilitates rather than hinder cell removal. Therefore the cell residence time in the ultrasonic filter is reduced, which is preferred for cell growth and productivity.

8.2 Materials and Methods

8.2.1 Three Chamber Ultrasonic Filter with Oblique Middle Chamber

As shown in Figure 8.1 the ultrasonic filter is composed of three chambers. The middle chamber is the functioning part of the device, with walls that are at oblique angles with the transducers. There are two outlet ports at one end of the middle chamber; one is for concentrated particles and the other is for clarified fluid. Water is pumped through the two side chambers to prevent heat accumulation generated by the acoustic transducers.

The body of the chamber is made of 2 mm thick transparent polycarbonate sheet and 0.13 mm transparent polycarbonate film is used to separate the cooling chambers and the middle chamber. The transducer carriers are made of 2 mm thick borosilicate glass. The
inner dimension of the ultrasonic filter is 150 x 43 x 18 mm (height x width x thickness). The longest width of the cooling chamber is 23 mm and

![Figure 8.1 Schematic of ultrasonic filter with oblique middle chamber](image)

The narrowest width is 5mm, the width of the middle chamber in horizontal direction is 15 mm. This means the angle of the inclination of the middle chamber for the tested ultrasonic filter is 6.8 °. Four pieces (two on each side) of 55 x 18 x 1 mm piezoelectric lead zirconate titanate transducers (APC 880, American Piezo Ceramics, Inc.,
Mackeyville, PA) with fundamental frequency around 2.1 MHz are used for the excitation to maintain resonance in the acoustic filter, which are glued on the glass carrier. The top edge of the ultrasonic transducer is 10 mm to the top end of the filter and the lower edge is 30 mm to the low end of the ultrasonic filter.

Figure 8.2 Force analyses of a particle in the filter, where $G$ is gravity force, $F_B$ is buoyancy force, $F_{ac}$ is the primary acoustic force, $F_H$ is hydrodynamic drag force.

Primary acoustic force is calculated using the following equation:

$$F_{ac} = 4\pi R^3 \kappa E_{ac} \sin(2\kappa x) F$$

where $\kappa$ is the wave number across the acoustic filter, $E_{ac}$ is energy density within the suspension, $x$ is the distance of the cell from the nearest pressure nodal plane, $R$ is the...
particle radius, and $F$ is the acoustic contrast factor. This factor is characteristic of the suspension and is given by:

$$F = \frac{1}{3} \left( \frac{5\rho_p - 2\rho_f}{\rho_f + 2\rho_p} - \frac{\gamma_p}{\gamma_f} \right)$$  

Where $\rho_p$ is the density of the particle, $\rho_f$ is the density of carrying fluid, $\gamma_f$ is the compressibility of carrying fluid, $\gamma_p$ is the compressibility of particle. The primary acoustic force moves the particles to the pressure nodal planes or antinodal planes determined by the particle and carrying fluid physical property in the ultrasonic standing wave field.

Hydraulic drag force is calculated using the following equation:

$$F_H = 6\pi\mu(v_f - v_p)R$$  

where $\mu$ is the fluid viscosity, $v_f$ is the practical velocity, $v_p$ is the particle velocity, and $R$ is the radius of the spherical particle.  

Let’s first consider particles that are homogeneous and have higher compressibility than the carrying fluid, then the primary acoustic force moves the particles to the nearest pressure nodal planes. As shown in Equation 8-1, the primary acoustic force is zero when the particle is within a nodal plane and it increase to the maximum at the middle point between two adjacent nodal planes. The hydraulic force has the tendency to push the particles away from a nodal plane, causing primary acoustic force increase along with the distance from the nodal plane. The forces in horizontal direction on the particle reaches a balance When:
\[ F_{Ac} + F_D \sin \theta = 0 \] 8.4

Thus particles will collect in a plane a distance \( x \) away from the nodal plane as shown in Figure 8.2 B. For particles with density greater than the carrying fluid, the gravity force is larger than the buoyancy force. Therefore a particle in the middle chamber within the setup as shown in Figure 8.1, will move down until reaching the lower surface of the separator. Then collected particles on the separator surface will slide down along the lower surface until they exit via port I. Here the hydraulic drag force parallel to the separator surface will facilitate the particle removal since it has the same direction as the particle movement as well as the gravity force. And relatively clarified fluid will leave the middle chamber via Port II.

