LETHAL AND SUB-LETHAL EFFECTS OF HYDRODYNAMIC FORCES ON ANIMAL CELL CULTURE

DISSERTATION

Presented in Partial Fulfillment of the Requirements for
the Degree Doctor of Philosophy in the Graduate School of the Ohio State University

By


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The Ohio State University
2008

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ABSTRACT

Biotechnology-derived protein drugs, usually referred as biologics, represent a significant part of the whole pharmaceutical market. Typically, biologics are produced in genetically modified animal cells, which are regarded by many engineers and practitioners in the pharmaceutical industry as extremely sensitive to hydrodynamic forces. Since during research or normal operation in the biopharmaceutical industry cells are exposed to a range of hydrodynamic forces, this “shear sensitivity” idea often leads to very mild, sub-optimal designing and operating conditions.

To determine the actual levels of hydrodynamic stress capable of affecting the metabolism or viability of a cell line in bioprocessing or analytical devices, a microfluidic contracting-expanding device was developed in our group that exposes cells to controlled, well-defined hydrodynamic forces by means of keeping the flow in laminar conditions. Using this device, changes in cell behavior can be determined as a function of the local energy dissipation rate (EDR). EDR is a scalar value that is intrinsic to any moving fluid, is independent of the flow regime (turbulent/laminar) and accounts for both shear and extensional components of three-dimensional flow. It represents the rate at which work is done on a fluid element or a cell. If laminar flow is maintained, EDR can...
be reliably calculated using well-established equations for simple geometries or computational fluid dynamics (CFD) software for more complex problems.

The microfluidic device, consisting of a micro-channel bored in a stainless steel sheet in sandwich between two polycarbonate plates, was used in different setups to imitate the environment cells will experience in both bioprocessing and analytical equipment. As a model for analytical devices, it was selected a Fluorescent Activated Cell Sorter (FACS), where cells are forced through a nozzle and interrogated by a laser beam. This instrument was mimicked by passing the cells once through the microfluidic device; on the other hand, as a model for bioprocessing equipment, the hydrodynamic forces a cell experiences in a bioreactor were simulated by recirculating the cells through the microchannel, in an intent to reproduce the cyclic passage of the cells through the high EDR zone around the impeller to zones of relative low EDR intensity away from it.

Several cell lines of industrial, research and medical interest were tested using the just described methodology. For single passage, in every case the elicited response was mainly an increase of cell necrosis with larger EDR while only a small fraction of cells became apoptotic when exposed to the highest levels of EDR tested. Changes in medium composition or genetic modifications did not affect this behavior.

The response to repeated, chronic exposure to moderate levels of EDR was case-specific. A research CHO cell line (CHO6E6) stopped growing at the lowest levels of chronic EDR evaluated \(2.9 \times 10^5 \text{ W} \cdot \text{m}^{-3}\) and started dying when the EDR intensity was increased. On the other hand, the growth curve of a GS-CHO industrial cell line that produces a fully human antibody was not affected at all even at the highest EDR tested \(6.5 \times 10^6\).
W·m⁻³), although the glycosylation pattern of the antibody suffered modifications. Single passage results for both cell lines showed a very similar behavior to previously tested cell lines.

Interestingly, results showed that at least some medical cell lines (THP1) might have an EDR threshold lower than industrial or research cell lines (i.e., more “shear” sensitive), which suggest a possible selection of the tougher individuals after continuous manipulation. This conclusion seems to be also supported by the chronic exposure of the industrial GS-CHO cell line at the highest chronic EDR tested. If this is the case, the microfluidic device could even become a tool for selection of stronger clones in the pharmaceutical industry.
In memory of
my father, José Rubén Godoy, and
my teacher, Carlos Alberto Marín.
I will miss you both my best friends…

Dedicated to my mother,
to my siblings
to my niece
and to my wife.
ACKNOWLEDGMENTS

I would like to start by thanking God for giving me the ability to finish this goal I started so long ago. Although I have had to overcome very hard tests through all this time, I have felt his presence through strange, subtle and incredibly perfect ways.

My great appreciation goes to Professor Jeffrey J. Chalmers, my adviser, for welcoming me in his research group, for letting me work in his laboratory and for his support both academic and financial. I really enjoyed the liberty he granted me to explore many different research fields at my own pace before committing to my particular project, and even then he gave me a large flexibility in conducting my research. I am also immensely grateful for his support in helping me to get the internship in Pfizer Inc, accomplishing one of my most cherished dreams.

At Ohio State I had the opportunity to meet many people who helped me in many ways to make it through this thesis. In particular, I would like to thank:

- Jing Ying, Wei-Wei Hu, Bavhya Mehta, Ignacio Sanz –Valero, Luis Vargas and Suwattana Pruksasri (Dear), for all the nice, unforgettable moments together.
All my colleagues within the Yang-Chalmers groups/laboratory/student’s office, making my life more pleasant and funny. I specially appreciate all the technical advice, and enjoyed all the long hours of work with Dr. Mike Mollet. Thanks for being so patient with my English…and my dumbness…none of them have improved so much…

Paul Green for all his technical support and advice in designing and making the Torture Chamber.

Angela Bennett, Lois Holliday and Lynn Flanagan for helping me to sort all the vicissitudes of my visas and my academic paperwork.

I wish to thank Pfizer Inc. for the financial support and the honor of allowing me to work at the Global Research and Development Laboratories, at St. Louis, Missouri. In there, I was given support by so many people that sadly I can not name them all. In particular I would like to thank:

- Dr. Sussan Casnocha for her guidance, constant support and friendship.

- Dr. Ningning Ma, for his friendship, for always being there to help me, for picking me up every day after my surgery, for taking the time to check my presentations and giving me advice about them; for helping me with the papers, for….and for…
Centy “rewarding” Okediadi, Amber “nervous” Hartleroad and JoAnn “my hero” Ellet-Habing, for all their help and advice, but especially for their friendship and for always having a really encouraging smile in their faces. Recuerden que están invitados a visitarme en Colombia cuando quieran….

Jennifer Pierce, thank you for the pleasure of your company and nice conversations along many hours of late work.

Li Huang: thanks for spending time sharing with me our very different points of view about so many things in life. I am going to miss you and Dahli.

Financial support from the Fulbright-DNP-COLCIENCIAS program during the initial three years of this work is acknowledged. I also would like to thank the Universidad Nacional de Colombia for allowing time to continue my academic formation.

Quisiera agradecer a mi Papá, por haberme enseñado todas las cosas valiosas que vale la pena aprender en la vida. Seguirás siendo mi guía y el mejor de mis amigos. Nos volveremos a ver...aunque ojalá no muy pronto...

También quisiera agradecer a Carlos Alberto Marín, profesor y amigo. Gracias por llenar mi infancia de enseñanzas, consejos y alegrías...y uno que otro reglazo.
Mami: gracias por tu amor incondicional y constancia infinita con las que has logrado mantener unido ese hogar tan bonito que constituye nuestra esencia e identidad.

Especial gratitud a mi hermano Henry, quien ha tenido que sufrir la desdicha de crecer aguantándose a su hermano mayor. Ojalá podamos graduarnos juntos y quemar una copia del diploma el mismo día para que el viento lo lleve junto a papá...él se pondrá tan contento...

Quisiera dar gracias especiales a mi hermana Natalia, por constituirse en la complice inseparable de mi mami durante este tiempo que yo no pude estar físicamente a su lado. Sigue adelante, ningún lugar está lejos...

A la ciruelita, Paula Ariadna, quien llegó enviada por Dios para recordarnos la alegría de la inocencia y del asombro, quisiera escribirle unas líneas para que cuando algún día pueda leerlas, se entere que desde que nació tiene la capacidad asombrosa de hacer enormemente feliz a la familia con solo sonreír.

Solo me queda brindarles mi alma a todas las personas que se han quedado a mi lado a lo largo del camino. A Rosa Piedad e Iván. A Sandra, Jacqueline, Sandra Constanza, Mario y Germán. A Mariela. Gracias por todos sus risas, bromas, consejos, regaños, palabras de consuelo y sabiduría y por el privilegio de su tiempo.
Finalmente, estoy en deuda y agradezco infinitamente a mi esposa, Claudia, quien ha sido, es y seguirá siendo una constante fuerza impulsora, una llama eterna. Negrita: gracias por todo esa paciencia, ese amor incondicional y todo lo que me hemos compartido...
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FIELDS OF STUDY

Major Field: Chemical Engineering

Minor Field: Biotechnology, Biochemical Engineering, Cell Culture.
TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Chapter</th>
<th>Title</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>Introduction</td>
<td>1</td>
</tr>
<tr>
<td>1.1</td>
<td>Cell culture</td>
<td>1</td>
</tr>
<tr>
<td>1.2</td>
<td>Objectives</td>
<td>3</td>
</tr>
<tr>
<td>1.3</td>
<td>Scopes of this study</td>
<td>4</td>
</tr>
<tr>
<td>1.3.1</td>
<td>Acute hydrodynamic forces and apoptosis: a complex question (chapter 3)</td>
<td>4</td>
</tr>
<tr>
<td>1.3.2</td>
<td>Cell damage in a fluorescence activated cell sorter (chapter 4)</td>
<td>5</td>
</tr>
<tr>
<td>1.3.3</td>
<td>Experimental evaluation of the effect of chronic hydrodynamical stresses on cultures of suspended CHO cells (chapter 5)</td>
<td>6</td>
</tr>
<tr>
<td>1.3.4</td>
<td>Quantification of the sensitivity of an industrial GS-CHO cell line to chronic repetitive energy dissipation in a scale-down fed-batch bioreactor (Chapter 6)</td>
<td>7</td>
</tr>
<tr>
<td>1.3.5</td>
<td>An improved methodology for the modeling and simulation of the effect of energy dissipation rate on animal cells in microfluidic devices (chapter 7)</td>
<td>8</td>
</tr>
<tr>
<td>1.4</td>
<td>References</td>
<td>9</td>
</tr>
<tr>
<td>2.</td>
<td>Literature review: mixing, aeration and hydrodynamics in bioreactors</td>
<td>13</td>
</tr>
<tr>
<td>2.1</td>
<td>Abstract</td>
<td>13</td>
</tr>
<tr>
<td>2.2</td>
<td>Introduction</td>
<td>15</td>
</tr>
<tr>
<td>2.3</td>
<td>Aeration</td>
<td>18</td>
</tr>
<tr>
<td>2.3.1</td>
<td>surface aeration</td>
<td>24</td>
</tr>
<tr>
<td>2.3.2</td>
<td>perfluorocarbons</td>
<td>26</td>
</tr>
<tr>
<td>2.3.3</td>
<td>oxygen carriers</td>
<td>28</td>
</tr>
<tr>
<td>2.3.4</td>
<td>Membrane aeration</td>
<td>28</td>
</tr>
<tr>
<td>2.3.5</td>
<td>Sparging</td>
<td>29</td>
</tr>
<tr>
<td>2.3.6</td>
<td>Sparger design</td>
<td>30</td>
</tr>
<tr>
<td>2.3.7</td>
<td>CO₂ accumulation and removal</td>
<td>32</td>
</tr>
</tbody>
</table>
4.3.4 Micro-fluidic contraction device ................................................. 193
4.3.5 Cell damage analysis ............................................................... 194
4.4. Results .................................................................................. 195
4.4.1 FACS flow rate measurements .............................................. 195
4.4.2 CFD simulations .................................................................. 195
4.4.3 Torture chamber studies on cells .......................................... 197
4.4.4 FACS studies ....................................................................... 198
4.5. Discussion ............................................................................ 199
4.6. References ........................................................................... 205
5. Experimental evaluation of the effect of chronic hydrodynamical stresses on cultures of suspended CHO cells ................................................. 226
5.1 Abstract ................................................................................ 226
5.2 Introduction .......................................................................... 227
5.3 Materials and methods .............................................................. 229
5.3.1 Cell line ............................................................................. 229
5.3.2 Cell adaptation and maintenance ........................................ 230
5.3.3 Chronic exposure studies .................................................... 232
5.3.4 Cell concentration and viability .......................................... 234
5.3.5 Glucose and lactate .............................................................. 234
5.3.6 Antibody production ............................................................ 234
5.3.7 Lactate dehydrogenase (LDH) ............................................ 235
5.3.8 Bioreactor ........................................................................ 236
5.3.9 Microfluidic channel ............................................................ 237
5.3.10 Recirculation system ......................................................... 238
5.4 Results .................................................................................. 239
5.4.1 Acute exposure to high EDR ................................................. 239
5.4.2 Chronic exposure to high EDR .............................................. 240
5.5 Discussion ............................................................................ 242
5.6 References ........................................................................... 248
6. Quantification of the sensitivity of GS-CHO cells to chronic repetitive energy dissipation in a scale-down fed-batch bioreactor ................................................................................. 267
6.1 Abstract ................................................................................ 267
6.2 Introduction .......................................................................... 270
6.3 Materials and methods .............................................................. 276
6.3.1 Cell line ............................................................................. 276
6.3.2 Cell culture process ............................................................ 277
6.3.3 Single exposure experiments .............................................. 279
6.3.4 Chronic exposure experiments .......................................... 280
6.3.5 Analytical assays ................................................................. 281
6.3.5.1 pH, dissolved gas and metabolites .................................... 281
6.3.5.2 Cell density and viability ................................................. 282
6.3.5.3 Product titer .................................................................. 282
6.3.5.4 Osmolality .................................................................... 282
6.3.5.5 Necrosis ....................................................................... 283
## LIST OF TABLES

<table>
<thead>
<tr>
<th>Table</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.1</td>
<td>Specific oxygen uptake rates ($q_{O_2}$) reported for animal cell lines</td>
<td>95</td>
</tr>
<tr>
<td>2.2</td>
<td>Examples of empirically derived equations for $k_{l,a}$ estimation found in</td>
<td>96</td>
</tr>
<tr>
<td></td>
<td>literature</td>
<td></td>
</tr>
<tr>
<td>2.3</td>
<td>Power number for selected impellers for turbulent regime</td>
<td>97</td>
</tr>
<tr>
<td>2.4</td>
<td>Relative Oxygen carrying capacities ($k$) of water, perfluorochemicals</td>
<td>98</td>
</tr>
<tr>
<td></td>
<td>and gas vesicles (Modified from Sundararajan and Ju, 2006)</td>
<td></td>
</tr>
<tr>
<td>2.5</td>
<td>Benefits and drawbacks of PFCs (modified from Lowe et al., 1998)</td>
<td>99</td>
</tr>
<tr>
<td>2.6</td>
<td>Correlations derived by Cui et al. (1996) for power drawn in systems</td>
<td>100</td>
</tr>
<tr>
<td></td>
<td>with multiple Rushton impellers</td>
<td></td>
</tr>
<tr>
<td>2.7</td>
<td>Methodologies reported in literature for measurement of mechanical</td>
<td>101</td>
</tr>
<tr>
<td></td>
<td>properties of animal cells and/or determination effect of hydrodynamical</td>
<td></td>
</tr>
<tr>
<td></td>
<td>forces on animal cells</td>
<td></td>
</tr>
<tr>
<td>2.8</td>
<td>Some parameters reported in literature to correlate the effect of</td>
<td>102</td>
</tr>
<tr>
<td></td>
<td>hydrodynamical forces on cells</td>
<td></td>
</tr>
<tr>
<td>3.1</td>
<td>Table of physical and geometric characteristics used for the calculation</td>
<td>157</td>
</tr>
<tr>
<td></td>
<td>of EDR in the microfluidic device</td>
<td></td>
</tr>
<tr>
<td>3.2</td>
<td>Physical and geometrical characteristics of the bioreactor used in the</td>
<td>158</td>
</tr>
<tr>
<td></td>
<td>calculation of EDR</td>
<td></td>
</tr>
<tr>
<td>3.3</td>
<td>Table of physical and geometric characteristics used for the calculation</td>
<td>159</td>
</tr>
<tr>
<td></td>
<td>of EDR in the spinner flask</td>
<td></td>
</tr>
<tr>
<td>3.4</td>
<td>Average and maximum energy dissipation rate in a 250-mL, 7.2-cm internal</td>
<td>160</td>
</tr>
<tr>
<td></td>
<td>diameter spinner flask (Adapted from Venkat, 1995)</td>
<td></td>
</tr>
<tr>
<td>4.1</td>
<td>Highest level of EDR that a particle would experience in a BD FACSVantage</td>
<td>209</td>
</tr>
<tr>
<td></td>
<td>for different operating conditions in a 70 and 100 μm nozzle</td>
<td></td>
</tr>
<tr>
<td>Section</td>
<td>Title</td>
<td>Page</td>
</tr>
<tr>
<td>---------</td>
<td>----------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>4.2</td>
<td>Comparison of the FLUENT simulation core radius (in μm) and the core radius calculated from Equation 4.2 (also in μm) for different nozzle diameters and operating conditions in a BD FACS Vantage</td>
<td>210</td>
</tr>
<tr>
<td>4.3</td>
<td>Cell damage results in the flow contraction device of THP1 and CHO cells subjected to different levels of EDR</td>
<td>211</td>
</tr>
<tr>
<td>4.4</td>
<td>Cell damage results in the FACS Vantage of THP1 and CHO cells subjected to different experimental conditions</td>
<td>212</td>
</tr>
<tr>
<td>5.1</td>
<td>Characteristics of the bioreactor used in this study, and comparison of specific characteristics of the current vessel to that used by Venkat et al. (1996)</td>
<td>252</td>
</tr>
<tr>
<td>5.2</td>
<td>Flow characteristics in the recirculation system</td>
<td>253</td>
</tr>
<tr>
<td>5.3</td>
<td>List of experiments performed in the recirculation system</td>
<td>254</td>
</tr>
<tr>
<td>6.1</td>
<td>Experimental flow rates and corresponding calculated energy dissipation rates</td>
<td>334</td>
</tr>
<tr>
<td>6.2</td>
<td>Effect of chronic exposure to hydrodynamic force on product charge profile and methionine oxidation. Acidic and basic species are normalized to the corresponding control bioreactor. Data on the percentage of mAb with oxidized methionine for both the test and control experiments are shown</td>
<td>335</td>
</tr>
<tr>
<td>7.1</td>
<td>Table of physical and geometric characteristics used for the calculation of EDR in the microfluidic device</td>
<td>374</td>
</tr>
<tr>
<td>7.2</td>
<td>Mesh summary for the FLUENT® simulations of the torture chamber</td>
<td>375</td>
</tr>
<tr>
<td>7.3</td>
<td>Parameters of the fitted model for (% Damage) in the torture chamber</td>
<td>376</td>
</tr>
<tr>
<td>7.4</td>
<td>Comparison between the median maximum EDR from Mollet et al. (2007) and this study</td>
<td>377</td>
</tr>
</tbody>
</table>
LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Description</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.1</td>
<td>Approval of mammalian cell culture generated products over time. Source: Molowa (2001)</td>
<td>11</td>
</tr>
<tr>
<td>1.2</td>
<td>Manufacturing demand of biopharmaceutical industry. Source: Molowa (2002)</td>
<td>12</td>
</tr>
<tr>
<td>2.1</td>
<td>Geometrical configuration, flow patterns and total non-gassed power drawn into the liquid as a function of impeller spacing for a mixing system with multiple Rushton turbines (Adapted from Hudcova et al., 1989)</td>
<td>103</td>
</tr>
<tr>
<td>2.2</td>
<td>Impeller configurations commonly employed in animal cell culture....</td>
<td>104</td>
</tr>
<tr>
<td>2.3</td>
<td>Regions of highest shear rate and highest energy dissipation rate behind the blades of a Rushton turbine</td>
<td>105</td>
</tr>
<tr>
<td>2.4</td>
<td>Effect of the gas flow rate on the power drop under gassed conditions for two impeller geometries in Newtonian, water-like fluids. (Adapted from Galindo and Nienow, 1993)</td>
<td>106</td>
</tr>
<tr>
<td>2.5</td>
<td>Effect of the number and size of the baffles on the power drawn by an impeller in a cylindrical stirred tank reactor (Adapted from Oldshue, 1983)</td>
<td>107</td>
</tr>
<tr>
<td>2.6</td>
<td>Change in the tank diameter and impeller diameter as the volume of the vessel increases from 0.5 to 10,000 liters keeping constant the geometrical ratios H/T = 1 and T/D = 3</td>
<td>108</td>
</tr>
<tr>
<td>2.7</td>
<td>Lines of constant, maximum EDR in a vessel as a function of impeller rotational speed and diameter for Rushton turbine in water. H/T = 1 and T/D = 3</td>
<td>109</td>
</tr>
<tr>
<td>2.8</td>
<td>Average EDR for the whole vessel as a function of impeller diameter and RPM using a Rushton turbine in water. H/T = 1 and T/D = 3</td>
<td>110</td>
</tr>
<tr>
<td>2.9</td>
<td>Calculated maximum and average energy dissipation rate as a function of RPM for an Applikon bioreactor containing four baffles. The single points correspond to experimental measurements without baffles (Adapted from Mollet et al., 2004)</td>
<td>111</td>
</tr>
</tbody>
</table>
2.10 Molecular signaling and response cascade in endothelial and smooth muscle cells (A) before and (B) after stimulation by hydrodynamic forces. ................................................................. 112