If the magnitude of horizontal direction of the hydraulic drag force is higher than the largest primary acoustic force can occur at a given power input, then the particles just will be carried out with the carrying fluid via Port II and no separation induced by the acoustic force will happen. So there is a flow rate limit below which the separation occurs.

As shown in Equation 8-1 and 8-3, the primary acoustic force is proportional to the cube of the particle radius and hydraulic force is proportional to the first power of the particle radius. Therefore the primary force decrease more than the hydraulic force along with the reduction of particles radius. This relative sensitivity to radius is the basis of separating particle suspension with different diameter particles. The separation of a population of particles by size was investigated using this device.
8.2.2 Polystyrene Particles

Monodisperse standard polystyrene particles (Sigma, St. Louis, MO) with diameter 8, 10, and 15 um diameter and 1.05 g/cm³ density were used to test the device. Particles with 8 um and 15 um diameter are translucent with no color stain. The particles with 10 um diameter are made of polystyrene with blue color. This arrangement makes it easy to distinguish the particles with 8 um and 10 um diameter when counting particle under microscope. The particles were suspended in DI water supplemented with 0.1% Triton X-100 (Sigma, St. Louis, MO), which helps prevent the particles from aggregating. The concentration of each type of particle in the mixture is from 1 to 2 million particles per milliliter, which is similar to the cell concentration used in this study.

8.3 Cell or particle retention test setup
8.2.3 Cell line and Medium

HB-159 Hybridoma cells (ATCC) are used for the cell retention tests conducted within the ultrasonic filter. A serum free medium, BD Cell Mab Medium Serum Free, (BD Biosciences - Advanced Bioprocessing) supplemented with 0.1% Pluronic F68 (Sigma, St. Louis, MO) is used for cell culture. Cells were cultured in T-flasks first, then transferred to a 2-L B.Braun stirred bioreactor and the working volume was 1.5 L.

8.2.4 Experimental Setups

As shown in 8.3, particle or cell suspension is pumped into the ultrasonic filter via the top inlet, the concentrated stream leaving the port I will be returned back to bioreactor and sample will be taken from the stream leaving port II after at least 4 residence times (of fluid) in the filter. The flow direction of liquid in the filter is downward. For both particle and cell retention tests, the flow rates via port I and port II are equal.

8.2.5 Analysis Methods

Cell density and viability were determined using hemocytometer count and trypan blue exclusion method. Samples from the stream leaving the outlet ports were gathered during quasi-steady period, at least after four residence times. The number of polystyrene
particles in the effluent is determined using microscope with hemocytometer. The particle identity is determined by both color and size.

The cell or particle retention rate, $R$, is defined as:

$$R = \frac{X_R - X_O}{X_R} \times 100\%$$  \hspace{1cm} 8.5

where $X_R$ is the cell or particle concentration in the bioreactor; $X_O$ is the cell or particle concentration in the relatively clarified stream that exits port II from the filter.

8.3 Results and Discussion

8.3.1 Polystyrene particle separation

Polystyrene particle suspension with three diameters was tested at first to confirm that the device is not only able to separate particles from the carrying fluid but also has the potential to separate the particles from each other based on size difference.

As shown in 8.4, near the top inlet area the cloud of particles in the suspension evenly filled the middle chamber. Then the “cloud of particle” became thinner towards the middle region of the filter. And when the clusters becomes large enough they were observed to drop down to the lower surface of the middle chamber and slide down to the port I.
In the following studies, the performance of the ultrasonic filter with oblique middle chamber was quantitatively evaluated. As shown in 8.5 for all three particles, the retention rate goes down as flow rate increases. When the flow rate increase, the hydraulic drag force increases as well, which results in small particle clusters accumulated in the vertical plane. The particle retention capacity decreases accordingly. The particle retention efficiency increases with particle diameter, as expected, although the average difference is only about 5% between the 15 um and 8um diameter particles.
Apparently the particles cannot be separated thoroughly but at least the particles with certain size range can be enriched by pumping particle suspension with mixed diameters repeatedly through the ultrasonic filter.