2.11 Effect of agitation rate on cell concentration, viability and aggregate diameter of murine NSC in batch suspension in a 125 mL spinner flask. Data points are average of duplicate runs. (a) Cell concentration: (●) 60 rev·min⁻¹; (■) 100 rev·min⁻¹. Viability: (○) 60 rev·min⁻¹; (□) 100 rev·min⁻¹. (b) Average aggregate diameter: (●) 60 rev·min⁻¹; (■) 100 rev·min⁻¹. Standard deviation: (○) 60 rev·min⁻¹; (□) 100 rev·min⁻¹. (Adapted from Sen et al. 2001)........ 114

2.12 Effect of stirring speed on cell concentration after 7 days of culture of Vero cells on Cytodex microcarriers on 250 mL spinner vessels (Data from Hirtenstein and Clark, 1980)................................. 115

2.13 Experimental curves for the percentage of damage experienced by cells in a custom-design microfluidic device for single abuse experiments. Adapted from Ma et al. (2002), Mollet et al. (In Press) and Mollet et al., (submitted)......................................................... 116

2.14 Summary of the reported energy dissipation rate at which cells are affected as well as the reported levels of energy dissipation rate in various bioprocess environments. Adapted from Ma et al. (2002) and Mollet et al. (2004).................................................................. 117

2.15 A three dimensional plot of the number of cells associated with each bubble as a function of cell concentration (cell·mL⁻¹) and Pluronic F-68 concentration. The dots indicated experimental data and the surface is the plot of a multiple variable regression. (Adapted from Ma et al., 2004)................................................................. 119

3.1 Summary of the reported energy dissipation rate at which cells are affected as well as the reported levels of energy dissipation rate in various bioprocess environments. Adapted from Ma et al. (2002) and Mollet et al. (2004).................................................................. 161

3.2 Views of the microfluidic channel. (a) Photograph of the machined stainless steel sheet. (b) Photomicrograph of the neck region; arrows indicate the surface roughness and actual dimensions.............. 163

3.3 Various plots presenting the simulated velocity profiles in planes in (a) the entrance region (x=10 mm) and (b) in the beginning of the throat (x=15 mm). The flow rate of 90 mL·min⁻¹ simulated was the maximum used in this study......................................................... 164

3.4 Simulation of the pathlines of two particles along the microfluidic channel, the range of color corresponding to levels of magnitude of EDR (in W·m⁻³)................................................................. 166
Photographs ((a) and (c)), of cells flowing through the entrance and exit regions of the flow contraction device at a flow rate of 30 mL·min⁻¹. Parts (b) and (d) present fluent simulations of particle pathlines through the entrance and exit regions of flow contraction device at a flow rate of 30 mL·min⁻¹. Part (e) is a computer simulation of the energy dissipation rate, in W·m⁻³, that a particle would experience at a flow rate of 90 mL·min⁻¹.

Histogram of the maximum EDR experienced by each simulated particle injection into the simulated flow in the microfluidic channel. The numeric value presented is the median value for each population.

Cell damage/lysis as a result of being subjected to a single, high level of hydrodynamic stress (in units of EDR). Cells had been previously cultured in T-flasks. The cell damage/lysis was determined (a) from visual counting, (b, c) from LDH assays.

Bivariate plot distribution of 7-AAD fluorescence vs. Annexin V-PE fluorescence for WT CHO-K1 cells cultured in T-flasks with 10% FBS. Quadrant I: 7-AAD negative, Annexin V negative (healthy cells); quadrant II: 7-AAD negative, AV-PE positive (cells undergoing early apoptosis); quadrant III: 7-AAD positive, AV-PE positive, (necrotic and/or late apoptotic cells); quadrant IV: 7-AAD positive, AV-PE negative, likely naked nuclei/cell debris that bind 7-AAD but lack the membrane-bound PS.

Time course experiment of the percent of the cell population exhibiting early (a) and late apoptosis/necrosis (b) as a function of time after exposure to a given level of EDR. The cell population used was WT-CHO cultured in T-flasks with 10% FBS and harvested in late exponential growth.

Time course experiment of the percent of the cell population exhibiting (a) early and (b) late apoptosis/necrosis as a function of time after exposure to a given level of EDR. The cell population used was CHO–bcl-2 cultured in T-flasks with 10% FBS and harvested in late exponential growth.

Time course experiment of the percent of the cell population exhibiting (a) early apoptosis and (b) late apoptosis/necrosis as a function of time after exposure to given level of EDR. WT CHO-K1 cells were cultured in a spinner flask with 0% FBS and harvested in late exponential growth.

Time course experiment of the percent of the cell population exhibiting (a) early apoptosis and (b) late apoptosis/necrosis as a function of time after exposure to given level of EDR. The cell population...
population used was WT CHO cultured in a bioreactor with 0% FBS.

3.13 Time course experiment of the percent of the cell population exhibiting (a) early apoptosis and (b) late apoptosis/necrosis as a function of time after exposure to given level of EDR. The cell population used was CHO-bcl-2 cultured in a bioreactor with 0% FBS.

3.14 Histogram overlays of FCM analysis of bcl-2 over expressing cells stained with an anti-human bcl-2 antibody conjugated to PE and an isotype control antibody conjugated to PE. (a) is a histogram of a cell sample taken from a spinner vessel and (b) is a histogram of cells taken from the bioreactor.

4.1 Photograph (a), top view (b), and a perspective view (c) of the flow contraction device. All measurements are in millimeters.

4.2 Photograph of BD FACSVantage nozzle through drop collection tube (a); an enlarged photograph of the nozzle and reflection of laser beam off the exit stream indicating point of laser interrogation (b); an enlarged photograph of the region in which the stream breaks into individual drops and that are subsequently deflect by the charge on the deflection plates (c).

4.3 Schematic of flow cytometry fluidics: enlargement of the nozzle area (a); locations where pressure measurements were made to determine flow rate: the sheath tank area (b), and the sample injection area (c).

4.4 Exit sheath flow rate as a function of sheath pressure for the 70 μm and 100 μm nozzles (a), and sample flow rate as a function of sample differential and sheath pressure for a 70 μm (b) and a 100 μm (c) nozzles.

4.5 View of the nozzle geometry and mesh used for the simulation (a), and Fluent output of the simulations of particles flowing through the nozzle (b). For the specific simulated conditions, the sheath pressure was 17 psi and the sample differential pressure was 2 psi. The color coded figures correspond to the levels of EDR, in units of W·m$^{-3}$.

4.6 Example histograms of maximum EDR that simulated particles would experience flowing through a 70 μm diameter FACS nozzle operating at 25 psi sheath pressure and sample pressures ranging from 0.5 to 2.0 psi.

4.7 Percent cell damage, based on LDH assay, as a result of a single pass of the specific cell line indicated through the flow contraction device at the given level of EDR. The data for the TPH1 cell line is new for this study while the data for the CHO, Sf-9, MCF-7, and hybridoma cell lines are from Ma et al. (2002).
4.8 Typical dot plot of 7-AAD (FL3-H) versus Annexin V-PE (FL2-H), data taken 7.5 hours after the cells sorted with a sample differential of 0.5 psi……………………………………………………………… 224

4.9 Early apoptosis (a), and late apoptosis/necrosis (b), as a function of time for a CHO cell suspension passed through the FACSVantage with a 100 μm nozzle at 17 psi sheath pressure and a sample differential of 0.5 psi (dotted) or 1.5 psi (dashed). The solid line is a control………………………………………………………………… 225

5.1 Summary of the reported energy dissipation rate at which cells are affected as well as the reported levels of energy dissipation rate in various bioprocess environments. Adapted from Ma et al. (2002) and Mollet et al. (2004)…………………………………………………… 255

5.2 Torture Chamber (TC). (a) Photograph (b) Scheme detailing the dimensions (lengths in mm)……………………………………………………………………………………………………………… 257

5.3 Diagram of the experimental setup for continuous, chronic exposure of suspended animal cells to high levels of hydrodynamic forces…… 258

5.4 Response of CHO cells used in this study to an acute exposure to the hydrodynamic forces created by the TC and characterized by the specific level of EDR. As reference points, data from previously published studies with CHO K1 cells, MCF-7 and THP1 cells are presented (Mollet et al. 2007, 2008, Ma et al. 2002). Dashed lines indicate the flow rate for every EDR…………………………………… 259

5.5 Control experiments of the normalized growth, (a), and viability, (b), for three runs in the bioreactor and/or recycle system. TE4 and TE8 runs are typical growth in the system without recycle, while TE5 is with recirculation without the TC. The dashed line corresponds to the point at which recycling was initiated………………………………… 260

5.6 Normalized growth, (a), and viability, (b), of two experimental runs in which the cell suspension was subjected to recycle through the TC at 10 mL·min⁻¹, corresponding to 2.9×10⁵ W·m⁻³. The point of initiation of recycle is indicated with the dashed lines………………………………………………………………………… 261

5.7 Five experimental runs in which the cell suspension was subjected to recycle through the TC at 30 mL·min⁻¹, corresponding to 2.27×10⁶ W·m⁻³. The point of initiation of recycle is indicated with the dashed lines………………………………………………………………………………………… 262

5.8 The concentration of glucose (g·L⁻¹), and lactate (g·L⁻¹) as a function of time for (a) the controls, and (b) the five experiments run at a recycle of 30 mL·min⁻¹………………………………………………………… 264

5.9 The specific rate of glucose consumption for the control experiments, (a), and selected experimental runs with recycle, (b)………………………… 265

xxiii
5.10 Secreted antibody concentration as a function of time for selected experimental runs

6.1 Characteristics of the microfluidic device (Torture chamber). (a) Photograph. (b) Scheme of the stainless steel plate (dimensions in mm)

6.2 Equipment setup for single pass-experiments

6.3 (a) Viable cell concentration in a fed-batch process and the time points at which cells were taken for energy dissipation sensitivity test. Arrows indicate the sampling time (b) Sensitivity of GS-CHO cells at different growth stages to EDR

6.4 Equipment setup for chronic exposure

6.5 Comparison of cell growth and antibody production curves for both chronically exposed to EDR and corresponding control bioreactors. (a) 10 mL·min⁻¹ – 2.9×10⁵ W·m⁻³, (b) 30 mL·min⁻¹ – 2.3×10⁶ W·m⁻³, (c) 50 mL·min⁻¹ – 6.5×10⁶ W·m⁻³. Product titer was normalized to the highest titer of all bioreactor runs

6.6 Comparison of LDH concentration in the medium and cell size between control and test bioreactors. (a) 10 mL·min⁻¹ – 2.9×10⁵ W·m⁻³, (b) 30 mL·min⁻¹ – 2.3×10⁶ W·m⁻³, (c) 50 mL·min⁻¹ – 6.5×10⁶ W·m⁻³

6.7 Comparison of glucose metabolism in control and test bioreactors. (a) 10 mL·min⁻¹ – 2.9×10⁵ W·m⁻³, (b) 30 mL·min⁻¹ – 2.3×10⁶ W·m⁻³, (c) 50 mL·min⁻¹ – 6.5×10⁶ W·m⁻³. Glucose metabolism was based on cumulative volumetric glucose consumption and lactate concentration. All data are normalized to the highest lactate concentration and glucose consumption of all bioreactors

6.8 Comparison of apoptotic and necrotic population (measured by flow cytometry) between test and control bioreactors. (a) 10 mL·min⁻¹ – 2.9×10⁵ W·m⁻³, (b) 30 mL·min⁻¹ – 2.3×10⁶ W·m⁻³, (c) 50 mL·min⁻¹ – 6.5×10⁶ W·m⁻³

6.9 Comparison of product aggregation and fragmentation in control and test bioreactor exposed at chronic energy dissipation of 6.5×10⁶ W·m⁻³. Lanes 3 and 7: molecular weight markers; Lane 4: standard antibody. Lane 5: Test bioreactor material. Lane 6: Control bioreactor material. (a): Reducing conditions. (b): Non-reducing conditions

6.10 Effect of chronic exposure to energy dissipation in glycosylation. (a): 2.3×10⁶ W·m⁻³; (b) 6.5×10⁶ W·m⁻³

6.11 Summary of the reported energy dissipation rate at which GS-CHO and other cell lines are affected as well as the reported levels of
energy dissipation rate in various bioprocess environments. Adapted from Ma et al. (2002) and Mollet et al. (2004)………………….. 332

7.1 Torture Chamber (TC). (a) Photograph (b) Scheme detailing the dimensions (lengths in mm) ......................................................... 364

7.2 Sketch of the boundary layer growth modeling along the edges of the constriction in the Torture Chamber (TC)............................... 365

7.3 Torture Chamber Mesh details.................................................... 366

7.4 Results of velocity profiles for the region x = 15 mm (throat) and y = 0 (centerline of the plane) after simulation of the torture chamber at two different flow rates using a standard (Mollet et al., in press) and a finer (present study) meshing........................................... 367

7.5 Velocity and EDR profile in plane x = 15 mm (Throat) for a flow rate = 90 mL·min⁻¹. Position of the plane (gray-color top or bottom plane in y-z plane) is indicated by the red zone in the small TC sketch……… 368

7.6 Velocity “digitization” of the grid in plane x = 15 mm (Throat). Flow rate = 90 mL·min⁻¹.............................................................. 369

7.7 % Damage as a function of EDR in the throat calculated for every flow rate tested in this study....................................................... 370

7.8 Comparison between the predictions of the model and the experimental results for single abuse experiments............................. 371

7.9 Comparison between the predictions of the model and the experimental results for single abuse experiments............................. 372

7.10 Comparison of the profiles of EDR in plane x = 15 mm (Throat) for a flow rate = 90 mL·min⁻¹. Position of the plane (gray-color top or bottom plane in y-z plane) is indicated by the red zone in the small TC sketch. Left panel: Mollet et al. (2007). Right panel: this study…. 373
CHAPTER 1

INTRODUCTION

1.1. CELL CULTURE

Biotechnology-derived protein drugs, usually referred as biologics, represent a significant part of the whole pharmaceutical market. The Figure 1.1 shows the exponential increase on the number of FDA’s approved biologics since 1986, when the first monoclonal antibody (Mab), ORTOCLONE OKT3 (muromonab-CD3) was introduced as a treatment for solid transplant rejection. Since then, 29 Mabs and more than 36 recombinant-DNA-derived proteins and therapeutic agents have been approved for marketing in the USA for the diagnosis or treatment of diverse diseases; these figures do not include other biology-based products such as virus vaccines, hormones, enzymes, growth and blood factors, (Said et al., 2007; Ozturk, 2006; Thomson Centerwatch, 2007). In monetary terms, the growth also has been remarkable: by 2003, the world market of MAbs was somewhat bigger than 4 billion dollars, while by 2005, the estimated world market for antibodies was 14 billion dollars and such amount was expected to grow to more than 16 billion by 2006 (Research and Markets, 2007). In fact, the accelerated growth in biopharmaceuticals is expected to continue since 67% of the world clinical pipeline of new products in
development and/or under clinical trials are held by small biotechnology-based companies (Tollman et al., 2004). As a result of those expectations, the industry has been expanding the size of bioreactors and production facilities in an exponential rhythm (see Figure 1.2) and implementing strategies for resource and process optimization, in order to maximize productivities and economical profits.

Typically, biologics are produced in genetically modified animal cells, which have an innate ability for proper folding and post-translational processing of proteins, making them preferable over bacteria or other eucaryotic hosts. However, as a result of early reports in the literature, there exists a generalized belief among many engineers and practitioners in the pharmaceutical industry according to which mammalian cells are extremely sensitive to hydrodynamic forces; the rationale behind this idea is that because of their big size and lack of cell wall, animal cells can not resist mechanical deformation and therefore should be easily injured. Since during normal operation, cells are exposed to a range of hydrodynamic forces in bioprocessing equipment such as pumps, bioreactors (i.e., mixing, bubbling), tubing, filtration systems, etc., this “shear sensitivity” idea often leads to very mild, sub-optimal designing and operating conditions; a typical example involves the decrease in productivity of MAbs as the scale increases, very probably resultant from mixing problems, leading to oxygen limitation, high levels of lactate and increased broth osmolality.

Despite the culture of animal cells has been extensively studied for about one hundred years (earliest attempts were published by Harrison in 1907 and Carrel in 1912), no
generalized method of scaling up has been developed yet, and the accumulated knowledge regarding the effect on cell metabolism and death as a result of exposure to mechanical forces is case-particular and fragmented. As culture and processing of cells expose them to a range of hydrodynamic forces capable of affect their behavior in a series of different ways; it is precisely such spectrum of effects what conforms the main subject of this study.

1.2. OBJECTIVES

The overall objective of this research was to study the effect of hydrodynamic forces on animal cells of research, medical and industrial interest. To lend practical relevance to that objective, this study targeted the development and use of a methodology capable of subject cells to single and multiple exposures of controlled hydrodynamic stress in a easy, accurate, reproducible, and aseptic manner.

For that purpose, a convergent-divergent microchannel, routinely designed as the torture chamber (TC), originally devised by Ma et al. (2002) and improved by Mollet (2004) was used to obtain well defined flow. The Energy Dissipation Rate, (EDR), that represents the rate at which work is done on a fluid element or, in the context of this study, a biological cell, was selected to assess the level of hydrodynamic stress. The design and operating conditions of the TC allowed for a proper calculation of EDR. Using this device, we intended to simulate the real environment inside analytical devices an industrial bioreactors. This work reports the research collaboration with Dr, Mike Mollet as well as
further steps in the evaluation of the effects of hydrodynamic forces on animal cells. Traditionally, such effects have been assessed in terms of cell death whether necrosis (accidental death) or apoptosis (cell suicide). However, non-lethal effects including metabolism changes and product formation have been much less studied and may result even more interesting from an industrial point of view.

1.3. SCOPES OF THIS STUDY

1.3.1. Acute hydrodynamic forces and apoptosis: a complex question (Chapter 3)

The most studied effect in literature regarding hydrodynamical cell damage is cell death, especially via necrosis. However, one common complaint about such studies is the wide variety of employed methods and instruments and the difficulty to relate results derived from them to normal engineering conditions inside bioprocessing equipment. This study characterizes the development and modeling of a second-generation flow-contraction device (TC), originally developed by Ma et al. (2002); this device allows cells to be subjected to well-defined hydrodynamic forces, with the additional advantage of being steam-sterilizable. Studies were conducted with this system on wild-type Chinese Hamster Ovary cells (CHO-K1) and a strain of CHO cells which expresses the human Bcl-2Δ gene (CHO-bcl-2). Our primary goal was twofold: first, developing an easy, cheap and reproducible method of exposing cells to well-controlled hydrodynamic stresses that could be related to engineering conditions; second, finding out if a single exposition to a very high intensity hydrodynamic stress could not only induce cell death
by necrosis (as typically reported) but also via apoptosis, and how such induced cell-death could be affected by the culture history, culture medium and genetic characteristics of the cell.

1.3.2. Cell damage in a fluorescence activated cell sorter (Chapter 4)

Fluorescence activated cell sorting, FACS, is a widely used method to sort subpopulations of cells to high purities in both research and clinical assays. To achieve relatively high sorting speeds, FACS instruments operate by forcing suspended cells to flow in a single file line through a laser(s) beam(s). Subsequently, this flow stream breaks up into individual drops which can be charged and deflected into multiple collection streams.

A common complaint from the users of FACS facilities is that the number and viability of the cells obtained through sorting is usually much lower than expected; this problem is usually attributed to a lack of dexterity of the machine operator. However, a further analysis of the fluidics inside the FACS nozzle reveals that cells might be exposed to high levels of hydrodynamic stress and this could be the reason behind the problems previously mentioned.

This study concentrates on an experimental evaluation of the effects of passing several cell lines of commercial and medical interest through a FACS device and compares the
results with those from passing the same cells through the TC developed in the previous chapter.

1.3.3. Experimental evaluation of the effect of chronic hydrodynamical stresses on cultures of suspended CHO cells. (Chapter 5).

A common criticism to many of the studies reported in literature concerning hydrodynamical sensitivity of animal cells rely on the fact that the results from single exposures of cells to hydrodynamic forces in a particular device are used to infer conclusions about the behavior of the same cells along culture and bioprocessing. In a bioreactor the cells go back and forth from a very turbulent zone with a high stirring intensity just around the impeller to zones away from the impeller with a much lower stirring intensity. This movement exposes cells to recurrent high hydrodynamic forces that are not well modeled with a single-exposure experiment.

In this study the use of the TC is extended to a system in which a particular strain of suspended CHO cells, grown in a typical bioreactor, are subjected to chronic exposure of moderately high levels of hydrodynamic forces by way of a continue recycle loop between the bioreactor and the TC. The behavior of cells was inspected to observe the influence of chronic exposures to hydrodynamic stress in terms of cell death (necrosis and apoptosis) and metabolism changes.
1.3.4. Quantification of the sensitivity of an industrial GS-CHO cell line to chronic repetitive energy dissipation in a scale-down fed-batch bioreactor (Chapter 6)

So far, the TC has been applied to cells of medical or research interest. However, two questions remain unanswered:

- Is the sensitivity to hydrodynamic forces a function of the age of the culture?. Previous research experiments performed in batch mode, lasted for about one week; however, industrial cultures are normally fed-batch and may go on for two weeks with cells remaining a larger period in the stationary phase. This work explores the resistance of cells to hydrodynamic stress at different phases of the culture for longer cultures.

- Are industrial cell lines stronger than research or medical cell lines? Since industrial cell lines have been selected as a result of their good behavior in bioreactor, we were wondering if the sensitivity observed in THP1 cells for single exposure and CHO6E6 cells in chronic exposure to hydrodynamic stresses was a result of their lack of adaptation to high levels of EDR/turbulence. In this paper, we characterize the response of an industrial, monoclonal antibody producing GS-CHO cell line to a similar chronic-exposure experimental set-up as CHO6E6. As response variables we included cell growth rate, viability, necrosis, apoptosis, carbon metabolism, productivity, and product quality. This study clearly proves the applicability of the
methodology developed to evaluate hydrodynamic sensitivity in an industrial environment, and suggests that more intensive agitation should be evaluated in large-scale bioreactors to favor better mixing.

1.3.5. An improved methodology for the modeling and simulation of the effect of energy dissipation rate on animal cells in microfluidic devices. (Chapter 7).

The levels of EDR that cells experience in the TC are obtained through simulation using the commercial software FLUENT; the validity of the results depends on the accuracy of the assumption of laminar flow inside the microchannel and in the proper treatment of data generated by FLUENT. In this work we present an improved methodology for the analysis of the data delivered by the software, based on considerations of momentum balance and the sound assumption of a physical limit of stress beyond which the cell membrane loses its integrity and cell dies. This methodology allows the theoretical prediction of the trend of observed breakthrough curves and could lead to further refinement in the design of bioprocess equipment since it provides a methodology to establish a more accurate threshold, above which animal cells subjected to hydrodynamic forces could be destroyed.

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1.4. REFERENCES


Figure 1.1. Approval of mammalian cell culture generated products over time. Source: Molowa (2001).
Figure 1.2. Manufacturing demand of biopharmaceutical industry. Source: Molowa (2002).
CHAPTER 2

LITERATURE REVIEW: MIXING, AERATION AND HYDRODYNAMICS IN BIOREACTORS

The content of this chapter is currently under the process of peer review as a chapter contribution for the *Wiley Encyclopedia of Industrial Biotechnology*. The chapter was written in conjunction with Claudia Berdugo, also a PhD student at Dr. Chalmers’ group.

2.1. ABSTRACT

Animal cell culture has been used extensively for the production of a wide variety of substances with biological and therapeutic activity. In spite of being a mature area of development, no universally accepted method of design or scale-up of animal cell culture has been developed.

When designing or scaling-up a new process, mixing and aeration are among the most important operations to be considered since they achieve some of the most fundamental objectives carried out in a bioreactor such as keeping certain level of homogeneity in the
physicochemical parameters of the culture and providing oxygen to the cells. On one side, oxygen is a crucial nutrient involved in growth and energy production in animal cell culture; however, the low solubility of oxygen in water-based media forces its continuous supply to the culture, usually through direct sparging. Foam and cell-bubble interactions resulting from gas sparging may be problematic or even catastrophic for the culture but they are usually controlled through the use of antifoaming substances and protective surfactants.

Mixing, on the other hand, is absolutely necessary in animal cell culture to keep cells suspended and improving mass and heat transfer. While some degree of mixing is obtained through air sparging, particularly in the case of air-lift bioreactors, most bioreactors rely on some kind of mechanical device (impeller) to draw kinetic energy into the broth. Even though the choice of type and geometric characteristics of the impeller are highly subjective, the optimum stirring speed is commonly evaluated experimentally in bench scale bioreactors; even so, a large proportion of under mixed, large scale animal cell culture bioreactors are currently in operation in many facilities as a result of a wrongfully perceived “shear sensitivity” of animal cells. Although it has been conclusively shown that certain culture conditions can lead to cell damage and death, such damage is very dependent on the particular cell line, the culture characteristics such as surface-dependent vs. free suspension growth and the presence of any gas-medium interface where bubbles can rupture, especially when protective surfactants are not present. While experimental evaluation is advisable, for common industrial cell lines and
culture media “shear” sensitivity is not an issue and therefore scaling up conditions
should be focused on improving mass transfer limitations.

2.2. INTRODUCTION

Animal cells have an innate ability for proper folding and post-translational processing of
proteins that makes them preferable as host for producing biological components of
therapeutic and diagnostic interest; as a result, the culture of mammalian cell have been
used extensively for the production of biologics, including virus vaccines, monoclonal
antibodies (MAbs), hormones, enzymes, growth and blood factors. Although the
industrial exploitation of animal cell cultures started over five decades ago with the
production of Salk polio virus vaccine in primary monkey kidney cells (Griffiths, 2000),
it has been during the last 21-year period when there has been a rapid increase in the
number of FDA approved products produced in mammalian cell culture, starting in 1986
with the production of recombinant tissue plasminogen activator (tPA) with genetically
engineered CHO cells and the simultaneous introduction of the first Mab, ORTOCLONE
OKT3 (muromonab-CD3) as a treatment for solid transplant rejection. Since then, 29
MAbs and many other therapeutic agents have been approved for marketing in the USA
for the diagnosis or treatment of diverse diseases, a majority of which are produced in
animal cell cultures (Ozturk, 2006; Thomson Centerwatch, 2007). By 2005, one estimate
of the world market for antibodies produced in animal cell culture was 14 billion dollars
and this number was expected to grow to more than 16 billion by 2006 (Research and
Markets, 2007). While the specific, and or volumetric productivity continues to improve,
i.e. greater than 5 g/L of product is now considered the norm, this rapid growth in the number and demand of biologics has driven the industry to expand the size and number of production facilities and lead to statements that shortfalls in manufacturing capacity exist (Molowa and Mazanet, 2003). All of this positive growth puts significant pressure to continually improve the size and productivity of commercial animal cell culture systems.

Despite the fact that reports exist showing that animal cell culture has been conducted for over a hundred years (Harrison, 1907; Carrel, 1912), no universally accepted method of design or scale-up of animal cell culture has been developed. Diverse culture methodologies including static cultures (T-flask), roller bottles, cultures on microcarriers and freely suspended cells in batch, fed-batch or perfusion systems, among others, have evolved. While freely suspended, fed-batch processes have emerged as the predominate ones, much of this evolution has been guided by the particularities of the process, the cell line and, most importantly, the in-house expertise of the researchers specific to each organization. Although there are reports of large scale (1000 to 2000 liter) airlift bioreactors for protein/antibody production (Birch et al., 1985; Varley and Birch, 1999; Hesse et al., 2003), at least 70% of the licensed processes for recombinant proteins, antibodies and vaccines using microcarriers or freely suspended cultures use traditional stirred tank bioreactors with reported capacities up to 20,000L (Chu and Robinson, 2001; Butler, 2005; Meier, 2005). Several reasons account for this preference including the vast empirical knowledge accumulated for the design, scale-up and operation of this type of reactor in the chemical and biochemical industries over the last century, the versatility of
this reactor type allowing its adaptation to several different processes with minor or no modifications and, finally, the relative simplicity. As a result, most guidelines for bioprocesses design and scale-up are based on stirred tank reactors and as such will be the primary focus for the remainder of this review.

All animal cell processes are aerobic; oxygen is a crucial nutrient involved in growth and energy production in such cells. Unfortunately, because of its low solubility in water-based media, dissolved oxygen is consumed quickly, requiring a continuous supply in order to keep cells alive. The easiest way of providing oxygen to a stirred cultured is through surface aeration; however, surface aeration is not sufficient for cultures beyond ~100 L. Other aeration methods (i.e. perfluorocarbons, membrane aeration, oxygen carriers) have been used successfully at scales up to 500 L but their use is limited by high cost and downstream concerns (perfluorocarbons) or by limited design data and difficulties in maintenance (membrane aeration). These limitations have placed air and oxygen-enriched air sparging as the most common and simplest method for continuously providing oxygen in bioreactors.

Agitation, on the other hand, is essential for satisfactory mass transfer and homogeneity in a bioreactor with or without sparging; this homogeneity is crucial for process control. Although sparging provides some degree of agitation by itself, most animal cell cultures rely on some sort of mechanical device to impart enough kinetic energy to the fluid so a certain degree of homogeneity is reached.
Both agitation and sparging are essential for the success of industrial cultures of animal cells; yet, both are associated with cell damage as a result of cell-bubble, liquid-cell and/or solid-cell interactions. Significant progress has been made over the last two decades in understanding of cell damage mechanisms and in this chapter we will attempt to present an overview of the current practices and protocols for aeration and mixing in bioreactor design and operation as well as their connection to hydrodynamical damage in animal cell processes.

2.3. AERATION

Oxygen is a key substrate in animal cell cultures; unfortunately, its sparing solubility in water-based media (7.8 mg·l⁻¹ when bubbling air in water at 760 mm Hg and 25°C) requires a continuous supply throughout the culture for most larger scale situations. At high cell concentrations, the rate of oxygen consumption may exceed the rate of oxygen supply; in those conditions, the dissolved oxygen (DO) concentration falls down to a point (the critical oxygen concentration) where it becomes limiting. Reported critical oxygen concentration for animal cells ranges typically between 1 and 10% of air saturation (Gotoh et al., 2001; Zeng and Bi, 2006). Below such range, typically the oxygen consumption rate as well as the mitochondrial activity decrease, while the specific glucose and glutamine consumption rates increase (to compensate for ATP production) with the corresponding increase in the lactate and ammonium formation rates (Miller et al., 1987; Ozturk and Palsson, 1990; Lin and Miller, 1992). Together with a reduced mitochondrial activity, there is a reduction in the specific secretion rate of
proteins and volumetric accumulation of product which translates into higher cell viability, delayed death and lower chromosomal damage but also into low product yield (Ozturk and Palsson, 1990; Packer and Fuehr, 1977).

On the other hand, elevated oxygen concentration (~100% of air saturation) may stimulate the generation of reactive oxygen species (ROS) that can alter cellular macromolecules such as DNA, proteins and lipids, impairing cell growth or even causing death. (Zeng and Bi, 2006).

As a result, cell culture should be constrained within an optimal range of dissolved oxygen that must be determined experimentally, taking into account that optimal DO values for cell growth may be different than optimal values for protein production. Oh et al. (1989) reported satisfactory cultivation of mammalian cells from approximately 5 to 100% of air saturation. Jan et al. (1997) studied the effect of dissolved oxygen from 10% to 150% of air saturation on the growth of a murine hybridoma cells at steady state in serum-free continuous culture; while no significant effect on cell viability, growth rate or specific antibody production rate was found, an increase in the amount of glucose utilized at higher oxygen concentrations was detected. It was speculated that this increased glucose consumption rate was associated with an increased activity of antioxidant enzymes needed to reduce the cytotoxic effect of ROS. Using the same system, Kunkel et al. (1998) described an increase in the level of galactosylation of the mAb chains with increased dissolved oxygen.
For common animal cell lines under favorable oxygen supply, reported specific oxygen uptake rates \( (q_{O_2}) \) range between \( 6.4 \times 10^{-18} \) and \( 4.5 \times 10^{-16} \) mol oxygen·cell\(^{-1}\)·s\(^{-1}\) (See Table 2.1). Given that currently cell densities of up to \( 10^7 \) cells·ml\(^{-1}\) are common place at the commercial scale (deZengotita et al. (2000); Wurm, 2004; Nienow, 2006), the actual oxygen uptake rate in a typical culture may range from \( 6.4 \times 10^{-5} \) to \( 4.5 \times 10^{-3} \) mol oxygen·m\(^{-3}\)·s\(^{-1}\); this value is at least one or two orders of magnitude lower compared to yeast (i.e. \( 2.2 \times 10^{-2} \) mol oxygen·m\(^{-3}\)·s\(^{-1}\) for \textit{Candida guilliermondii} (Gimenes et al., 2002)) and bacteria (i.e. \( 3.3 \times 10^{-1} \) mol oxygen·m\(^{-3}\)·s\(^{-1}\) for \textit{E. coli}, corresponding to \( q_{O_2} = 12 \) to 21 mmol ·gDW·h\(^{-1}\) (Fong et al., 2005) and up to 190 gDW·l\(^{-1}\) (Shiloach and Fass, 2005)). This low oxygen requirement may help to explain why even with an agitation intensity for animal cell cultures about 100-fold lower than for microbial or fungi cultures (average power drawn per unit volume ~1000 W·m\(^{-3}\) vs. ~10 W·m\(^{-3}\) respectively) current bioreactors are still able to provide enough oxygen to meet animal cell needs (Varley and Birch, 1999; Ma et al., 2006). However, with the advent of perfusion cultures, fortified media and improved fed-batch culture strategies, it is expected that cell concentrations will increase by an order of magnitude (Kompala and Ozturk, 2006). Therefore, an equivalent increase in oxygen transfer rate (OTR) must be achieved to supply enough oxygen to the culture.

The OTR is determined from the product of the mass transfer coefficient, \( k_La \) [s\(^{-1}\)], and the driving force, which is the difference between the saturated dissolved oxygen
concentration in the medium, $C_{O_2}^*$, and the actual dissolved oxygen concentration in the broth, $C_{O_2}$:

$$\text{OTR} = k_{L}a \cdot (C_{O_2}^* - C_{O_2})$$  \hspace{1cm} (Eq. 2.1)

The saturating dissolved oxygen concentration in the medium in equilibrium with the gas phase, is determined by:

$$C_{O_2}^* = \frac{y_{O_2} \cdot P_T}{H}$$  \hspace{1cm} (Eq. 2.2)

where $y_{O_2}$ is the molar fraction of oxygen in the gas (0.21 for air), $P_T$ is the total pressure of the gas and $H$ is the Henry’s constant which is a function of temperature and the composition of the medium. A simple inspection of Eq. (2.1) reveals that in order to increase OTR it is necessary to increase either $k_{L}a$ or $C_{O_2}^*$ since $C_{O_2}$ can only been reduced to a minimum threshold to avoid productivity issues as explained before. An increment of $C_{O_2}^*$ can be achieved by an increase in pressure of the gas (Eq. 2.2), but this action also results in an increment in the concentration of dissolved $CO_2$ and this may result in inhibitory effects. OTR also may be increased up to 5 times by increasing the molar fraction of oxygen in the gas using oxygen-enriched air or even a separate stream of pure oxygen into the broth. While this strategy is commonly used in bench bioreactors, it will lead to increased levels of dissolved $CO_2$ originating additional concerns about pH control and osmolality (Nienow, 2006). Therefore, probably the most prudent way to further increase the OTR is by increasing $k_{L}a$. 

21
Although \( k_L a \) can be affected by several different factors, most proposed correlations in literature (some of them presented in Table 2.2) adopt the form of an equation dependent on two parameters that are independent of the impeller type, media composition, and scale:

\[
\begin{align*}
  k_L a &= A \cdot \left[ (\bar{\epsilon}_g)^\alpha \right] \cdot [V_s]^\beta \\
\end{align*}
\]  

(Eq. 2.3)

where \( \alpha \) and \( \beta \) are approximately \( 0.5 \pm 0.1 \), respectively (Nienow, 2006). \( V_s \) is the surface velocity of the gas, defined by Eq. (2.6), and \( \bar{\epsilon}_g \) is the average total energy dissipation rate drawn into the liquid by both the impeller \( \bar{\epsilon}_g \) and the gas sparging \( \bar{\epsilon}_s \):

\[
\bar{\epsilon}_g = \bar{\epsilon}_g + \bar{\epsilon}_s
\]  

(Eq. 2.4)

The energy delivered to the system from air sparging can be calculated from:

\[
\bar{\epsilon}_s \approx V_s \cdot g \cdot \rho
\]  

(Eq. 2.5)

where \( g \) is the acceleration due to gravity (9.81 m/s\(^2\)), \( \rho \) is the liquid density and \( V_s \) is the superficial velocity, given by:
where \( Q \) is the air flow rate \([\text{m}^3 \cdot \text{s}^{-1}]\), \( T \) is the vessel diameter \([\text{m}]\), \( V_R \) is the volume of medium in the reactor \([\text{m}^3]\) and \( \phi \) is the air flow rate expressed as v.v.m. Since cell damage as a result of bubble bursting (see cell damage section) is typically minimized at low air flow rates, \((\varepsilon_T)_s\), can often be neglected for stirred tank reactors used for animal cells.

The energy drawn into the liquid by one impeller under gassed conditions, \((\varepsilon_T)_{lg}\), can be estimated as:

\[
(\varepsilon_T)_{lg} = P_{0g} \cdot \frac{\rho \cdot N^3 \cdot D^5}{V_R} \tag{Eq. 2.7}
\]

where \( N \) is the impeller speed \( [\text{s}^{-1}] \), \( D \) is the impeller diameter \([\text{m}]\) and \( P_{0g} \) is the power number under aerated conditions, which depends on the Reynolds number, the impeller type and the gas flow rate. For typical culture conditions, the properties of the fluid are quite similar to those of water, so the flow is essentially turbulent \((\text{Re} = \rho_L \cdot N \cdot D^2 / \mu \sim 10^5)\); in that case, \( P_{0g} \) becomes independent of the Reynolds number; additionally, as the gas flow rate \( (\phi) \) is very low, typically ranging from 0.005 to 0.01 v.v.m. (Nienow et al., 1996), \( P_{0g} \) is essentially equal to the Power number under non-gassed conditions \((P_0)\). In
spite of that, the relationship between $P_{0g}$ and $P_0$ is typically important in impeller selection. Values for $P_0$ are presented in Table 2.3 for impeller typically used in animal cell bioreactors (Nienow, 2006). Further details about $P_0$ and $P_{0g}$ are presented in the mixing section.

The term $A$ in Equation (2.3), and therefore $k_{L,a}$, are very sensitive to composition. For example, by introducing ions in water up to a limiting concentration of $\sim 10$ g/L NaCl, Van’t Riet (1979) showed that $k_{L,a}$ can easily increase by a factor of 100% respect to the value for distilled or tap water at the same conditions of $(\sigma_T)_{lg}$ and $V_S$; the presence of ions strongly changes the coalescent characteristic of pure water, increasing drastically the interfacial area. In contrast, Metz et al. (1979) showed that in a stirred bioreactor with otherwise constant conditions (400 rpm, $V_s = 30.6$ m·h$^{-1}$) the addition of 0.5 ppm of a surfactant reduced the $k_{L,a}$ from $\sim 180$ to $\sim 115$ h$^{-1}$. Similar results were presented by Morão et al. (1999) for three different types of antifoaming substances. Addition of Pluronic F-68 also lowers $k_{L,a}$ (Lavery and Nienow, 1987); therefore, caution should be taken when choosing a particular correlation for designing purposes.

### 2.3.1. Surface aeration

Oxygen transfer through the headspace is, obviously, the most common and easy method of gas exchange used for small vessels at the laboratory scale, including T-flasks, spinners, roller bottles and small bench-scale reactors. The maximum volume at which surface aeration can be used alone depends on a series of factors such as cell
concentration, whether or not the headspace gas is enriched with oxygen and whether or not bubble entrainment is allowed (if the system is agitated). However, a general principle which holds for scale-ups is that the area per unit volume for mass transfer decreases with the reciprocal of the diameter of the vessel. To alleviate this problem at moderate scale, the use of surface impellers (an impeller close to the liquid-gas interface) can significantly increase the OTR as a result of an increased renewal of the liquid directly in contact with the gas. If bubble entrainment occurs, a further increase in OTR can be achieved as a result of the significant increase in the interfacial surface. Working with a 500 ml spinner vessel, Aunis et al. (1989) reported a 50% increase in the OTR by moving the impeller within 0.13 times the impeller diameter of the liquid surface. Hu et al., (1986) reported an increase in the oxygen supply up to 4 times in their reactor by using an additional surface impeller. Another approach for high values of surface OTR involves the use of special impeller types inducing bubble entrainment such as helical and double helical ribbons together with surface baffles (Kamen et al., 1992).