Over 90% retention was achieved, at the given power input level up to 23L/day, with 10 and 15 um particles. These two diameters are close to the mammalian cell size with density similar to mammalian cells, so we speculate that mammalian cells also can be efficiently retained, with selective retention of viable over nonviable cells.
8.3.2 Cell Retention Test

As shown in 8.6 viable cell retention was always higher than that of nonviable cells. If we consider the middle chamber as an inclined gravity settler, this device has a cell retention capacity that is about 22 times that of an inclined gravity settler with the same surface area but without the ultrasonic enhancement (see Figure 5.3 in Chapter V).

![Cell retention vs. flow rate with signal generator voltage of 2.8Vrms and average power input on the transducers of 13.9 W. The cell line used is the HB-159 hybridoma.](image)

The difference of retention between viable cells and nonviable is less than 5% at most of sampling points and the average is less 10%, while for the inclined gravity settler the difference is above 20%. This means the selectivity of the ultrasonic filter is significantly smaller than the gravity settler. Smaller particle in the filter has more chance to be
washed out but it is the situation before they form a large multiple cell cluster with other
particles. The closer the small particle is to the lower surface the less chance it can be
washed out since it need travel across more layer of nodal planes than the particles in
further locations. In the contrast, in gravity settler the separation is mostly to deal with
single cells. There is little chance the smaller nonviable cell can form a cluster with other
cells during the settling process. For the similar reason, pervious developed upward-flow
ultrasonic filters have smaller selectivity than inclined gravity settler, in which the small
particles need travel upwardly through the filter working chamber across the ultrasonic
standing waves. The longer the path is the less chance the small particles can be washed
out. Therefore, if the culture is very sensitive of viability, then the inclined gravity settler
should be selected as cell retention device even its capacity is smaller than the ultrasonic
filter based on unit settling surface area.
In this study, modifications and characterizations of three cell retention approaches were presented: inclined gravity settler, acoustic filter filled with porous mesh, and acoustic filter with an oblique middle chamber.

9.1 Gravity Settler

The major advantages of using an inclined gravity settler for cell retention are: its easy of use; and economical to manufacture. The major advance of the downward flow inclined gravity settler presented here, is that for a unit working surface area, our downward flow inclined gravity settler has a capacity that is about three-fold greater than that of the commercially available inclined gravity settlers (e.g., from Biotechnology Solutions, Inc.). One factor affecting the performance of this device is the cell settling velocity. The settling velocity differs significantly among cell lines and it changes substantially when
cell viability drops for same cell line. The cell settling velocity measurement should thus be performed routinely for each cell line both before and during the perfusion culture when a gravity settler is used for cell retention. In this way a gravity settler with enough capacity can be selected \textit{a priori} to maximize viable cell retention efficiency. Furthermore, the time-dependency of the cell size and thus the settling velocity indicates that a gravity settler with real-time adjustable capacity is preferred for optimal cell retention. The settling velocity measurement device developed and presented here is unique and is a simple and inexpensive method for measuring the velocity of the viable and nonviable cells.

\textbf{9.2 Acoustic Filter Filled with Porous Mesh}

An acoustic filter with a porous mesh designed for non-cellular particle filtration was characterized for use in mammalian cell retention. This approach presents an improvement of the commercial acoustic filters by including a porous mesh in the acoustically-active chamber. It was found that the power input could be reduced up to 39\% for the same cell retention capacity. This represents an improvement in acoustic filtration for cell retention, especially when cell growth and productivity are sensitive to power input levels. For long-term, large-scale applications, this power reduction can result in significant cost-savings.

As with the commercial acoustic filters, this acoustic filter filled with porous mesh still needs on-and-off cycles.\textsuperscript{17, 108, 118} This on-and-off cycle not only requires the complexity of specific control system, but it also lowers the capacity of the device, since when the filter is turned off, it is not retaining cells but permitting the retained cells to return to the
bioreactor. Thus the filter has a nonworking period within each cycle. In order to solve this issue we developed a novel acoustic filter which can be operated continuously.

### 9.3 Acoustic Filter with an Oblique Middle Chamber

Cells in the carrying fluid can be effectively separated under the combined effects of acoustic, gravity and hydraulic drag forces using this novel ultrasonic filter with an oblique middle chamber. This design has an advantage over the previously developed ultrasonic filters for particle recovery or concentration because the moving direction of collected particles or aqueous droplets is same as the carrying fluid. Experiments proved that the new design is able to be operated continuously, while the on-and-off cycles have to be arranged for the previously developed ultrasonic filters, which simplified the operation and lowered hardware requirements.