A variation in the surface aeration approach is the disposable bag reactors (i.e. the Wave® bioreactor), which can range in size from 100 ml to 300 L. This system achieves sufficient OTR by means of a rocking motion that induces a wave within the bag that is sufficient to suspend the cells, mix the medium and provides a virtually bubble-free oxygen transfer through the rapid turnover of the medium surface. Singh (1999) presents data for Wave reactors from 100 ml up to 100 liter, with corresponding $k_{L}a$ values ranging from 1.6 to 4 h$^{-1}$. While for small scale these values of $k_{L}a$ are in the same range as for other surface aerated reactors, it is in the lower end of the range for sparged,
mechanically stirred reactors (Lavery and Nienow, 1987). However, for volumes larger than 10 liter the $k_{L}a$ in a Wave reactor can still up to one order of magnitude larger than for a spinner or reactor of comparable size; consequently, it is reported to be able to support cell growth up to concentrations of $7 \times 10^6$ cell ml$^{-1}$ (Singh, 1999).

### 2.3.2. Perfluorocarbons

Perfluorocarbons (PFCs) are hydrocarbons whose hydrogen atoms are partly or totally replaced by fluorine. They are hydrophobic and immiscible in water based media; therefore, the addition of PFCs yields a four-phase (two liquid, one solid and one gas phase) mixture in sparged bioreactors. Because of the much higher solubility of oxygen in PFCs than in water (see Table 2.4), there has been an active interest in using them as gas vectors to improve oxygen transfer and CO$_2$ removal in bioreactors. The benefits of using PFCs are summarized by Lowe et al. (1998) and presented in Table 2.5, together with their main disadvantages.

Several studies have been conducted with animal cell cultures in which the medium was supplemented with perfluorocarbon emulsions. Cho and Wang (1988) employed perfluoromethyldecalin to oxygenate mouse-hybridoma cells, resulting in increased cell density and rate of Mab synthesis. Using the PFC FC40™, Ju and Armiger (1992) found that the exponential growth phase of mouse hybridoma cells was prolonged, along with a more than six-fold increase in viable cell concentration, which was consistent with theoretical predictions based on the enhanced oxygen transfer. Although the high
concentration of surfactants needed to keep PFCs in suspension might be deleterious for
cells or for downstream operations, such effects have not been reported. It has also been
suggested that the formation of emulsions itself might be problematic for recycling and
re-oxygenation. Gotoh et al. (2001a; 2001b) proposed two interesting alternatives to
solve this potential problem with emulsions: they developed a loop column reactor
having a wetted-wall of PFCs to culture insect cells (Sf9). With this arrangement, the
oxygen was transferred by direct contact of the broth with the liquid film created on the
annular wall of the column. They reported a two-fold increase in the viable cell density
and specific growth rate of the cells as well as a five-fold reduction in the specific lactate
yield. In the second permutation, the same authors grew cell suspensions using a planar
layer of PFC at the medium-PFC interface in a spinner flask and the PFC was
recirculated and re-oxygenated in an outer aeration unit. With this arrangement, the
maximum cell density attained in the PFC-mediated aeration culture was higher than that
in a surface aeration control culture; also, the recombinant protein production by virus-
infected insect cells was significantly increased. The PFC itself did not seem to cause any
negative effects on insect cell growth, viral infection, or recombinant protein production.
However, in spite of all the previous advantages, PFCs introduce additional cost and the
potential problems in downstream processing have kept PFC so far as only a research
curiosity (Varley and Birch, 1999).
2.3.3. Oxygen carriers

At least two other oxygen carriers, in addition to PFC, have been suggested for use to enhance oxygen supply in cell culture: cyanobacterial gas vesicles (GVs) and hemoglobin (Sundararajan and Ju, 2006). While the use of hemoglobin as an oxygen carrier has only been tried in microbial cultures and it has been limited by its high cost and by its high affinity for oxygen (i.e. it requires long residence times in the reactor to effectively release the bound oxygen), GVs have actually been tested in animal cell culture. GVs in cultures of Vero cells on microcarriers have been reported to increase by 30% the glucose uptake rate although no effect on cell concentration was described. GVs can be produced at a relatively low cost from simple cyanobacterial cultures, they do not require any suspension stabilizing agent (unlike PFCs), and they have more than twice the oxygen carrying capacity as PFCs (See Table 2.4). However, they can not be autoclaved; consequently, sterilization is cumbersome and time-consuming (Sundararajan and Ju, 2006). It remains to be seen if a commercial application will be found for GVs.

2.3.4. Membrane aeration

In membrane aeration, the membrane provides enough interfacial area for oxygen diffusion while it acts as a physical barrier that keeps cells away from gas-liquid interfaces, eliminating any cell damage related to bubble-bursting phenomena or cell entrapment in foam. This methodology has been reported to give high oxygen transfer rates (i.e.~0.5 g O₂·l⁻¹·h⁻¹) that are comparable to sparging, although they require higher
gas pressure and flow rates (Moreira et al., 1995; Lütz et al., 2006). Ma et al. (2006) categorize the different types of membranes into two groups: a microporous type and a diffusional type. The microporous type typically is a thin membrane made up of nylon, polyvinylidene fluoride, polyester, etc., with pore sizes ranging from 0.01 μm to about 20 μm and where the medium is in direct contact with air in the micropores of the membrane. The liquid-gas interface is held stationary via both hydrophobic forces and gas pressure. In the diffusional type of membrane, the oxygen diffuses through the actual membrane material. Some of the most common examples are silicon tubing and silicon hollow-fiber membrane oxygenators.

There are reports of the use of membranes for oxygen transfer up to 150 L (Lehmann et al., 1988; Vorlop et al., 1989); however, their use at larger scales is problematic. While there is always the risk of rupture, clogging, and fouling of the membranes, these risks significantly increase during scale-up since one is faced with classical surface-to-volume ratio problems, i.e. as the volume of the vessel increases, one needs to increase the surface area of the membranes, typically tubing, even faster. At some point, the amount of tubing required becomes unmanageable, especially when one considers the challenge of cleaning and sterilizing large vessels (Kretzmer, 2002; Ma et al., 2006).

2.3.5. Sparging

Despite all of the alternatives reviewed above, the most commonly used way to introduce and remove gaseous nutrients and byproducts from animal cell culture, after simple head
space mass transfer, is by sparging the gas directly into the medium. While potentially catastrophic effects can occur (i.e. cell death of the whole culture in a few hours), through the use of specific additives the destruction of a majority of the cells can be prevented. More on both the mechanism(s) of death and prevention will be presented later.

2.3.6. Sparger design

Proper selection of a gas sparger involves a compromise between maximizing gas transfer (O₂ and CO₂), minimizing cell damage and foam formation, and complying with GMP considerations (easily cleanable, steam sterilizable and free draining). Ideally, spargers should be designed to disperse bubbles sufficiently to avoid coalescence into larger bubbles as they rise within the vessel (Marks, 2003); however, excessive pressure drop across the orifice may create sufficient hydrodynamic forces to inhibit net cell growth (Murhammer and Goochee, 1990) and therefore should be avoided.

There are three commonly used designs in cell culture: the point sparger, ring sparger and frit sparger (Marks, 2003). Point sparger is the simplest design consisting of a pipe ending in a nozzle through which air is introduced into the vessel; its simplicity makes it ideal for cleaning and sterilization. However, the moderate control of the bubble size that can be achieved by changing the nozzle diameter, gas flow rates and position (depth) of the nozzle (Kulkarni and Joshi, 2005) results in the formation of large bubbles. Although Orton and Wang (1991) suggested the use of macrospargers producing large bubbles (diameter in the range of 6-8 mm) for minimizing foaming and bubble-related cell death,
the low surface per unit volume created with these spargers results in the use of higher flow rates in order to provide enough oxygen to the culture.

To address the high flow rates needed when a single point sparger is used, yet still follow GMP guidelines, one might add a number of air nozzles drilled at the bottom of a ring, allowing for free drainage from the tube. While an improvement with respect to a reduction in air flow through a single hole, it has been argued that this enhancement is not sufficient to justify the added expense and cleaning difficulties (Marks, 2003). Recently, Harris et al. (2005) presented a new approach for a multi-point sparger design called the multiple slot disperser which consists of an assembly of flat, parallel arrays of slot nozzles. In water it produced a dense, three-dimensional plume of air bubbles with a consistently narrow bubble size distribution and a relatively sharply defined median bubble diameter. Most importantly, the plume attributes may be modified within wide limits by changing the slot width, plate and array dimensions as well as the gas flow rate. Additionally, the method of construction is highly adaptable, avoiding the need for pores or drilled holes and it is relatively easy to clean and autoclave.

A significant alternative to the single or multiple hole disperser, is the well known and used frit sparger which utilizes porous materials (ceramic, sintered stainless steel or Teflon) to create an almost unmeasureable amount of very small bubbles within the bioreactor. These bubble are nearly ideal for gas transfer because of the large interfacial gas-liquid area they provide and the extended time of contact as a result of lower rising velocities of the smaller bubbles. However, disadvantages of these spargers include
cleaning difficulty, more-stable foam and reduced CO₂ stripping capability, forcing in some cases the use of both a frit and a large bubble sparger and the use of antifoaming chemicals (Marks, 2003). The cleaning challenge can be so significant that the authors know of at least one commercial process in which the frit sparger is replaced after every batch. Documenting some of these reported advantages and disadvantages, Nehring et al. (2004) tested both a stainless steel porous sparger and a porous ceramic (TiO₂) microsparging system for the cultivation of MDCK cells from a 2 to 100 liter scale. The ceramic system produced bubble diameters of 100 – 500 μm, and values of kₜ,a up to 50 h⁻¹, 3 times higher than those produced by the stainless steel sparger at the same gas flow rate; however, formation of compact, stable foam and pH problems were observed. Similarly, Chisti (1993), found the use of a multi-hole sparger to be preferable over a porous metal sparger for scale-up of a bioreactor as a result of foaming problems caused by the smaller bubble size generated by the fritted sparger.

2.3.7. CO₂ accumulation and removal

Somewhat ironically, as animal cell culture progressed from a research scale to relatively large scale, commercial process, the addition of oxygen became less of a concern and the removal, and in some cases the initial addition, of CO₂ became more problematic. This results from a number of issues as a consequence of the complex role CO₂ plays in cell culture. Carbon dioxide is a nutrient needed for synthesis of nucleic acid components such as purines and pyrimidines (Smith et al., 2005); therefore, low levels of this component may inhibit cell growth. Alternatively, CO₂ is also a byproduct of aerobic
metabolism and high levels of dissolved CO\textsubscript{2} are inhibitory for cells and have been reported to strongly affect cell growth and metabolism (deZengotita \textit{et al.}, 1998; Mostafa and Gu, 2003; Zhu \textit{et al.}, 2005). To further complicate the situation, carbon dioxide forms part of a complex buffer system given by the next set of equilibrium reactions:

\[ CO_2(g) \leftrightarrow CO_2(\text{dissolved}); \quad [CO_2]_{\text{dissolved}} = \frac{pCO_2}{\gamma} \quad (\text{Eq. 2.8}) \]

\[ CO_2(\text{dissolved}) + H_2O \leftrightarrow H_2CO_3; \quad K_h = \frac{[H_2CO_3]}{[CO_2]_{\text{dissolved}}} = 1.70 \times 10^{-3} \quad @ 25^\circ C \quad (\text{Eq. 2.9}) \]

\[ H_2CO_3 \leftrightarrow H^+ + HCO_3^-; \quad K_{a1} = \frac{[HCO_3^-][H^+]}{[H_2CO_3]} = 2.5 \times 10^{-4} \text{ mol l}^{-1} \quad @ 25^\circ C \quad (\text{Eq. 2.10}) \]

\[ HCO_3^- \leftrightarrow H^+ + CO_3^{2-}; \quad K_{a2} = \frac{[CO_3^{2-}][H^+]}{[HCO_3^-]} = 5.61 \times 10^{-11} \text{ mol l}^{-1} \quad @ 25^\circ C \quad (\text{Eq. 2.11}) \]

Since sodium bicarbonate is typically used as a pH buffer in the culture medium, from the previous set of reactions it is easy to deduce that a change in the dissolved CO\textsubscript{2} concentration could lead to a change in the pH through the following relationship:

\[
[H^+] \approx \frac{10^{-14}}{[H^+]} + \frac{K_h \cdot K_{a1} \cdot pCO_2}{[H^+]} + 2 \cdot K_{a1} \cdot K_{a2} \cdot pCO_2 \cdot [H^+] \approx \sqrt{10^{-14} + K_h \cdot K_{a1} \cdot pCO_2 \cdot \gamma} \quad (\text{Eq. 2.12})
\]
where pCO$_2$ is the partial pressure of CO$_2$ in the exit gas in equilibrium with the medium. A rise of pCO$_2$ at a controlled pH typically leads to an associated increase in the medium osmolality as a result of both an increased dissolved CO$_2$ concentration and the addition of a base, such as sodium hydroxide, to maintain a constant pH. The combined influence of high osmolality and pCO$_2$ might conduce to an even more significant change in the metabolism of the cell. Finally, to add a final level of complexity to the situation, CO$_2$ is much more soluble than O$_2$, and this CO$_2$ solubility is sufficiently sensitive to pressure such that a significant gradient in CO$_2$ can exist between the top and bottom of a 1000 L, weakly mixed, animal cell culture bioreactor.

Several published examples of the effect of the concentration of dissolved CO$_2$ on a cell culture are reported next. In 1990, Drapeau et al. studied the effect of increasing dissolved CO$_2$ concentration from 53 to 165 mm Hg on recombinant CHO cells for the production of hM-CSF in a 2500 L reactor. At the highest pCO$_2$, the specific cell growth rate decreased by 52% and the specific production rate of hM-CSF dropped by 56% relative to the culture with the lower pCO$_2$. While the dissolved oxygen and pH were constant and equal in both cultures, it is unclear if the osmolality was kept constant. In 1996, Gray et al. also described an inhibitory effect in CHO cell density and specific production rate with reported reductions of 33% and 44%, respectively, in a system in which pure oxygen microbubbles using a fritted sparger or silicone diffusion aerators were used to transfer oxygen to the culture. Mostafa and Gu (2003) also reported a 40% drop in the specific production rate of a therapeutical glycoprotein produced by CHO cells when pCO$_2$ increased from 68 mm Hg at bench (1.5 l) scale reactors to 179 mm Hg
in pilot-plant (1000 l) bioreactors. In the last two cases, osmolality was not controlled so the real cause of the dramatic effects observed is potentially a combination of the effects of increased pCO₂ and osmolality.

To isolate the impact of pCO₂ and osmolality on CHO cell growth, Zhu et al. (2005) performed experiments in bench-scale bioreactors where they changed only one variable at a time. Raising pCO₂, from 50 to 150 mm Hg, under a controlled osmolality of approximately 350 mOsm·kg⁻¹ resulted in a 9% reduction in specific cell growth rate. Increasing osmolality from 316 to 450 mOsm·kg⁻¹ resulted in a linear reduction in specific cell growth rate (0.008 h⁻¹ per 100mOsm·kg⁻¹) up to 60%. The combined effects of high pCO₂ (140-160 mm Hg) and osmolality (400-450 mOsm·kg⁻¹) caused a 20% drop in viable cell density.

In contrast to CO₂ buildup as a result of low levels of gas-medium exchange, the high rates of aeration in bubble columns or airlift reactors may lead to excessive CO₂ stripping, which can have equally inhibitory effects (Birch et al., 1987). To solve this problem, the gas for aeration usually includes a certain amount of CO₂ to both keep the pH in a proper value and avoid a reduction of pCO₂ below 40-50 mm Hg, range usually considered optimal for animal cell culture (Gódia and Cairò, 2006).
2.4. MIXING

Mixing can be defined as “the operation in which two or more materials (gas, liquid and/or solid) are distributed throughout a mass in varying degrees of uniformity dependent upon the change and state to be accomplished” (Uhl and Gray, 1967). In animal cell bioreactors, this mixing is typically accomplished by the addition of energy to the broth in the form of mechanical agitation and gas sparging. Ideally, this energy should be delivered to the culture broth in a completely homogenous manner such that any fluid element, or in this case any cell, will experience a similar distribution of energy in a similar period of time. Also ideally, this energy delivery should be sufficient to distribute both the cells, and molecularly sized nutrients and by-products, in such a manner that no “process effecting concentration gradients” exist in the vessel. The term, “process effecting concentrations” is used since a concentration gradient is also a time dependent phenomena in a dynamic environment such as a bioreactor and the characteristic time scales need to also be considered. As it will be discussed below, far from ideal situations exist in most animal cell bioreactors.

In general, the hydrodynamic environment experienced by a particular cell in a culture is a function of its spatial position in the interior of the bioreactor, of the characteristics of the mixing system, and of time. With the exception of fungal and some microbial cultures, the rheological characteristics of the broth do not change dramatically with time, remaining essentially Newtonian and very similar to water. Depending on the final cell concentration and the potential release of nucleic acids into the culture, a slight increase
of the viscosity at the end of the culture may become apparent, but typically this is not considered important. However, in spite of the low viscosity, a poor design of the mixing system may lead to catastrophic consequences. If the mixing is not intense enough, cells will settle at the bottom of the reactor creating zones with limitations of mass and energy transfer and unsatisfactory homogeneity, rendering the control systems of the reactor inefficient and ending up with low viability and productivity. Even if agitation is sufficient to maintain a suspended culture, a number of reports exist of undermixed commercial animal cell culture process in which significant dissolved CO$_2$ concentration exist between the top and bottom of the bioreactor, and “zones” of relatively unmixed, high-pH “clouds” exist in the vessel after base addition for considerable periods of time. Langheinrich and Nienow (1999) measured pH in three different zones of an 8 m$^3$ bioreactor equipped with a 0.225$T$ Rushton turbine intended for the culture of mammalian cells; they found pH gradients close to 1 unit between the alkali addition zone and the impeller discharge when addition of the Na$_2$CO$_3$ was done on the liquid surface. Ozturk (1996) observed “snow-ball” aggregation of mammalian cells, corresponding to alkaline cell lysis, close to the headspace addition point of base in a poorly agitated bioreactor: the concept of an animal cell suspended in a “pool” of pH 9 or 10 is not a desirable thought!

On the other hand, it has long been feared, but substantiated with little data, that if suspended animal cell cultures are mixed too aggressively in typically designed bioreactors, the hydrodynamic forces experienced by the cells may inhibit cell growth, productivity, or have other undesirable effects. Unfortunately, historically, no universally
accepted algorithm or protocol allowing for the proper design of mixing systems for animal cell cultures has existed; therefore, an empirical, case-by-case process has typically been conducted at each organization (company) as an animal cell process was scaled up. Also, many (majority?) of these established processes are under mixed. While this approach has resulted in a number of organizations having significant “in-house” expertise, well documented, fundamentally based, peer reviewed scale-up protocols are still lacking. However, a sufficient amount of experimental data is beginning to accumulate which allows for the development of such scale-up algorithms.

The importance of mixing to animal cell culture is far from unique; in fact, specific professional societies, such as the North American Mixing Forum, are dedicated solely to understanding and optimizing mixing in the chemical process industry. Taken in a proper perspective, the challenge of mixing in animal cell culture is not nearly as complex as mixing in many other industries. Consequently, much can be learned from this community.

2.4.1. Stirred tank reactors

As mentioned before, for a number of reasons, not the least of which is the relative simplicity and considerable knowledge/data base, the classical stirred tank reactor is the predominate method to culture animal cells from bench scale up through large scale, commercial systems. While a complete review of stirred tank bioreactors is beyond the scope of this chapter, a quick summary will be presented. The interested practitioner is
encouraged to read more complete discussions on the topic (Oldshue, 1983; Hempel, 1988; Tatterson, 1991).

2.4.2. Geometry

A typical, stirred tank, animal cell bioreactor is a mixing vessel with an aspect ratio of tank height (H) to tank diameter (T) of approximately (H/T) = 2-3 (See Figure 2.1). This tank is penetrated by a shaft which extends down the center line of the tank; the outside end of this shaft is attached to a motor while the other end to one or more impellers which are suspended in the culture broth. Typically, the impeller diameter (D) is about \( \frac{1}{3} \) to \( \frac{1}{2} \) of the tank diameter. The tank is filled with broth up to a height (H_L) of ~ 0.7 H. The off-bottom clearance (C), is usually around ~ 0.1-0.3 T. In the case of multiple-impeller systems, the separation between impellers (ΔC) is highly variable as it will be discussed next, but normally is set around one impeller diameter.

2.4.3. Impeller

A number of different impeller designs exist and are used (see Figure 2.2), ranging from radial-flow impellers such as the traditional Rushton turbine used extensively in the mixing community to provide a high level of intense mixing by way of a high local shear rates, to axial-flow impellers like the marine propeller which provide better bulk movement of the broth, relative to the Rushton. Impeller selection is based on a series of considerations including its power number (P_0) and its pumping number (Nq). Impellers
with larger ratio $Nq/P_0$ are superior in producing better bulk mixing at lower shear rates. At the same average energy per unit volume drawn into the liquid $\left(\varepsilon_T\right)_I$, impellers with a larger diameter have a higher pumping capacity and therefore may have a better mixing performance. Impeller designs generating lower shear rates are thought to be superior in reducing alleged cell damage as a result of hydrodynamic forces. In this sense, hydrofoils are superior to pitched blade turbines (PBT) and PBTs are superior to Rushton turbines (Ma et al., 2006). Chen et al. (2003) presented a novel methodology to evaluate impellers based on dinoflagellate: when stimulated by hydrodynamic forces, these organisms generate light; at speeds corresponding to the same oxygen transfer coefficient, a Rushton turbine (150 rpm) generated a much higher luminescence (~20000 counts in 10 ms) compared to marine propellers (170 rpm; ~2000 counts in 10ms) indicating that much higher forces are created in the bioreactor with the first impeller.

In non-gassed conditions, the power drawn by an impeller is a function of Reynolds number, impeller type and its geometry. For impeller Reynolds numbers over $10^4$ (normal conditions in culture), the power number becomes independent of Reynolds and depends only on variables such as blade angle, number and width, baffle number and width, and clearance ratio ($C/D$).

Equation (2.7) governs the power drawn by a single impeller under gassed conditions. In such circumstances, gas reaching the impeller is driven to the back of the blades (see Figure 2.3) of the impeller generating air cavities that are stabilized by the lower pressure in such zone and reduce the amount of power the liquid is able to transmit to the broth;
depending on the amount of gas, the size of such cavities may grow to a point where they
surround the whole impeller; this phenomena is known as flooding and reduces
drastically the amount of power the impeller is able to draw into the liquid because now
the impeller is practically rotating in air. Characteristic curves of $P_{0g}/P_0$ are presented in
Figure 2.4; observe that impellers like the Scaba, are much less prone to flooding than the
Rushton turbine and the reduction in power drawn is limited or non-existant as a result of
their design.

When the tank is sufficiently large, multiple impellers can be attached to a single shaft,
with the potential to use a combination of impellers (i.e. both a Rushton and a marine
type) to improve the mixing performance. Under non-sparged conditions, the power
drawn into the liquid by the different impellers depends on the distance between them.
Hudcova et al. (1989) established that in the case of Newtonian, low-viscosity media, the
power drawn in non-gassed conditions by two Rushton turbines is maximized when the
impellers are placed apart as a minimum ~1.8 times the impeller diameter because the
flow patterns generated by each impeller practically do not interfere each other (see
Figure 2.1); in such case, the total power drawn into the system is simply the sum of the
power drawn by each impeller. Similar results were obtained by Machon et al. (1985) for
a diversity of combinations of impellers. Under gassed conditions, however, there is an
uneven distribution of the gas between the impellers: the lower impeller draws less power
than the upper one and usually works as a gas disperser. A similar behavior (uneven
power drawn) is observed when the separation of the impellers is increased until a
maximum is reached when the impellers are apart a distance greater than $2D$; in this
situation, the prediction of the total power drawn into the liquid is much more complex. Correlations proposed by Cui et al. (1996) to calculate the power drawn by individual impellers in gassed systems with multiple (1-3) Rushton turbines are presented in Table 2.6; in this case, the bottom and top impellers are presented as drawing the same power. Linek et al. (1996) also developed gassed power correlations for a system with four Rushton turbines; the interested reader is advised to consult their work.

2.4.4. Baffles

To further improve the effectiveness of the impeller, baffles are used, typically up to four, in which case they are placed perpendicular to the tank wall at 90 degree increments around the vessel. The width of the baffles influences the power drawn into the liquid and therefore the mixing performance, as presented in Figure 2.5; a typical width of the baffle is on the order of 10% of the tank diameter.

2.4.5. Mixing times and energy dissipation rates

As mentioned before, many bioreactors have been designed assuming the high “shear sensitivity” of the cells, which translates on poor mixing performance and a correspondingly lack of homogeneity. In turbulent systems under non gassed conditions, the time required for mixing the content of a bioreactor up to a certain degree of homogeneity (usually 95% of the final response), which is referred to as the mixing time,
\( \theta_m \), is independent of the impeller type and can be related to design and operational parameters by the next set of equations (Ma et al., 2006):

\[
\theta_m = 5.3 \cdot \left[ N^{-1} \cdot P_0^{-1/3} \cdot \left( \frac{T}{D} \right)^{2} \right] \quad \text{for } H/L/T = 1 \quad \text{(Eq. 2.13)}
\]

\[
\theta_m = 3.3 \cdot \left[ N^{-1} \cdot P_0^{-1/3} \cdot \left( \frac{T}{D} \right)^{2.43} \right] \quad \text{for } H/L/T > 1 \quad \text{(Eq. 2.14)}
\]

where \( \theta_m \) will be in the units of the inverse of the impeller rotational speed. Eq. (2.13) correlates very closely for a wide variety of impellers with an almost identical equation derived by Kresta et al. (2006), except the coefficient changes from 5.3 to 5.8. For clarity purposes, Eq. (2.13) can be rearranged by assuming a cylindrical tank \( V_r = \left( \frac{\pi}{4} \right) \cdot T^2 \cdot H_L \) and using Eq. (2.7) to give:

\[
\theta_m = 57.4 \cdot \left( \frac{T}{D} \right)^{1/3} \cdot \left[ \left( \frac{\varepsilon_T}{g} \right)_g \right]^{1/3} \cdot T^{2/5} \quad \text{for } H/L/T = 1 \quad \text{(Eq. 2.15)}
\]

where \( \theta_m \) will be in seconds when the remaining variables are in SI units. For the derivation of equation (2.15), it was assumed that the density of the fluid is very close to that of water (1000 kg·m\(^{-3}\)) and that usual gas flow rates are so low in animal cell culture that \( P_0g \approx P_0 \) and \( (\varepsilon_T)_g \approx (\varepsilon_T)_g \). Equation (2.15) is identical to the obtained by Nienow (1997) and indicates that during scale up (increase of \( T \)) for geometrically similar tanks (\( T/D \) constant), the mixing time can be kept constant only if the power input per unit volume is increased. In contrast, the concern of potential hydrodynamic cell damage has
resulted in a majority of large scale animal cell bioreactors to be designed with power per unit volume significantly decreased relative to laboratory scale vessels, ending up in increased mixing times and relatively poor mixing.

One concept commonly used in the mixing industry is the energy dissipation rate, or $EDR$, which has units of power per unit volume (i.e. $W\cdot m^{-3}$). On a bulk, or average basis, this is a very appealing parameter since the average energy dissipation rate, $(\varepsilon_g)$, is just the rate of energy added to the system divided by the volume in which the energy is delivered. From a fundamental point of view, the $EDR$, $\varepsilon$, can be determined using the following, first principle, equation (Bird et al., 2006; Brodkey, 1995):

$$\varepsilon \equiv \tau : \nabla U$$

(Eq. 2.16)

where $\tau$ is the stress tensor and $\nabla U$ is the velocity gradient tensor. For an incompressible Newtonian fluid, Equation (2.16) becomes (Brodkey, 1995):

$$\varepsilon \equiv \tau : \nabla U = \mu \left[ \nabla U + (\nabla U)^T \right] : \nabla U = \mu \sum_i \sum_j \left[ \nabla U_g + (\nabla U)^T \right]_{ij} \nabla U_{ji}$$

(Eq. 2.17)

In Eq. (2.17), $\mu$ is the viscosity of the medium, $\nabla U^T$ is the transpose of $\nabla U$ and $\nabla U$ is the gradient of the velocity vector given by Eq. 2.18.
Equation (2.16) can be derived from first principles using the second law of thermodynamics. More specifically, Equation (2.16) emerges from the loss term in the energy balance equation. Much like the Navier-Stokes equation, Equation (2.16) is valid for any flow regime, be it laminar, transitional, or fully turbulent.

It is this dual nature, a pragmatic value (the power of the mixing motor turning the impeller shaft going into the bioreactor divided by the number of liters in the vessel) and the fundamental, first principle definition, that has lead to the use of EDR to characterize the flow conditions acting on cells for over thirty years (Blustein and Mockros, 1969) and has been extensively used in the fluid mechanical/mixing community for even longer (Kresta et al. 1998).

As it might be imagined, but not typically fully appreciated, the flow in a stirred tank reactor is highly inhomogeneous. Qualitatively, this was demonstrated in the 1970’s visually by demonstrating that very strong vortices emanate from the tips of the impeller blades; such vortices are responsible, for example, for gas dispersion in the impeller blades (See Figure 2.3). These qualitative observations have been quantified by experimental studies, using a variety of experimental techniques to demonstrate that the
Local energy dissipation rate in the impeller region, and specifically in these vortices, has been reported to be over $10^3$ times higher than the local EDR in the bulk fluid away from the impeller (Cutter et al. 1966; Costes and Couderc, 1988; Zhou and Kresta, 1996; Mollet et al. 2004).

Given the importance that high, local EDR levels have on rapid, molecular mixing, Zhou and Kresta (1996a, 1996b) conducted a significant number of quantitative studies on the maximum and distribution of EDR as a function of a number of variables in stirred tank vessels, including: tank volume, impeller type, and impeller rotational speed (RPM). These studies were, in general, consistent with other published studies. Several important conclusions that are relevant to animal cell bioreactors can be made:

1. The energy dissipation rate is always high in the impeller discharge stream. Specifically, 43.5% of mechanical energy added to the vessels is dissipated in the impeller discharge region of a Rushton turbine, and 70.5% in a pitched blade turbine (PBT). In addition, the “average” energy dissipation rate is over an order of magnitude higher in the impeller region when compared to bulk average for the whole vessel.

2. For a given impeller type, the maximum, local energy dissipation rate in the impeller region can be approximated using a non-dimensional constant and to a specific, non-dimensional location relative to the impeller. This non-dimensional constant, $\psi$, (our notation) is given by Eq. 2.19.
\[ \psi = \varepsilon_{\text{max}} / \rho \cdot N^3 \cdot D^2 \]  

(Eq. 2.19)

In Eq. 2.19, \( D \) is the impeller diameter and \( N \) is the impeller speed with units of \( s^{-1} \). If specific geometric ratios are held constant (including the type of impeller), \( \psi \) did not vary significantly over a large range of impeller speeds. If the \( D/T \) (impeller diameter/vessel diameter) or \( C/T \) (off-bottom clearance/vessel diameter) ratio changed, within certain limits, up to a 46% increase in the value of \( \psi \) was observed when working with a RT. These observations also held for the PBT impeller.

Using this relationship for the maximum EDR in a stirred tank vessel, along with the classical \( P_0 \) and \( P_{0g} \), one can approximate the mean and maximum, local EDR as one scales the stirred tank mixing, keeping the \( H_l/T, D/T, \) and \( C/T \) ratios nearly the same and using the same type of impeller. Figures 2.6, 2.7, and 2.8 present predictions of vessel size and dimensions, maximum local EDR, and average EDR. Specifically, Figure 2.6 presents the change in the tank diameter and impeller diameter as the volume of the vessel increases from 0.5 to 10,000 liters keeping geometrical ratios constant; Figure 2.7 presents lines of constant, maximum EDR as a function of RPM and impeller diameter, and Figure 2.8 presents the average EDR for the whole vessel as a function of impeller diameter and RPM.

Alternatively, Figure 2.9 presents the mean and the maximum EDR, calculated respectively with the power number (Eq. 2.7) and the maximum EDR relationship (Eq. 2.19, using a value of \( \psi = 13.7 \)), as a function of impeller rotational speed. This plot
corresponds, specifically to a 2L Applikon bioreactor which was mixed with a standard Rushton Turbine impeller. The circle and square data points correspond to specific values of EDR measured with a particle tracking technology (Mollet et al. 2004).

While all of this discussion on EDR is helpful, a significant question should be raised with respect to how EDR relates to cells. This will be addressed in the next section.

2.5. THE RELATIONSHIP OF ANIMAL CELLS TO HYDRODYNAMIC FORCES

There is probably no subject in animal cell culture cultivation that is more misunderstood and misquoted as being a “major limitation” to large scale animal cell culture than the “shear sensitivity” of the cells. This misunderstanding, at least partially, arose from early reports (Bryant et al., 1960; Telling and Elsworth, 1965) whose significance will be discussed later on. It also seems logical to think that a sufficiently intense force could disrupt the integrity of the cell membrane causing cell death; especially when animal cells lack a “cell wall” compared to robust bacteria. To further reinforce this perception, there are clear, well established studies which do demonstrate almost complete cell destruction as a result of purely hydrodynamic forces and forces associated with gas sparging.

This misunderstanding has had a significant, negative impact on the field in the past, and its legacy continues to this day in both the perception of many cell culturists, as well as in
the under mixed, large scale animal cell culture bioreactors that are currently in operation in many facilities. To begin establishing some structure to the notion of the “shear sensitivity” of animal cells, a number of concepts need to be further defined and refined:

1. The term “shear stress” is misleading. In reality, except for specific studies in specifically designed vessels, at the most fundamental level cells are subjected to time varying shear and elogational forces in typical bioprocessing equipment. In addition, it has been argued that elogational forces are more damaging to cells than shear forces (Garcia-Briones and Chalmers, 1994).

2. There is a big difference between the hydrodynamic forces needed to affect or kill a cell attached to a surface relative to a cell in free suspension. This difference will be quantified below.

3. There is big difference in the potential to damage cells when gas-medium interfaces are present relative to the absence of any gas-medium interface, and this is especially true when protective surfactants are not present. This also will be discussed later on.

4. The sensitivity of animal cells to hydrodynamic forces can vary from a cell line to another, and even within different clones of the same cell line; however these differences have not been documented to prevent most cells of commercial interest to be cultured in bioreactors.

5. Due to the complexity of hydrodynamics, and especially under turbulent conditions, fundamental, first principle, mathematical models describing the hydrodynamic forces operating on cells in most bioprocessing equipment do
not exist and probably never will. Consequently, we are left with continually improving empirical models and experimental studies that allow us to develop reasonable scale-up methodologies.

With this introduction, what is known on the effect of hydrodynamic forces on cells, both attached and in free suspension as well as a scale-up methodology will be presented next.

2.5.1. Molecular mechanism involved in the response of animal cells to mechanical forces

Liquid-cell interactions resulting from fluid flow have been extensively studied, predominately with cells that normally experience fluid flow, such as endothelial cells, EC. Particularly interesting are models of the interactions of fluid forces with EC and their subsequent response to small changes of shear stress in laminar flow. Related to the EC response is that of smooth muscle cells (SMC) to cyclic stretch resulting from the pulsatile nature of blood flow. These studies provide models to begin understanding the physiological responses to mechanical forces inside the human and animal circulatory and respiratory systems. While it is challenging to extrapolate the results from experiments with EC/SMC cells in laminar flow chambers to non-EC/SMC cells in the much more complex nature of turbulent flow in bioreactors, the significant, non-lethal effects of hydrodynamic forces provide insight into potential mechanisms and responses to investigate.

50
Several excellent reviews, including those of Malek and Izumo (1994), Li et al. (2005), Lehoux et al. (2006), Tzima (2006), Li and Xu (2007), Chien (2007) and Haga et al. (2007), document what is known about the response of vascular endothelial cells to sub-lethal hydrodynamic forces. One of the hallmarks of the EC response appears to be the rearrangement of the cell’s cytoskeleton under the influence of fluid flow (Papadaki and Eskin, 1997). The cytoskeleton is a three-dimensional intracellular network of several different types of protein filaments (like actin) that determines the shape, mechanical properties and movements of the cell, such as those involved in muscular contraction or phagocytosis. The filaments interplay closely with the cellular and nuclear membranes by anchoring to several specific membrane proteins. When the cell is exposed to mechanical forces, the cytoskeleton attempts to resist them and undergoes deformation, specially if the cell is attached to a solid surface. Such deformation implies spatial reorganization of the cytoskeletal lattice; it is presumed that this reorganization is able to transmit the mechanical force along the whole cytoplasm and the nucleus. This mechanism suggests that the deformation of the cell brings together different cell components (i.e. proteins, enzymes, transcription factors and nucleic acids) in both the cytoplasm and the nucleus, originating a cascade of reactions that may lead to a wide diversity of cell responses.

A second proposed mechanism that allows cells to respond to fluid flow involves molecules at the luminal cell surface since they are in direct contact with the fluid. These molecules can be activated by direct conformational change as a result of flow (i.e. rupture or formation of disulfide bonds or bending/stretching of protein domains) or indirectly through mass transfer gradients. Such molecules include (Li et al., 2005):
1. **Integrins**: a family of more than 20 transmembrane heterodimers normally connected to specific extracellular ligands such as fibronectin, vitronectin and collagen. After the onset of flow, the integrin activation starts within 1 minute and last for more than 6 hours, originating an intracellular cascade involving kinases and cytoskeletal proteins.

2. **Ion channels**: shear stress increases Ca\(^{2+}\) influx; higher Ca\(^{2+}\) concentration can lead to multiple Ca\(^{2+}\) dependent cellular responses, including regulation of other channels (Cl\(^{-}\) and K\(^{+}\)). The response of this receptor is fast (within a minute) but subsides \(\sim 5\) minutes after the beginning of the stress.

3. **Receptor tyrosine kinases**: A series of surface proteins (like Flk-1) transiently activated by shear stress, resulting in their oligomerization, tyrosine phosphorylation, association with other proteins (i.e. Shc) and a series of consequent gene transcription events.

4. **GPCRs and G proteins**: G proteins are activated within 1 second after the onset of shear stress and they are involved in shear-induced Ras-GTPase activity that serves as molecular switches for a variety of cellular signaling events.

5. **PECAM-1**: Shear stress causes the transient phosphorylation of PECAM-1 within 1 minute, a glycoprotein expressed in EC, platelets and leucocytes that plays an important role in leucocyte aggregation and EC-leukocyte interaction.

6. **Membrane lipid bilayer**: Application of shear stress to EC cells causes an immediate (< 10 sec) increase in the fluidity of their membrane lipid bilayer in
the upstream direction followed by a secondary, larger increase reaching a peak at 7 minutes. Alternatively, the fluidity of the membrane is reduced on the downstream side of the cell, indicating that the EC membrane lipid bilayer can sense the applied shear stress with spatial discrimination.

After sensing mechanical forces, the transduction of shear stress to the interior of the cell ends up in a series of fast signaling cascades that include opening of K⁺ and Ca²⁺ channels, activation of heterotrimeric G proteins, production of NO⁻, tyrosine phosphorylation of proteins such as Shc, c-src, and focal adhesion kinase (FAK), activation of mitogen-activated protein kinase (MAPK), protein kinase C (PKC), and C-Jun-N-terminal kinase (JNK), release of reactive oxygen species (ROS), and activation of transcriptional regulators such as c-fos, c-jun, c-myc, and nuclear factor (NF)-κB. Slower responses include increased expression of genes for intercellular cell adhesion molecule (ICAM)-1, nitric oxide synthetase (NOS), platelet-derived growth factor (PDGF), tissue factor, transforming growth factor (TGF)-β, and monocyte chemoattractant protein (MCP)-1 and decreased expression of the vasoconstrictor endothelin 1 (Et-1) (Tzima, 2006). Figures 2.10 A and B describe the signaling cascade in EC/SMC that ends up in effects such as increased gene expression, proliferation or apoptosis, cell migration and alignment, increased cell membrane permeability and changes in the mechanical properties of the cell.
While these results are interesting from a molecular biology point of view, few of these observations have been confirmed in suspended cells of biotechnological interest. However, they do provide potential responses and mechanisms to investigate.

2.5.2. Cell damage concept

The ambiguity in defining the concept of damage may arise in the fact that conditions that generate biological responses in cells may not be the same that generate negative effects on the process from the point of view of yield or productivity. Consider, for example, the culture of murine neural stem cells (NSC) in 125 mL spinner flasks (Sen et al., 2001). Obviously, neural stem cells are not selected in nature to stand hydrodynamical forces as they grow in still environments and we might anticipate that increased hydrodynamical forces could affect cells’ behavior. However, Figure 2.11 shows that increasing agitation rate (starting from static culture in T-flask) increases significantly the number of viable cells, probably as a result of improved mass transfer and control of environmental variables inside the spinner; at the same time, viability is notoriously increased at higher stirring speeds and the mean diameter of aggregates is reduced, which translates into avoiding limiting conditions (specially oxygen) in the core of aggregates that could conduce to apoptosis or necrosis. The combined effect of an improved mass transfer and a decreased resistance to the transport of nutrients to every cell overcomes any negative effect of cellular damage that may exist, resulting in an increment of cell productivity. Quite similar results were obtained by Moreira et al.
(1995) for BHK cells grown as suspended natural aggregates, where again the maximum stirrer speed evaluated was 100 rev·min$^{-1}$.