### 9.4 Selection of Cell Retention Approach

With further optimization of this design, it is believed that it can achieve separation efficiencies similar to commercial units, with simpler hardware/software design, lower power input per flow capacity, and smaller transducer area per flow capacity.

Both gravity settlers and acoustic filters are being successfully used in industrial applications. There is not doubt about their stability for long-term cell retention.
applications. The biggest difference between acoustic filters and inclined gravity settler is the construction cost. For 200 L/day capacity the price of a commercial acoustic filter system is more than 10-fold that of an inclined gravity settler. If the cell retention device will be used many times, the one-time investment of an acoustic filter system might be acceptable. However, for single-use disposable systems, of which the bioreactor market is increasing dramatically, only an inclined gravity settler can make economic sense.

The working volume of the acoustic filter is much smaller than an inclined gravity settler. Therefore if a cell line is very sensitive to growth environment, like pH and DO, then an acoustic filter is preferred for the cell retention task. Currently the largest available acoustic filter system is 200 L/day (Applikon Biotechnology), while the largest gravity settler available is 500 L/day (Biotechnology Solutions). The scalability of gravity settler is better than the acoustic filter. This is because the output of the acoustic transducer is limited and the cooling system requirement for removing the heat generated by acoustic transducers. The power consumption of acoustic filter system is significantly greater than that used in the pumps of the inclined gravity settler, but given the high cost of biotechnology products, this factor is not significant for the selection of cell retention devices for perfusion culture systems. On the other hand, for the production of biofuel from algae in very large-scale systems, the higher energy cost of the acoustic filter precludes the use of this system.

All the devices tested in this study were constructed in the lab manually. It is expected that better performance of the devices can be achieved by professional fabrication.
10.1 Gravity Settlers

10.1.1 Bench-Top Scale Gravity Settlers

The results of the culture tests show that this gravity settler is a reliable cell retention device for long-term (at least two months) high-density perfusion culture. The antibody productivities were stable during the first phase run of 0.8 day$^{-1}$ perfusion rate for both cell lines tested. This implies that perfusion culture using this device for cell retention can be conducted long-term with no sacrifice of productivity.

Our study shows that with a 1 L perfusion bioreactor, it is feasible to provide enough cells to seed a bioreactor with working volume of 250 liter. The expansion rate of 250-fold is tens times that of conventional processes. A total of 6.4 days was required to achieve this inoculum, counting from when the perfusion rate was gradually increased
until it was doubled. This indicates that less than 1/3 of the time for inoculum preparation was needed, compared to the conventional 3 to 4 week schedule.

### 10.1.2 Settling Velocity Measurement Column

The settling column described in Chapter IV provides an inexpensive, rapid, and accurate method for determining cell settling velocities and that can distinguish the settling velocities of viable and nonviable cells. The method was validated using polystyrene particles with known physical properties, and resulted in less than 4% error compared to the theoretical value obtained using Stokes’ law.

### 10.1.3 Gravity Settler Scale-up

With short-term cell retention tests, it was shown that this downward flow inclined gravity settler is scalable to industry level for mammalian cell perfusion cultures. When scaling up, there is a maximum feasible settler length, since an increase in settler length increases the cell retention time, which is not favored for cell growth and productivity. The efficiency of cell retention decreases with increase of settler length. With increased inlets to help the flow distribution the width of the settler can be increased with no foreseen limitation.
The primary algae dewatering tests showed that the inclined gravity settler can effectively assist in water removal task and it can be operated continuously for three weeks. From our preliminary results, algae culture can be concentrated at least 4-fold.

10.2 Acoustic Filter

10.2.1 Effect of Porous Mesh and Transducer Attachment Method

The collection or retention of fine particles, like hybridoma cells, from a flowing suspension can be enhanced using an acoustically driven porous polymer mesh having pore size two orders of magnitude larger than the particles. The power input with porous mesh acoustic filter can be significantly reduced compared to the control experiments, 36% reduction at 0.50 mm/min, 39% reduction at 0.75 mm/min and 14% reduction at 1 mm/min fluid velocity. Alternatively, with the same power input, the acoustic filter with mesh allows the retention of the same amount of cells in less time. Lower exposure time to ultrasonic field or lower power input is helpful for both the long-term cell viability and productivity.