On the other hand, several reports indicate that highly stirred environments can per se affect viability of suspended mammalian cell cultures. As an example, Hirtenstein and Clark (1980) showed that while agitation has a beneficial effect in the growth of Vero cells on microcarriers in spinner vessels, a further increase in stirrer speed after the optimum affected dramatically the cell concentration in the system (see Figure 2.12). Similarly, Kunas and Papoutsakis (1990) reported that stirrer speeds above 700 rev·min$^{-1}$ reduced cells’ viability on cultures of Hybridoma CRL-8018 when no bubbles are present, while not damage was seen at speeds less than 600 rev·min$^{-1}$. To further add complexity to the observations, Venkat et al. (1996) and Croughan et al. (1987) reported that 150-210 rev·min$^{-1}$ in spinner vessels can result in significant damage to CHO cells attached to microcarriers.

From the two previous paragraphs the obvious conclusion is the existence of a maximum threshold of stirring speed beyond which hydrodynamical stresses can have detrimental effects. However, how stirring speed relates to more fundamental properties of the fluid, the flow and the cells remains more of a experimental exercise than a theoretically one. For practical purposes, detrimental effects can extend beyond conditions causing cell death (reduced viability, increased necrosis or apoptosis) to poorer product quality or lower productivity as compared with an appropriate control.
2.5.3. Hydrodynamical cell damage

Reports detailing cell damage or death are abundant; the first reports appeared simultaneously with early attempts to scale-up cultures of mammalian cells (Bryant et al., 1960; Telling and Elsworth, 1965). Augenstein et al. (1971) passed cultures of HeLa S3 and mouse L929 through capillary tubes of different diameters. Cell death occurred during the residence time of cells in the capillary and it could be correlated to average wall shear stress (10-200 N·m⁻²) as well as the power dissipated. Midler and Finn (1966) reported that damage to shear-sensitive protozoa cells in a uniform shear device showed a two-phase behavior: a rapid primary damage followed by a slow decline of viable cells. This indicates that not only the magnitude of the hydrodynamic force but also the exposure time should be taken into account when considering cell damage.

A diversity of methodologies have been used in the literature to assess the effects of mechanical forces on cells; the most representative are presented in the Table 2.7. Rheometers and parallel-plates laminar flow chambers have been often used to study cell sensitivity to shear stress, as the stress tensor can be defined with great precision and can be kept constant for long periods in those devices; Joshi et al (1996) presents an extensive review of the use of rheometers on animal cells and outline a number of limitations of these devices, including:

- Limited or null oxygenation capacity restricts experiments to short periods not representative of long cell cultures.
- Settling of cells forces continuous mixing and also restricts experiments to short intervals.

- Normal stresses (i.e. extensional flow) can not be applied to cells, even though it has been suggested that they can be as harmful or even more damaging to cells than shear (Taylor, 1934; Garcia and Chalmers, 1994; Gregoriades et al., 2000).

- Hydrodynamic stress fluctuations, normally found in turbulent flows, may be even more damaging that steady shear stresses. Although laminar flow chambers are suitable to apply such fluctuations, the ability of rheometers to exert them is limited.

- It is difficult to apply the results obtained within these devices to figure out proper conditions for the cells inside bioreactors, given the much greater complexity of the turbulent flow and the wide range of hydrodynamic forces that a single cell may experience along the culture time.

Because of the previous limitations, studies performed in stirred tanks are often preferred from a design point of view (see Table 2.7). Unfortunately, the wide diversity on geometrical configuration of bioreactors and conditions used for such studies makes it extremely difficult to draw conclusions about what levels of hydrodynamic forces cells are able to withstand. As it was described previously, the quantitative determination of meaningful values of hydrodynamic forces in turbulent systems such as stirred tanks requires the measurement of velocity profiles along the whole bioreactor, which is a hard task to do because it requires expensive equipment and the measurements are limited to a few points inside the vessel. For that reason, authors normally resort to try to explain
their results based on a series of parameters, some of them based on fluid-dynamics theory, some of them strictly empiric. Some of those parameters are presented in Table 2.8 and a rationalization for their use will be presented in the next section.

Harmful effects other than cell lysis or death caused by hydrodynamic forces are often referred as sub-lethal; studies focusing on sub-lethal effects in bioprocessing equipment have been limited and sometimes contradictory. Al-Rubeai et al. (1990) found increased glucose consumption and mitochondrial activity under intense agitation and in the absence of air bubbles. Al-Rubeai et al. (1995) found that in addition to cell death under conditions of intensive agitation (1500 rpm), sub-lethal effects included lost of microvilli on the cell’s surface and changes in cycle distribution of the cell population. Al-Rubeai et al. (1993) and Lakhotia et al. (1992) reported changes in DNA synthesis rate at sub-lethal conditions; in contrast, Passini and Gooche (1989) did not find any effect of sublethal stress on DNA synthesis. Mufti and Shuler (1995) observed that human hepatoma cells attached to microcarriers and grown in 50 mL spinners, responded to moderate levels of agitation by inducing a cytochrome P450 monooxygenase (CYP1A1) activity; CYP1A1 is involved in the oxidation of arachidonic acid, a substance whose metabolism has been observed to be altered by hydrodynamic stress in endothelial cells cultured in parallel-plate flow chambers (McIntire et al., 1987).
2.5.4. Quantification of Cell Damage

The often contradictory nature of sub-lethal hydrodynamic effects may be the result of confusion as several different observed events are classified within the same category. As an example, cells growing attached to a surface (surface culture or microcarriers) could experience similar phenomena as those described for endothelial cells; however, cells growing freely in suspended culture should be less likely to be affected by shear stresses as the cells now are free to rotate, reducing considerably the deformation of the cytoskeleton and varying continuously the surface exposed to the hydrodynamical forces. Consistent with this concept, Ma et al. (2002) experimentally demonstrated that CHO cells in suspension are four to five orders of magnitude less sensitive to a measure of extensional and shear stress, energy dissipation rate, EDR, than CHO cells attached to 200 μm microcarriers. Cherry and Papoutsakis (1988; 1989) invoked collision between microcarriers and between microcarriers and solid parts of the bioreactor (impeller and walls) as the main factors behind the increased sensitivity of cells growing in microcarriers; as a logical consequence, new parameters (turbulence collision severity and impeller collision severity) were proposed by the authors to correlate cell damage, parameters that can not be used properly in freely suspended cells as the inertia of free cells is much lower than that of microcarriers.

The core of the confusion in methodologies and parameters to asses cell damage is threefold. First, the difficulty to relate results obtained with laminar flow devices with those results obtained at the turbulent conditions prevalent in industrial bioreactors as a
result of our inability to properly characterize and quantify the bulk and local hydrodynamic in the vessel; second, the lack of a biological model explaining how the cells are affected by hydrodynamical forces and how that effect changes depending on characteristics such as cell type, age and mode of growth (i.e. anchorage dependent or suspended). Third, the lack of a relationship or mathematical model relating the first two points; such relationship should link the lethal and sub-lethal effects of hydrodynamical forces to both a hydrodynamic descriptor or parameter and to the time of exposure to it.

Having a single parameter (or at least a reduced set of them) that can be related to overall yield, productivity, cell growth, etc., is highly desirable from an engineering point of view to facilitate design and scale-up of new processes. Garcia and Chalmers (1994) suggested that “ideal” parameters should be independent of the geometric characteristics of the culture system as cells do not “sense” the average conditions of the bioreactor but the characteristics of the microenvironment surrounding each one of them at each time. Consequently, criteria as mean power input, agitation rate, impeller tip speed, impeller-based Reynolds number or integrated shear factor should not be used as they represent average values derived from the particular geometric configuration of every system and because it has been shown that it is not possible to hold constant more than one of these parameters at the same time during scale-up (Kossen, 1994).

While the idea proposed by Garcia and Chalmers (1994) results theoretically appealing, its real implementation is currently an impossible task; first, the microenvironment surrounding every cell is not only dependent on the position (three-dimensional) but also
is time-dependent as most industrial cultures are batch or fed-batch and therefore their properties change constantly; second, the turbulent nature of the fluid dynamics inside an stirred bioreactor further complicates the panorama, as its description requires a second-order stress tensor consisting of nine distinct stress vectors. Third, the behavior of every cell is likely to be history-dependent: if two cells obtained from the same culture were placed in exactly identical conditions they probably will behave different as their previous history is expected not have been the same in the past. Finally, a precise characterization of turbulent phenomena is out of reach: from a theoretical point of view, the number of unknown variables is greater than the number of equations, so turbulent flow can not be solved mathematically from first principles; from a practical point of view, available techniques are limited to a very few points at a time (laser Doppler anemometry, constant temperature wire anemometry); increasing the number of points evaluated makes the cost prohibitive or imposes limitations in the resolution of the velocity profiles (particle tracking velocimetry). Therefore, in the end it is necessary to rely on some kind of average (time-average, spatial-average) or on a very gross distribution of some factor to describe hydrodynamic phenomena inside a bioreactor.

The most often used parameter for analysis of mixing phenomena is the Kolmogorov’s mixing microscale, $\eta$, which is a function of the ratio between the turbulence intensity (represented by the local energy dissipation rate, $\varepsilon$, and the kinematic viscosity of the liquid, $\nu$, is given by equation 2.20.
Kolmogorov’s mixing microscale (also denominated eddy size) was derived from classic dimensional analysis of turbulence by Andrey Kolmogorov in 1941; his theory of isotropic turbulence proposed that energy introduced into the liquid by the impeller undergoes a cascade from large, energy-containing scales to small, energy-dissipative scales. In the smallest scale, the fluid forms eddies where kinetic energy is ultimately converted into heat dissipation. As the Kolmogorov microscale depends only on fluid’s and flow’s properties and not on the system’s geometry or large-scale flow patterns, it is a good candidate for general parameter to correlate cell damage. In fact, it has been proposed that cell damage occurs whenever the eddy-length $\eta$, reaches the size of a freely suspended cell or the size of a microcarrier, depending on the culture type. Good predictions have been made in small-scale vessels; however, this parameter suffers from several weaknesses: it requires turbulent flow (so it can not be properly applied to laminar flow); additionally, it has been shown experimentally that turbulence is not really isotropic although one can always assume “local” isotropy. Finally, experimental determination of $\varepsilon$ is somewhat difficult; this last requisite usually has been overlooked by assuming that the energy transferred by the impeller is drawn into a limited volume around the impeller, although the precise size of that volume tends to be “determined” by every author so it matches his own results; since experimental measurements indicate that the energy dissipation is more concentrated in the very small volumes associated with the trailing vortices emanating from the impellers, as it was discussed before, Aloi and

$$\eta = \left(\frac{\nu^3}{\varepsilon}\right)^{\frac{1}{4}}$$  \hspace{1cm} (Eq. 2.20)
Cherry (1996) obtained a fairly good agreement between the results obtained in capillary flow and those obtained in bioreactors.

In contrast to the Kolmogorov’s mixing microscale, the Energy Dissipation Rate (EDR) is a scalar parameter that is independent of the flow regime (turbulent/laminar) and accounts for both shear and extensional components of three-dimensional flow. EDR represents the rate at which work is done on a fluid element or, for the purpose of this review, a cell (Clay, 1997). The EDR has been widely used to quantify local mixing performance in stirred tanks (Kresta, 1998) as well as laminar flow devices (Mollet et al., 2004; Mollet et al., 2007); even Kolmogorov’s mixing microscale is based on the local energy dissipation rate. Therefore, it seems quite appealing to use this parameter to correlate the hydrodynamical cell damage in diverse devices. Although EDR can not be measured directly, it can be calculated easily from Equations (2.17) and (2.18) provided that the broth behaves like an incompressible, Newtonian fluid (common situation in animal cell culture where the medium has water-like properties) and that the velocity vector has been accurately determined. This last requirement, however, limits the application of EDR to systems where the determination of the velocity vector $U$ has been done experimentally or it can be determined analytically (laminar flow).

Ma et al. (2002), designed and manufactured a microfluidic device in which suspended cells could be exposed to acute, high levels of EDR in a laminar flow environment; the advantages of this device include very well defined hydrodynamical characteristics and the possibility of emphasize the potentially more damaging extensional flow, although
some degree of shear is present. Using such device, they subjected an insect cell line (Sf-9), a Chinese Hamster Ovary cell line (CHO), a mouse hybridoma cell line (HB-24) and a human breast cancer cell line (MCF-7) to single exposures of EDR and measured the release of the cytoplasmic enzyme lactate dehydrogenase (LDH) as an indication of cell membrane rupture. Every one of the cell lines studied showed significant cell lysis beginning around an EDR of $10^7$ W·m\(^{-3}\) as observed in Figure 2.13. This is in agreement with previous results from Zhang and Thomas (1993), who needed average energy dissipation rates of $10^8 – 10^9$ W·m\(^{-3}\) to completely disrupt TB/C3 hybridoma and NS1 myeloma cells when passing in turbulent flow through capillary tubes.

To better express their results, Ma et al. (2002) developed a one-dimensional plot whose improved version is presented in Figure 2.14. As it can be appreciated, the values of EDR required to cause significant lysis in industrial cells are at least two orders of magnitude higher than the maximum EDR measured under normal conditions in bioreactors. Mollet et al. (2007) developed an improved, autoclavable second-generation microfluidic device to further investigate if besides the evident levels of necrosis already reported, the effect of single exposures to high levels of EDR could lead to apoptosis. In addition to being consistent with the previous work by Ma et al. (2002) in the levels of necrosis obtained after passing the cells once through the micro-device, Mollet et al. (2007) observed that only a small fraction of Chinese Hamster Ovary cells (CHO-K1) became apoptotic when exposed to sublethal levels of EDR.
In a related subject, Mollet et al. (In Press) used the improved microfluidic device as well as computer fluid dynamics (CFD) commercial software to simulate the single passage of cells through the nozzle of a commercial fluorescence activated flow sorter (FACS). In the FACS the cells experience high levels of EDR as a result of hydrodynamic focusing in the nozzle, with levels of cell necrosis on the order of 20 to 35% using CHO cells under typical operating conditions. They also found that non-adapted cells (THP1) may be much more sensitive to hydrodynamic stress than adapted, industrial cell lines (see Figure 2.13).

2.5.5. Detrimental effects of sparging

It is a well known fact the dramatic reduction in cell number and viability of animal cells caused by air bubbling in sparged reactors (Handa et al., 1987; Oh et al., 1989; Kunas and Papoutsakis, 1990a; Kioukia et al., 1996). Even under conditions of unusually high stirring speeds, Oh et al. (1989) and Kunas and Papoutsakis (1990a, 1990b) showed that as long as there is no air introduction into the culture medium (i.e. sparging or bubble entrainment from the central vortex), hybridomas could withstand stirring speeds as high as 600 rpm without detectable cell damage.

The specific cause responsible for cell damage as a result of cell-bubble interactions may be attributed to four different events: bubble formation and detachment at the sparger, bubble break-up and coalescence in the impeller trailing vortices, bubble rising through
the fluid or bubble disengagement at the surface of the culture. In the next lines we present a brief summary of the results of research on each of these potential events.

Significant cell damage occurring near the sparger was suggested by Tramper et al. (1987) who estimated that shear stress produced as a result of bubble formation and detachment could be up to one order of magnitude higher than that of bursting bubbles. Murhammer and Goochee (1990) noticed an inverse correlation between the pressure drop across the sparger and the net growth of insect cells in airlift reactors, and suggested that the damage could be concentrated in the sparger zone as a result of the turbulent oscillation of the detaching bubbles or the fast liquid movement to fill in the space vacated by them.

In a series of insightful experiments under identical aeration and agitation conditions, Oh et al. (1992) showed that bubble break-up as a result of the interaction of sparged air with the impeller is significant for cell damage. By comparing the results of placing the sparger in several positions, the authors showed that sparging air directly under the impeller led to an increase in the number of smaller bubbles and also to an increment in cell death, although it is difficult to say if it was caused by damage in the impeller region or just because increased bubble bursting in the medium-air interface.

Kioukia et al. (1992) grew hybridoma cells in a 2.5 L reactor stirred with a Rushton turbine and oxygenated only by surface aeration. In experiments with baffles in the reactor, successful growth and similar kinetic results were obtained at all speeds up to
400 rpm. In contrast, in experiments without baffles the peak cell density and growth rate decreased with increased speeds and cell death occurred from the start of the batch culture even though the average energy dissipation rate was only 20% of that for baffled conditions. The authors argued that the cause for this behavior was the entrainment of air bubbles in the bulk of the liquid from and their further disengagement through the vortex surface; furthermore, since the bubbles never reached the impeller region, the damage could not be related to bubble break-up/coalescence in the impeller and only was associated with the bubbles bursting at the medium-air interface.

Tramper et al. (1987) and Glasgow et al. (1992) showed that values of shear stress derived from rising bubbles are extremely low (~0.1 Pa) and should, therefore, be neglected. To confirm these results, Handa-Corrigan et al. (1989) and Jöbses et al. (1991) introduced air in bubble columns of different sizes. With all other variables being constant, increasing column height, and thereby increasing the distance a bubble had to travel to rise to the top of the column, did not result in a reduction of viability for several cell types tested (hybridoma, BHK-21, myeloma and lymphoblastoid cell lines).

Several researchers suggest that bubble-induced cell damage takes place only when a bubble, at the gas-liquid interface, ruptures (Handa et al. 1987; Bavarian et al., 1991; Trinh et al., 1994). Phenomenologically, a bubble at a gas-liquid interface rises to a particular height above the flat liquid surface while the remaining part is submerged; the extent of the submerged part depends on the diameter. During rupture, the liquid drains by gravity from the film covering the raised part of the bubble up to a point where the
film is so thin that a hole develops on the top and the bubble burst; then, the liquid rapidly moves down the walls of the bubble cavity until the liquid reaches the bottom of the cavity; the resulting impact creates simultaneously upward and downward jets. Several studies indicate that this process creates a very high energy dissipation rate over the very short period of time in which the rupture occurs. This bursting process was qualitatively presented by MacIntyre (1972), in a series of cinephotography-derived profiles of the liquid-gas interface during the bubble break-up event. Subsequently, Boulton-Stone and Blake (1993) and Garcia-Briones et al. (1994) reported the results of computer simulations of bubble break-up with simulated profiles very close to those photographed by MacIntyre (1972); their model indicates that the film at the top of the bubble retreats at speeds as high as ~8 m·s⁻¹. Of more significance, the calculated maximum energy dissipation rate is many orders of magnitude higher than those typically generated by an impeller. Additionally, such maximum EDRs are a function of the bubble radius being larger for smaller bubbles, indicating that smaller bubbles are potentially more damaging to cells. Interestingly, this result has been observed experimentally in several different studies (Handa-Corrigan et al., 1989; Jöbses et al., 1991).

The maximum energy dissipation rates (W·m⁻³) calculated by Garcia-Briones et al. (1994) during bubble break-up ranged from 9.52×10⁷ and 1.66×10⁷ for bubbles with diameters of 0.77 and 1.70 mm, respectively, to 9.4×10⁴ for a 6.32 mm bubble. Inspection of Figure 2.14 indicates that these higher EDR values are well within the range of EDR values that were able to kill cells in the flow contraction device. This consistency between the simulated values of EDR during a bubble rupture, the values of
EDR shown to kill cells in the flow contraction device and the significantly lower EDR values in the impeller region provide an overall consistent picture that cell damage occurs due to bubble rupture at the liquid surface and the foam layer. The remaining question is whether enough cells are killed due to bubble bursting to account for all cell death one can observe in a sparged culture.

To address this question a statistically significant study showed that on average ~10³ cells are killed per 2 mm bubble burst (Trinh et al., 1994) and the cell concentration collected from the upward jet is twice the bulk concentration in the culture. This result is in agreement with observations by Chalmers and Bavarian (1991) and García-Briones and Chalmers (1992), who using video microscopy detected cell attachment and accumulation on gas-liquid interfaces, especially in rising bubbles and the foam layer in the surface of the liquid in the reactor.

Parker and Barsom (1970) reported that the chemical composition of the film surrounding air-liquid interfaces contained more hydrophobic materials compared to the bulk water; therefore, the phenomena of cell attachment to such film (microlayer) could potentially be explained as a result of the hydrophobic characteristics of the cell membrane. Chattopadhyay et al. (1995) attempted to explain thermodynamically the cell adhesion to the gas-liquid interface and suggested a model for the variation of free energy as a function of the surface tension of the interfaces involved (gas-liquid-cells). According to their model, modifying the gas-liquid surface tension via chemical additives may create a condition where the change in free energy becomes positive and so the adherence stops.
being spontaneous. While a number of different surface active agents have been studied, including serum, dextran, hydroxyethyl starch, polyethylene glycol or polyvinyl alcohol, Pluronic F-68 continues to be the additive of choice. Pluronic F-68, is a block copolymer of Poly(oxyethylene) and poly(oxypropilene) and it is added to the culture medium in concentrations of 0.5-3 kg·m⁻³ (Chisti, 1999).

In addition to reduce or inhibit cell adhesion to the microlayer (Chalmers and Bavarian, 1991; Garcia-Briones and Chalmers, 1992), by changing either the bubble film (Jordan et al., 1994; Michaels et al., 1995) or the cell membrane properties (Wu, 1996; Wu et al., 1997), other mechanisms have been proposed to explain the reduction or virtual elimination by surfactants agents of cell death as a result of bubbling; they include:

1. Interaction of the surfactant with the cell membrane, reducing its fluidity and rendering it stronger to mechanical stresses. (Zhang et al., 1992; Ramirez and Mutharasan, 1990b).
2. Nutritive effects of the surfactant on the cells. As mentioned by Ma et al. (2006), the short time needed for detection of protective effects after addition of most chemical substances suggests that this mechanism of protection is not important.

In spite of the success of Pluronic F-68, a word of caution is in order: as cell densities increase, the effectiveness of F-68, even at high concentration, diminishes. A recent study by Ma et al. (2004) demonstrated that even at high concentrations, a significant number of cells can be retained in the foam layer. Figure 2.15 is a figure from that report and as it
can be observed, at cell concentrations greater than $10^7$ cells·ml$^{-1}$ (which can be routinely obtained in industrial cultures) and relatively high F-68 concentration, more than 200 cell/bubble are removed into the foam layer.

2.6. SUMMARY

As it has been reviewed above, much like many other technological advancements, the practical and/or commercial development of processes based on animal cells has preceded the more fundamental understanding. However, significant progress has been made on not only how those processes work but also on how to design and operate them based on either fundamental understanding or well justified correlations. Unlike in the early 1980s when industrialist had to scale up based on “rules of thumb”, it is now possible to design and operate bioreactors based on a significant amount of published, peer reviewed studies that, in general, present a consistent message.

Because of the relatively low mass transfer demands of, at least, the majority of past and current processes, industrial cell cultures have been “forgiving” of poor mixing conditions. However, just as fungal processes to make antibiotics have made tremendous increases in productivity over the last 60 years through both strain development and high density culture systems, there is every reason to believe the same will happen with industrial animal cell cultures. Therefore, it is entirely possible that the “forgiving” process of today will not be good enough for processes of the future. At some point, a situation will be reached when Pluronic F-68 will lose its effectiveness as the cell density
increases and the purely hydrodynamic conditions detrimental to suspended cells will need to be determined in order to adjust mixing conditions so the culture can be carried out successfully.
2.7. REFERENCES


<table>
<thead>
<tr>
<th>$q_{O_2}$ (mol oxygen·cell$^{-1}$·s$^{-1}$)</th>
<th>Cell line</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.8×10$^{-17}$ to 6.9×10$^{-17}$</td>
<td>Hybridoma NB1</td>
<td>Boraston et al. (1983)</td>
</tr>
<tr>
<td>5.3×10$^{-17}$ to 1.1×10$^{-16}$</td>
<td>Hybridoma AB20143.2</td>
<td>Miller et al. (1987); Miller et al. (1988)</td>
</tr>
<tr>
<td>4.2×10$^{-17}$ to 1.0×10$^{-16}$</td>
<td>Hybridoma KS1/4</td>
<td>Backer et al. (1988)</td>
</tr>
<tr>
<td>9.2×10$^{-17}$ to 1.0×10$^{-16}$</td>
<td>Hybridoma HB-32</td>
<td>Ramirez and Mutharasan (1990)</td>
</tr>
<tr>
<td>6.4×10$^{-18}$ to 2.4×10$^{-17}$</td>
<td>Hybridoma 167.4G5.3</td>
<td>Ozturk and Palsson (1990)</td>
</tr>
<tr>
<td>6.4×10$^{-17}$ to 1.2×10$^{-16}$</td>
<td>Hybridoma X-D</td>
<td>Hiller et al. (1991)</td>
</tr>
<tr>
<td>1.67×10$^{-16}$</td>
<td>Sf-9</td>
<td>Hensler and Agathos (1994)</td>
</tr>
<tr>
<td>8.6×10$^{-17}$ to 1.0×10$^{-16}$</td>
<td>Sf-9</td>
<td>Wong et al. (1994)</td>
</tr>
<tr>
<td>1.28×10$^{-16}$</td>
<td>Hybridoma MAK</td>
<td>Zhou and Hu (1994)</td>
</tr>
<tr>
<td>6.08×10$^{-17}$ to 1.13×10$^{-16}$</td>
<td>NS0</td>
<td>Yoon and Konstantinov (1994)</td>
</tr>
<tr>
<td>6.5×10$^{-17}$</td>
<td>Hybridoma C1a</td>
<td>Dorresteijn et al. (1994)</td>
</tr>
<tr>
<td>5.6×10$^{-17}$</td>
<td>Hybridoma HFN7.1</td>
<td>Eyer et al. (1995)</td>
</tr>
<tr>
<td>5.53×10$^{-17}$</td>
<td>CHO</td>
<td>Gray et al. (1996)</td>
</tr>
<tr>
<td>8.0×10$^{-17}$ to 1.6×10$^{-16}$</td>
<td>Trichoplusia ni BTI-Tn-5B1-4</td>
<td>Rhiel et al. (1997)</td>
</tr>
<tr>
<td>2.5×10$^{-17}$ to 4.5×10$^{-16}$</td>
<td>Sf-9</td>
<td>Rhiel et al. (1997)</td>
</tr>
<tr>
<td>3.3×10$^{-17}$ to 5.3×10$^{-17}$</td>
<td>Sp2/0-Ag14 derived cells (ATCC CRL-1581)</td>
<td>Sauer et al. (2000)</td>
</tr>
<tr>
<td>3.3×10$^{-17}$ to 1.2×10$^{-16}$</td>
<td>B-lymphocyte hybridoma (CC9C10)</td>
<td>Barnabé and Buttler (2000)</td>
</tr>
<tr>
<td>2.3×10$^{-17}$ to 1.7×10$^{-16}$</td>
<td>GS-NS0</td>
<td>deZengotita et al. (2000)</td>
</tr>
<tr>
<td>9.0×10$^{-17}$</td>
<td>CHO SSF3</td>
<td>Ducommun et al. (2001)</td>
</tr>
<tr>
<td>5.6×10$^{-17}$ to 2.2×10$^{-16}$</td>
<td>CHO K1 (ATCC CCL 61)</td>
<td>Carvalhal et al. (2003)</td>
</tr>
<tr>
<td>5.3×10$^{-17}$ to 8.9×10$^{-17}$</td>
<td>T-CHO ATIII</td>
<td>Deshpande and Heinzle (2004)</td>
</tr>
<tr>
<td>1.1×10$^{-16}$ to 2.6×10$^{-16}$</td>
<td>Sf9 (ATCC 1711)</td>
<td>Palomares et al. (2004)</td>
</tr>
</tbody>
</table>

**Table 2.1.** Specific oxygen uptake rates ($q_{O_2}$) reported for animal cell lines.
<table>
<thead>
<tr>
<th>Correlation</th>
<th>Units</th>
<th>System information</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>( k_{L, a} = 93.6 \cdot \left( \frac{\rho_T}{\rho_{L, g}} \right)^{0.4} \cdot \left( V_s \right)^{0.5} )</td>
<td>([k_{L, a}] : h^{-1}) &lt;br&gt;([\left( \frac{\rho_T}{\rho_{L, g}} \right)] : W \cdot m^{-3}) &lt;br&gt;([V_s] : m \cdot s^{-1}) &lt;br&gt;([V_R] : \text{liter})</td>
<td>Water &lt;br&gt;(2 &lt; V_R &lt; 4400) &lt;br&gt;(5.0 \times 10^2 &lt; \left( \frac{\rho_T}{\rho_{L, g}} \right) &lt; 1.0 \times 10^4) &lt;br&gt;(0.5 \times 10^{-2} &lt; V_s &lt; 4 \times 10^{-2})</td>
<td>Van’t Riet (1979)</td>
</tr>
<tr>
<td>( k_{L, a} = 7.2 \cdot \left( \frac{\rho_T}{\rho_{L, g}} \right)^{0.7} \cdot \left( V_s \right)^{0.2} )</td>
<td>([k_{L, a}] : h^{-1}) &lt;br&gt;([\left( \frac{\rho_T}{\rho_{L, g}} \right)] : W \cdot m^{-3}) &lt;br&gt;([V_s] : m \cdot s^{-1}) &lt;br&gt;([V_R] : \text{liter})</td>
<td>Water plus ions. &lt;br&gt;(2 &lt; V_R &lt; 4400) &lt;br&gt;(5.0 \times 10^2 &lt; \left( \frac{\rho_T}{\rho_{L, g}} \right) &lt; 1.0 \times 10^4) &lt;br&gt;(0.5 \times 10^{-2} &lt; V_s &lt; 4 \times 10^{-2})</td>
<td>Van’t Riet (1979)</td>
</tr>
<tr>
<td>( k_{L, a} = 0.255 \cdot (T)^{0.2} \cdot \Lambda \cdot \Gamma ) &lt;br&gt;( \Lambda = (H_L)^{1.3} \cdot \left( N \right)^{0.5} ) &lt;br&gt;( \Gamma = (D)^{2.3} \cdot \left( V_s \right)^{0.6} )</td>
<td>([k_{L, a}] : h^{-1}) &lt;br&gt;([T] : m) &lt;br&gt;([D] : m) &lt;br&gt;([H_L] : m) &lt;br&gt;([N] : \text{RPM}) &lt;br&gt;([V_s] : m \cdot h^{-1})</td>
<td>Water plus ions. &lt;br&gt;(100 \text{ liter} &lt; V_R &lt; 100000 \text{ liter})</td>
<td>Asay and Kono (1982)</td>
</tr>
<tr>
<td>( k_{L, a} = 125 \cdot \left( \frac{\rho_T}{\rho_{L, g}} \right)^{0.72} \cdot \left( V_s \right)^{0.7} ) (See Note)</td>
<td>([k_{L, a}] : h^{-1}) &lt;br&gt;([V_R] : \text{liter}) &lt;br&gt;([T] : m) &lt;br&gt;([H_L] : m) &lt;br&gt;(\left( \frac{\rho_T}{\rho_{L, g}} \right) : W \cdot m^{-3}) &lt;br&gt;([V_s] : m \cdot s^{-1})</td>
<td>2000 &lt; ( V_R &lt; 8000) &lt;br&gt;0.3 &lt; ( H_L &lt; 1.3) &lt;br&gt;( T = 2 \text{ m}) &lt;br&gt;( D = 0.45 \text{ m}) &lt;br&gt;( 1 \times 10^{-4} &lt; V_s &lt; 5 \times 10^{-4}) &lt;br&gt;(5.0 \times 10^2 &lt; \left( \frac{\rho_T}{\rho_{L, g}} \right) &lt; 1.0 \times 10^4)</td>
<td>Langheinrich et al. (2002)</td>
</tr>
</tbody>
</table>

**Note:** This correlation was derived by the authors of this review exclusively from data in Figures 3 and 5 of Langheinrich et al. (2002)

**Table 2.2.** Examples of empirically derived equations for \( k_{L, a} \) estimation found in literature.
<table>
<thead>
<tr>
<th>Impeller</th>
<th>Po&lt;sub&gt;t&lt;/sub&gt;</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>45° pitched blade turbine (4 blades, w/D=0.1. Baffled tank)</td>
<td>0.88</td>
<td>Tatterson (1991)</td>
</tr>
<tr>
<td>45° pitched blade turbine (4 blades, w/D=0.3. Baffled tank)</td>
<td>1.69</td>
<td>Tatterson (1991)</td>
</tr>
<tr>
<td>Marine Propeller (Square pitch, 3-bladed)</td>
<td>0.32</td>
<td>Chisti (2001)</td>
</tr>
<tr>
<td>Lightnin A310 hydrofoil</td>
<td>0.31</td>
<td>Chisti (2001)</td>
</tr>
<tr>
<td>Rushton (6 blades, central disk, 10% baffled tank)</td>
<td>5.0</td>
<td>Tatterson (1991)</td>
</tr>
<tr>
<td>Paddle (2 blades)</td>
<td>1.70</td>
<td>Chisti (2001)</td>
</tr>
<tr>
<td>Scaba 6SRGT. (D/T=0.44)</td>
<td>1.6</td>
<td>Amanullah et al. (1998)</td>
</tr>
<tr>
<td>“Elephant ear” impeller (3 blades, unbaffled)</td>
<td>0.006</td>
<td>Menisher et al. (2000). (calculated)</td>
</tr>
<tr>
<td>Prochem Maxflo T. (6 blades, D/T=0.44)</td>
<td>1.3</td>
<td>Amanullah et al. (1998)</td>
</tr>
</tbody>
</table>

*Table 2.3. Power number for selected impellers for turbulent regime.*
<table>
<thead>
<tr>
<th>Substance</th>
<th>k (at 37°C) [mol O₂ · m⁻³ · MPa⁻¹]</th>
<th>k/k\textsubscript{water}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>9.7</td>
<td>1.0</td>
</tr>
<tr>
<td>Perfluorodecaline</td>
<td>164</td>
<td>16.9</td>
</tr>
<tr>
<td>Perfluorotripropylamine</td>
<td>176</td>
<td>18.1</td>
</tr>
<tr>
<td>Perfluorotributylamine</td>
<td>156</td>
<td>16.1</td>
</tr>
<tr>
<td>Gas vesicles</td>
<td>388</td>
<td>40</td>
</tr>
</tbody>
</table>

**Table 2.4.** Relative Oxygen carrying capacities (k) of water, perfluorochemicals and gas vesicles (Modified from Sundararajan and Ju, 2006).
<table>
<thead>
<tr>
<th>Benefits</th>
<th>Drawbacks</th>
</tr>
</thead>
<tbody>
<tr>
<td>High oxygen carrying capacity.</td>
<td>Dispersion is unstable requiring high amounts of surfactants and energy.</td>
</tr>
<tr>
<td>Ease of sterilization (e.g., by autoclave).</td>
<td>Surfactants may become toxic to cells.</td>
</tr>
<tr>
<td>Provide a two-phase (perfluorocarbon-liquid and aqueous medium) interface and physical support for cell division (in stationary cell culture).</td>
<td>Once suspension is formed it is difficult to separate PFCs for reoxygenation.</td>
</tr>
<tr>
<td>Chemically and biologically inert</td>
<td>Downstream concerns.</td>
</tr>
<tr>
<td>scavengers of gaseous cellular products (e.g. ethylene)</td>
<td>Expensive.</td>
</tr>
<tr>
<td>Recoverable and recyclable.</td>
<td></td>
</tr>
</tbody>
</table>

**Table 2.5.** Benefits and drawbacks of PFCs  (modified from Lowe et al., 1998).
<table>
<thead>
<tr>
<th>Condition</th>
<th>Impeller</th>
<th>Equation</th>
<th>Mean deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Q \cdot N^{0.25} \cdot D^{-2} \leq 0.055$</td>
<td>Bottom</td>
<td>$1 - \frac{P_{og}}{P_0} = 9.9 \cdot (Q \cdot N^{0.25} \cdot D^{-2})$</td>
<td>&lt;6 %</td>
</tr>
<tr>
<td>$Q \cdot N^{0.25} \cdot D^{-2} &gt; 0.055$</td>
<td>Bottom</td>
<td>$1 - \frac{P_{og}}{P_0} = 0.52 + 0.62 \cdot (Q \cdot N^{0.25} \cdot D^{-2})$</td>
<td>&lt;6 %</td>
</tr>
<tr>
<td>$Q \cdot N \leq 0.013$</td>
<td>Middle and Top</td>
<td>$1 - \frac{P_{og}}{P_0} = 37.6 \cdot (Q \cdot N)$</td>
<td>2.3 %</td>
</tr>
<tr>
<td>$Q \cdot N &gt; 0.013$</td>
<td>Middle and Top</td>
<td>$1 - \frac{P_{og}}{P_0} = 0.375 + 8 \cdot (Q \cdot N)$</td>
<td>1.4 %</td>
</tr>
</tbody>
</table>

Q: Gas flow rate (m$^3 \cdot $s$^{-1}$); N: Impeller rotational speed (s$^{-1}$).
Conditions for correlated data: 0.238<T<1.83 m; 1-3 impellers; 0.92<N<10 s$^{-1}$; D/T = 1/3–1/2; Fl = 0 - 0.26

Table 2.6. Correlations derived by Cui et al. (1996) for power drawn in systems with multiple Rushton impellers.
<table>
<thead>
<tr>
<th>Method</th>
<th>Selected references</th>
</tr>
</thead>
<tbody>
<tr>
<td>Laminar Flow in capillary tubes</td>
<td>Thomas et al. (1994)</td>
</tr>
<tr>
<td>Turbulent flow in capillary tubes</td>
<td>Augenstein et al. (1971)</td>
</tr>
<tr>
<td>Magnetic twisters</td>
<td>Wang et al. (1993)</td>
</tr>
<tr>
<td>Optical deformation</td>
<td>Guck et al. (2001)</td>
</tr>
<tr>
<td>Micromanipulation (poking) devices</td>
<td>Zhang et al. (1992); Goldman (2000)</td>
</tr>
<tr>
<td>Stretching devices</td>
<td>Graf et al. (2003)</td>
</tr>
<tr>
<td>Aspiration devices</td>
<td>Discher et al. (1994); Jones et al. (1999)</td>
</tr>
<tr>
<td>Cone and plate rheometers</td>
<td>Goldblum et al. (1990); Born et al. (1992); Graf et al. (2003)</td>
</tr>
<tr>
<td>Concentric cylinder rheometers</td>
<td>Schürch et al. (1988); Joshi et al. (1996); Mardikar and Niranjan (2000)</td>
</tr>
<tr>
<td>Laminar flow between parallel plates</td>
<td>Motobu et al. (1998); Keane et al. (2003)</td>
</tr>
<tr>
<td>Contracting flow devices</td>
<td>Gregoriades et al. (2000); Ma et al. (2002)</td>
</tr>
<tr>
<td>Stirred tanks</td>
<td>Telling and Elsworth (1965)</td>
</tr>
<tr>
<td></td>
<td>Hirtenstein and Clark (1980)</td>
</tr>
<tr>
<td></td>
<td>Croughan et al. (1987); Oh et al. (1989)</td>
</tr>
<tr>
<td></td>
<td>Kunas and Papoutsakis (1990a,b)</td>
</tr>
<tr>
<td></td>
<td>Oh et al. (1992); Thomas et al. (1994)</td>
</tr>
<tr>
<td></td>
<td>Sen et al. (2001)</td>
</tr>
<tr>
<td>Free jets</td>
<td>McQueen et al. (1987)</td>
</tr>
<tr>
<td>Bioluminescence</td>
<td>Chen et al. (2003)</td>
</tr>
<tr>
<td>Atomic force microscopy</td>
<td>Hoh and Schoenenberger (1994)</td>
</tr>
<tr>
<td></td>
<td>Mahaffy et al. (2000)</td>
</tr>
<tr>
<td>Pressure probe</td>
<td>Tomos (2000)</td>
</tr>
</tbody>
</table>