The flexible attachment of the acoustic transducer to the filter frame can also significantly reduce the power input needed for maintaining the same level of cell retention capacity, compared to that achieved with a rigidly affixed transducer. The significance of using the porous mesh is not only about energy saving but also the increase of operating range.
10.2.3 Impact of Ultrasonic Field on Cell Growth and Productivity

Long-term exposure to ultrasonic standing waves negatively impact cell growth and antibody productivity at the levels of power input that are needed to retain cells and even at power levels significantly less. Damage to cells was found to be proportional to power input and exposure time. Serum had a small mitigating effect on the damage. Therefore it is not feasible to use an ultrasonic filter as a means to culture cells like a regular cell culture vessel.

10.2.4 Acoustic Filter with An Oblique Middle Chamber

The design of this device combines features of both the gravity settler and the ultrasonic systems to efficiently recover particles or cells. With assistance of the ultrasonic standing waves, particles in the suspension are gathered before they reach the settling surface. It virtually increases the size of the aggregate to be settled resulting in much shorter settling time. For the same unit surface area, the cell retention capacity of the acoustic filter is 22-fold that of the inclined gravity settler.

10.3 Recommendations

Small to medium scale of tests were conducted with gravity settler for both applications with mammalian cells and algae culture. Further scale-up research should be conducted based. The first scale-up approach, in actual systems coupled with bioreactors, will involve putting multiple channels together as shown in Figure 10.1. This settler should
have minimum total capacity of 50 L/day. The multiple channel design not only increases total handling capacity but also gives flexibility of operation especially for cell retention purpose. As shown in Figure 10.1, each channel is attached with a valve, which means the number of channels that will be used in the task can be controlled. By adding channels one by one during the task, the perfusion rate can be increased gradually without sacrificing cell retention efficiency. This design will be able to handle capacity from 50-200 L/day.

**Figure 10.1** Multiple channel gravity settler with minimum 50 L/day capacity

Next step, scale-up can be accomplished by stacking multiple plates of settlers to further increase the handling capacity as shown in Figure 10.2. The capacity of this type of settler is over 200 L/day. Future work on this settler is to make sure the flow distributes equally on each level. Flow distribution throttle might be needed for the even distribution of fluid in each layer.

For the ultrasonic filter with oblique middle chamber, the future emphasis lies in three aspects, optimization, actual perfusion culture tests, and scale-up. The optimization includes material design and material optimization. The inclination angle of the middle chamber was chosen randomly for preliminary tests. There is much room for further
optimization by mathematical modeling and experimentation. The aim is to maximize the particle handling capacity for a given working volume, which is the middle chamber volume. Another optimization is the material selection. Polycarbonate film was used to insulate the middle chamber from the cooling chambers. During tests, it was observed that the film tended to deform when there is imbalanced pressure on its two sides, which will cause extra resistance for cells to leave the middle chamber. Glass or stainless steel may have better performance.

Figure 10.2 1000 L/day capacity multi-layer inclined gravity settler with shared plates (side view)
After design optimization, the filter should be tested with actual perfusion culture to evaluate its reliability during long term use and to determine if there is negative impact on cell growth and productivity. There are two approaches to scale-up of ultrasonic filter. The first one is simply increase the working volume by increasing the width of the filter. All the recommended work is in conceptual phase and actual size need to be determined by further tests.
REFERENCES


32. Zi-Jun Zhang, J. H., Clair A. Francomano and Richard G. S. Spencer, , The Influence of Pulsed Low-Intensity Ultrasound on Matrix Production of Chondrocytes at


76. Shackel, I.; Bass, A.; Brewer, A.; Brown, P.; Tsao, M.; Chang, L., Comparison of manufacture technologies for rav12 monoclonal antibody in production medium


APPENDICES
APPENDIX A ELISA PROCEDURE

Elisa kit (Alpha Diagnostic International, San Antonio, TX) Anti-Mouse IgG1 coated strip plate (96 wells) was used for the IgG1 antibody concentration measurement. The plate was pre-coated with purified anti-Mouse IgG1 antibodies.

BLOCKING

1. Dispense 250 ul blocking buffer per well and Incubate plates for 2-6 h at room temperature or overnight at 4°C with gentle mixing.

2. Aspirate blocking buffer solution and tap over paper towels to remove all solution. The plates can be used directly for the assay or kept at 4°C in sealed bag for future use.

ANTIBODY ASSAY

1. Dilute samples, standard and control in Antibody/Conjugate Diluent (Serial dilution's of 1:10, 1:100, up to 1:100000K for standard IgG1 antibody). Dispense 100 ml to the pre-coated wells
2. Cover the plates with adhesive films. Incubate at room temperature for 30 min with gentle orbital shaking.

3. Aspirate solution and wash plates 5X with wash buffer (200-300 ul wash buffer should be used for each washing).

4. Dilute Anti-Rabbit IgG-HRP Conjugate (1:5000-1:20000) in Antibody Conjugate diluent. Dispense 100 ul of conjugate per well.

5. Cover the plates with adhesive films. Incubate at room temperature for 30 min with gentle orbital shaking.

6. Aspirate plates and wash plates 5X with wash buffer. Tap over paper towels to remove all solution.

7. Dispense 100 ul/per well of ready-to-use tetramethylbenzidine (TMB) substrate solution.

8. Cover the plates with adhesive films. Incubate at room temperature for up to 15 min with gentle orbital shaking. A blue color will develop in positive wells.

9. Dispense 100 ul diluted stop solution per well. The blue color will turn into yellow.

10. Read absorbance at 450 nm.
APPENDIX B OXYGEN CONSUMPTION ESTIMATION

Concentration of oxygen in culture medium at 37 degree Celsius saturated with air is 0.21 mmol/L. Oxygen consumption rate (q) of hybridoma is $0.22 \times 10^{-12}$ mol/cell/hour.

We assume the all cells in the spinner flask were retained in the ultrasonic filter.

Then the mass balance of oxygen

$$X \cdot q \cdot V = F (C_i - C_o)$$

where $t$ is the experiment duration, $X$ is cell concentration in spinner flask before the cell retention process started, $V$ is working volume of the spinner flask, $F$ is culture medium flow rate through the ultrasonic filter, oxygen concentration in influent stream at the inlet of the ultrasonic filter is $C_i$ and in the effluent stream at the outlet is $C_o$. Concentration of oxygen in spinner flask is $C_i = 0.21 \times 0.75 = 0.157$ mmol/L when DO is 75%.

So
\[ Co = Ci - \frac{X \cdot q \cdot V}{F} \]

X is equal to \(5 \times 10^5\) cells/mL and V is equal to 140 mL.

\[ Co = 0.126 \text{mmol/L which is 60\% in terms of DO} \]
APPENDIX C CELL EXPOSURE TIME ESTIMATE IN THE ULTRASONIC FILTER

At the same level of voltage input (150 mVpp) of the signal generator, the residence time in the spinner flask system and the 1 L bioreactor system are estimated as the following:

The working volume for the spinner flask is 140 mL and the traditional bioreactor is 1100 mL. The acoustic chamber volume is 13 mL. The cell suspension flow rate through the acoustic chamber is 30mL/min.

Hypothesis I: No cells are retained in the ultrasonic filter.

Then the average exposure time:

For cells in the bioreactor system is 24*13/1100=0.27hours

Hypothesis II: The retention is 100% in the spinner flask and the cells were retained with even speed.
In the 1L bioreactor culture vessel, the cell concentration was $5.4 \times 10^5$ cells/mL. After 24 hours, the retention is 40%. We assume cell accumulation in the filter does not influence the retaining efficiency.

The total average exposure time for the cells grown in 1 L bioreactor system should be:

$$24\times 40%/2+0.27-0.27\times 40%/2= 5.02 \text{ hours.}$$

Then, the retaining rate at 150 mVpp input of signal generator is $540000 \text{ cell/mL}\times 1100\text{mL} \times 40%/24 \text{ hour}=9.9\times 10^6 \text{ cells/hour.}$

Cell concentration in the spinner flask is $4.6\times 10^5 \text{ cells/mL}$. The retention will be finished in $460000\times 140/9900000=6.5 \text{ hours.}$

Suppose that no cells were retained in the first 6.5 hours, and then the average exposure time is $6.5\times 13/140=0.6 \text{ hours.}$

The total average exposure time in the spinner flask system should be:

$$(24-6.5)+6.5/2+0.6/2=21.7 \text{ hours.}$$
The exposure time evaluation is performed with two hypotheses, so it is not absolutely accurate but it can give clues for showing the difference in exposure time between the two systems.