**Table 2.7.** Methodologies reported in literature for measurement of mechanical properties of animal cells and/or determination effect of hydrodynamical forces on animal cells.
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Definition</th>
<th>Selected references</th>
</tr>
</thead>
<tbody>
<tr>
<td>Agitation rate</td>
<td>$N$</td>
<td>Kunas and Papoutsakis (1990a)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Moreira et al. (1995)</td>
</tr>
<tr>
<td>Impeller tip speed</td>
<td>$\pi ND$</td>
<td>Middler and Finn (1966)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Croughan et al. (1987)</td>
</tr>
<tr>
<td>Mean power input</td>
<td>$\varepsilon = \frac{P}{V} = \frac{N_p \rho N^3 D^5}{V}$</td>
<td>Ujcová et al. (1980)</td>
</tr>
<tr>
<td>Integrated shear factor</td>
<td>$ISF = \frac{2\pi ND}{T-D}$</td>
<td>Sinskey et al. (1981)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Croughan et al. (1987)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chen et al. (2003)</td>
</tr>
<tr>
<td>Volumetric integrated shear factor</td>
<td>$VISF = \frac{2\pi ND \left( \frac{V_x}{V} \right)}{T-D \left( \frac{V}{V} \right)}$</td>
<td>Chen et al. (2003)</td>
</tr>
<tr>
<td>Kolmogorov eddy size</td>
<td>$\eta = \left( \frac{v^3}{\varepsilon} \right)^{1/4}$</td>
<td>Croughan et al. (1987)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cherry and Papoutsakis (1988)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Croughan et al. (1989)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Oh et al. (1989)</td>
</tr>
<tr>
<td>Local energy dissipation rate</td>
<td>$\varepsilon = \tau : \nabla U$</td>
<td>Bluestein and Mockros (1969);</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ma et al. (2002)</td>
</tr>
<tr>
<td>Shear stress tensor</td>
<td>$\tau$</td>
<td>Keane et al. (2003)</td>
</tr>
<tr>
<td>Turbulent collision severity</td>
<td>$TCS = (\varepsilon V)^{3/4} \left( \frac{\pi^2 \cdot \rho_b \cdot \alpha \cdot d_h}{72} \right)$</td>
<td>Cherry and Papoutsakis (1988)</td>
</tr>
<tr>
<td>Impeller collision severity</td>
<td>$ICS = \left( \frac{9 \pi^4 \cdot \rho_b \cdot n_b \cdot n^3 \alpha \cdot D \cdot d_h^4}{512 \cdot V} \right)$</td>
<td>Cherry and Papoutsakis (1988)</td>
</tr>
<tr>
<td>Energy dissipation-circulation function</td>
<td>$EDCF = \frac{\varepsilon}{t_e}$</td>
<td>Jüsten et al. (1998)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Rocha-Valadez et al. (2005)</td>
</tr>
</tbody>
</table>

**Table 2.8.** Some parameters reported in literature to correlate the effect of hydrodynamical forces on cells.
**Figure 2.1.** Geometrical configuration, flow patterns and total non-gassed power drawn into the liquid as a function of impeller spacing for a mixing system with multiple Rushton turbines (Adapted from Hudcova et al., 1989).
Figure 2.2. Impeller configurations commonly employed in animal cell culture.
Figure 2.3.  Regions of highest shear rate and highest energy dissipation rate behind the blades of a Rushton turbine.
Figure 2.4. Effect of the gas flow rate on the power drop under gassed conditions for two impeller geometries in Newtonian, water-like fluids. (Adapted from Galindo and Nienow, 1993).
Figure 2.5. Effect of the number and size of the baffles on the power drawn by an impeller in a cylindrical stirred tank reactor (Adapted from Oldshue, 1983).
Figure 2.6. Change in the tank diameter and impeller diameter as the volume of the vessel increases from 0.5 to 10,000 liters keeping constant the geometrical ratios H/T = 1 and T/D = 3.
Figure 2.7. Lines of constant, maximum EDR in a vessel as a function of impeller rotational speed and diameter for Rushton turbine in water. H/T = 1 and T/D = 3.
Figure 2.8. Average EDR for the whole vessel as a function of impeller diameter and RPM using a Rushton turbine in water. H/T = 1 and T/D = 3.
Figure 2.9. Calculated maximum and average energy dissipation rate as a function of RPM for an Applikon bioreactor containing four baffles. The single points correspond to experimental measurements without baffles (Adapted from Mollet et al., 2004).
Figure 2.10. Molecular signaling and response cascade in endothelial and smooth muscle cells (A) before and (B) after stimulation by hydrodynamic forces.
Figure 2.11. Effect of agitation rate on cell concentration, viability and aggregate diameter of murine NSC in batch suspension in a 125 mL spinner flask. Data points are average of duplicate runs. (a) Cell concentration: (●) 60 rev·min⁻¹; (■) 100 rev·min⁻¹. Viability: (○) 60 rev·min⁻¹; (□) 100 rev·min⁻¹. (b) Average aggregate diameter: (●) 60 rev·min⁻¹; (■) 100 rev·min⁻¹. Standard deviation: (○) 60 rev·min⁻¹; (□) 100 rev·min⁻¹. (Adapted from Sen et al. 2001).
Figure 2.12. Effect of stirring speed on cell concentration after 7 days of culture of Vero cells on Cytodex microcarriers on 250 mL spinner vessels (Data from Hirtenstein and Clark, 1980).
Figure 2.13. Experimental curves for the percentage of damage experienced by cells in a custom-design microfluidic device for single abuse experiments. Adapted from Ma et al. (2002), Mollet et al. (In Press) and Mollet et al., (submitted).
Figure 2.14. Summary of the reported energy dissipation rate at which cells are affected as well as the reported levels of energy dissipation rate in various bioprocess environments. Adapted from Ma et al. (2002) and Mollet et al. (2004).
### Hydrodynamic Conditions

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Process</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Agitation</td>
<td>Volume average in typical animal cell bioreactors.</td>
<td>Varley and Birch (1999)</td>
</tr>
<tr>
<td>2</td>
<td>Agitation</td>
<td>Volume average in a 10 L mixing vessel (RT, 700 RPM)</td>
<td>Zhou and Kresta (1996)</td>
</tr>
<tr>
<td>3</td>
<td>Agitation</td>
<td>Maximum in the 10 L mixing vessel (RT, 700 RPM)</td>
<td>Wernersson and Tragardh (1999)</td>
</tr>
<tr>
<td>4</td>
<td>Agitation</td>
<td>Volume average in a 22,000 L mixing vessel (RT, 240 RPM)</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>Agitation</td>
<td>Maximum in the 22,000 L mixing vessel</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Agitation</td>
<td>Maximum in spinner vessel (200 RPM)</td>
<td>Venkat et al. (1996)</td>
</tr>
<tr>
<td>7</td>
<td>Bubble rupture</td>
<td>Pure water, bubble diameter: 6.32mm</td>
<td>Garcia-Briones et al. (1994)</td>
</tr>
<tr>
<td>8</td>
<td>Bubble rupture</td>
<td>Pure water, bubble diameter: 1.7mm</td>
<td>Boulton-Stone and Blake (1993); Garcia-Briones et al. (1994)</td>
</tr>
<tr>
<td>9</td>
<td>Flow through a pipe</td>
<td>Pure water, 100 mL/min, 1 mm diameter</td>
<td>Mollet et al. (2004)</td>
</tr>
<tr>
<td>10</td>
<td>Flow through a micropipette tip</td>
<td>Flow through a 200 μL micropipette tip in 0.2 sec</td>
<td>Mollet et al. (2004)</td>
</tr>
<tr>
<td>11</td>
<td>Agitation</td>
<td>Volume average in a highly agitated animal cell bioreactor</td>
<td>Oh et al. (1992)</td>
</tr>
</tbody>
</table>

*Figure 2.14 (Continued).*
Figure 2.15. A three dimensional plot of the number of cells associated with each bubble as a function of cell concentration (cell·mL⁻¹) and Pluronic F-68 concentration. The dots indicated experimental data and the surface is the plot of a multiple variable regression. (Adapted from Ma et al., 2004).
CHAPTER 3

ACUTE HYDRODYNAMIC FORCES AND APOPTOSIS: A COMPLEX QUESTION

The content of this chapter was published previously: Mollet, M.; Godoy-Silva, R.; Berdugo, C. and Chalmers, J. J. (2007). Acute Hydrodynamic Forces and Apoptosis: A complex Question. Biotechnology and Bioengineering. 98(4):772-788. This document is founded on the original draft entitled “Does Acute Hydrodynamic Forces Induce Apoptosis?” written by Dr. Mike Mollet based on the Chapter 4 of his own thesis: “Physiological Effects of Hydrodynamic Forces on Animal Cells”. Sections Introduction (xxx), Contractional flow device (xxx), Cell culture (xxx), Contractional flow device experiments (xxx), Cell analysis studies (xxxx), LDH studies (xxx), FCM studies (xxx), Immunostaining for BCl-2 overexpressing cells (xxx), Necrosis/Lysis studies (xxx), as well as Figures 3.1, 3.2, 3.6, 3.7 and 3.8 were largely unchanged from Dr. Mollet’s manuscript. Claudia Berdugo produced the results, pictures and video showed in Figure 5 and Flow in entrance and exit on contraction device section (xx). Ruben Godoy’s contribution to this work includes:

1. Participation in all the experiments of the flow cytometry studies.
2. Dr. Mollet performed simulations only of ¼ of the microfluidic device because of its symmetry. Since the reviewers asked for it, the whole geometry of the microfluidic device was then built, simulated in Fluent and reanalyzed.

3. Whole design and writing of all Tables, Figures 3.3, 3.4, 3.9-3.14 and sections Abstract, Computational fluid dynamics simulations, Reynolds number, Estimations of mean and maximum EDR in bioflo reactor, Estimations of mean and maximum EDR in spinner vessel and References, as well as a significant portion of Apoptosis/Necrosis studies using FCM and Discussion sections.

4. Editing of the document and rebuttal to the reviewers.

3.1. ABSTRACT

A second generation flow contraction device was developed and modeled which allows cells to be subjected to well-defined hydrodynamic forces. Studies were conducted with this system on wild-type Chinese Hamster Ovary cells (CHO-K1) and a strain of CHO cells which expresses the human Bcl-2Δ gene (CHO-bcl2). In this study, the following questions were asked: (1) Does an acute hydrodynamic force induce apoptosis in wild-type CHO and CHO-bcl2 cells? (2) Does the type of culture media make a difference with respect to the induction of apoptosis or necrosis?, and (3) Does culture history affect induction of apoptosis or necrosis?. The results obtained with this new flow contraction device and corresponding computer simulations are consistent with previously published studies with respect to the level of Energy Dissipation Rate (EDR) required to create significant cell lysis. Second, while detectable relative to the control in the T-flask
experiments, only a small fraction of the cells become apoptotic when exposed to a sub-
lysis level of EDR (< $10^8$ W·m$^{-3}$). Third, cells cultured in suspension with serum free 
media do not exhibit any higher or lower sensitivity (with respect to apoptosis) to various 
levels of EDR when compared to control cultures grown in T-flask and serum containing 
media; on the other hand, necrosis is significantly increased in experiments performed on 
suspended cells without serum. Fourth, the addition of the Bcl-2 gene product might 
slightly reduce the occurrence of apoptosis in T-flask culture; however the baseline 
response is so low that the difference is insignificant.

3.2. INTRODUCTION

To maintain a highly productive cell culture, one obviously wants to prevent cell death 
(Mastrangelo et al., 2000a). Necrosis, or passive cell death, is usually caused by 
excessively high concentrations of toxic agents or by some external force which suddenly 
destroy(s) the cell’s membrane. Apoptosis, on the other hand, is active cell death whereby 
a cell carries out a process that ultimately leads to its death. It has been generally 
suggested that one of the primary causes of cell death in bioreactors is apoptosis or 
programmed cell death (Al-Rubeai et al., 1995; Goswami et al., 1999; Zanghi et al., 
2000; Mastrangelo et al., 2000a; Mastrangelo et al., 2000b; Laken and Leonard, 2001).

Apoptosis is understood to be triggered by a specific signal, such as the binding of a 
cytokine to a receptor, or by events such as nutrient starvation, exposure to toxic 
chemicals, extreme pH or virus infection. In addition, several researchers have reported
that cells can also become apoptotic under mechanical stress (Al-Rubeai et al., 1995; Perani et al., 1998). To prevent apoptosis, a number of gene products that are capable of blocking the signal transduction pathway which initiates apoptosis have been cloned and overexpressed in cells, including the Bcl-2 and Bcl-xL gene products (Chinnaiyan et al. 1996; Suzuki et al., 1997; Mastrangelo et al., 2000a; Mastrangelo et al., 2000b). A practical application of the overexpression of these gene products is prolonging the length of viability of mammalian cells used for product production in bioreactors (Simpson et al., 1997; Mastrangelo et al., 2000b).

A number of methods exist that allow for the detection of both necrosis and apoptosis and these methods range from qualitative to quantitative. Most of the methods that are used to measure necrosis are based on tests of cell membrane integrity. Typically, a dye is selected that does not enter healthy cells. After a small amount of dye (i.e. trypan blue dye) is added to the cell suspension, the number of stained and unstained cells is counted in a hemacytometer using a light microscope. If a flow cytometer (FCM) is used, an impermeant, fluorescent viability dye such as propidium iodide (PI), 7-aminoactinomycin D (7-AAD) or TO-PRO-3 can be employed (Shapiro, 2003). A different method of detecting necrosis measures the activity of the intracellular enzyme lactate dehydrogenase (LDH) in the suspending fluid (i.e. the presence of LDH corresponds to cell lysis; Legrand et al., 1992).

Qualitative methods for apoptosis detection include visual observation of blebs on the cell membrane using a light microscope. Similarly, DNA stained with DAPI (4'-6-
diamidino-2-phenylindole) or Hoechst® 33342 and visually examined with a fluorescence microscope reveals intensely stained nuclei in apoptotic cells due to chromatin condensation, in contrast to normal cells whose nuclei appears more diffusely stained (Mastrangelo et al., 2000a; Mastrangelo et al., 2000b). A third method involves the extraction and analysis of the cells’ DNA using an agarose gel; if the cells were apoptotic, then a DNA “ladder” would be present (Cotter and Al-Rubeai, 1995). While these techniques are excellent methods for detecting apoptosis qualitatively, analysis of large numbers of cells to obtain statistically relevant data is difficult.

In contrast to the labor-intensive, qualitative techniques, there are several FCM techniques that allow the rapid analysis of apoptosis in large numbers of cells. One of the primary methods involves fixing the cells in cold, 70% ethanol, staining with PI, and performing a cell cycle analysis on the cell population. Apoptotic cells in this analysis would appear just before the G1 peak of the cell cycle; unfortunately cells that became apoptotic during the G2-M phase or are part of an aneuploid or tetraploid population would not appear as part of the sub-G1 peak (Sherwood and Schimke, 1995; Allen and Newland, 2001). Also, unless a large percentage of the cells are apoptotic, determining where the sub-G1 peak occurs can be highly subjective, even with appropriate peak deconvoluting software, such as Modfit™. Another FCM apoptosis detection method involves measuring the mitochondrial activity using dyes such as Rhodamine 123 or MitoTracker from Molecular Probes (Eugene, OR), because mitochondrial activity decreases during apoptosis (Darzynkiewz et al., 1997). However, a large number of cells need to be apoptotic to see a noticeable decrease in overall Rhodamine 123 fluorescence.
A third FCM method to detect apoptosis takes advantage of phosphatidylserine (PS) translocation (van Engeland et al., 1998). Specifically, for non-receptor mediated apoptosis, cytochrome c is released from the mitochondria, which initiates the caspase cascade and PS translocates from the interior of the cell membrane to the exterior. The caspase cascade ultimately initiates endonuclease activity within the nuclei and enzymatic degradation of the cytoskeleton. Annexin V (AV) conjugated to a fluorochrome such as fluorescein isothiocyanate (FITC) or phycoerythrin (PE), binds to the translocated PS which can subsequently be detected on a FCM. When using this method, cells should also be stained with a fluorescent viability dye such PI or 7-AAD to distinguish necrotic cells from early apoptotic cells. The biggest drawback to this technique is the fact that the expression of PS on the exterior of the cells’ membrane is transient; typically cells enter later stages of apoptosis within 24 hours of expressing PS in the external surface of the membrane. Because of this, a time course experiment is highly recommended to perform this analysis.

The primary goal of this study is to determine if hydrodynamic stress can also trigger apoptosis. Garcia and Chalmers (1994) suggested that an “ideal” parameter to characterize hydrodynamic stress should be independent of the geometric characteristics of the culture system as cells do not “sense” the average conditions of the bioreactor but their local microenvironment. Consequently, criteria such as mean power input, agitation rate, impeller tip speed, impeller-based Reynolds number or integrated shear factor are
not appropriate as they represent average values derived from the geometric configuration of each system. Nevertheless, they have been widely used to correlate cell damage in a series of reports (Middler and Finn, 1966; Ujcová et al., 1980; Sinskey et al., 1981; Croughan et al., 1987; Kunas and Papoutsakis, 1990; Moreira et al., 1995; Chen et al., 2003).

Unfortunately, while the concept proposed by Garcia-Briones and Chalmers (1994) might appear appealing, at the most fundamental level it is impossible to implement at the current state of experimental and simulations capabilities. To attempt to approach the ideal parameter to characterize the potential of hydrodynamic stresses to damage cells, several researchers have suggested the use of energy dissipation rate, $EDR$. First suggested by Bluestein and Mockros (1969), the $EDR$ is a scalar value that is intrinsic to any moving fluid, is independent of the flow regime (turbulent/laminar) and accounts for both shear and extensional components of three-dimensional flow. It has units of energy per unit time per unit volume (i.e., J·s$^{-1}$·m$^{-3}$), or power per unit volume (i.e., W·m$^{-3}$). The EDR represents the rate at which work is done on a fluid element or, for this research, a cell (Clay, 1997). Bird et al. (2001) define the EDR as the irreversible rate of internal energy increase per unit volume. Depending on the system geometry and the flow regime, the EDR can be obtained via computer simulations, experimental determination, or analytical solutions of the Navier-Stokes equations (Mollet et al., 2004). Because all of these advantages, it is proposed that the $EDR$ can be used as a predictor of cell damage.
For an incompressible, Newtonian fluid, such as water (main component of cell culture media), the following equation can be used to calculate the local $EDR$ ($\varepsilon$):

$$
\varepsilon = \tau : \nabla U = \mu \left[ \nabla U + (\nabla U)^T \right] : \nabla U = \mu \sum_i \sum_j \left[ \nabla U_i + (\nabla U_i)^T \right]_{ji} \nabla U_{ji}
$$

(Eq. 3.1)

where $\mu$ is the viscosity, $\nabla U$ is the velocity gradient tensor and $\nabla U^T$ is the transpose of $\nabla U$.

To characterize the effect that well-defined $EDR$ has on cells, Ma et al. (2002) designed and manufactured a microfluidic device in which suspended cells could be subjected to acute, high levels of $EDR$ and the effect that this exposure had on the cells could be evaluated. With this device they subjected a Chinese Hamster Ovary cell line (CHO), an insect cell line (Sf-9), a human breast cancer cell line (MCF-7) and a hybridoma cell line (HB-24) to intense $EDR$ and measured the release of LDH as indication of cell membrane rupture. Every one of the cell lines studied showed significant cell lysis beginning around an $EDR$ of $10^7 \text{ W} \cdot \text{m}^{-3}$. To put this value of $EDR$ in perspective, Ma et al. (2002) created a one dimensional graph which summarizes some of the $EDR$ values observed in various bioprocess environments as well as some of the effects that $EDR$ have on cells. That graph, with more recent data added in presented in Figure 3.1.

In this manuscript, we discuss the development of a second generation contractional flow device, its characterization, and our studies to determine if an acute hydrodynamic force can induce apoptosis in wild type (WT) CHO cells and a CHO line overexpressing a Bcl-
2 gene product. Specifically, the following questions were addressed: (1) Does transient hydrodynamic forces induce apoptosis?, (2) Does culture history affect induction of apoptosis? (T-flask, spinner, bioreactor), and (3) Does the type of culture media make a difference with respect to the induction of apoptosis?

### 3.3. MATERIALS AND METHODS

#### 3.3.1. Contractional flow device

To overcome some of the challenges of the photoresist technology employed in the development of the contractional flow device by Ma et al. (2002), the photoresist layer was replaced with a 30 gauge stainless steel sheet (304 μm thickness) sandwiched between two PMMA transparent plates. In this stainless steel sheet the flow channel was bored using wire electrical discharge machining (WEDM). WEDM works by guiding, via computer, a piece of metal submersed in de-ionized water into a thin (100 μm diameter), electrically charged wire; as the metal approaches the wire, an arc is produced and a small amount of the metal is vaporized. Using this machining technique, an accuracy of ± 5 μm can be achieved with a cut surface that is nearly perfectly vertical. Figure 3.2a is a picture of the stainless steel sheet used in the device, while Figure 3.2b is a photomicrograph used to determine the surface roughness and the actual dimensions of the channel after machining.
3.3.2. Computational Fluid Dynamics (CFD) simulations

The flow through the contractional flow device was simulated using the commercial computational fluid dynamics program FLUENT®, Ver. 6.2, 3d, dp. The dimensions of the stainless steel contractional device determined from microscope images (See Figure 3.2b) were entered into Gambit®, the preprocessor included with FLUENT® to create the geometry and mesh of node points of the contractional device. The whole microchannel was modeled with a mesh optimization which created approximately 691,000 node points in a grid size of approximately $5.7 \times 7.6 \, \mu m$ at the constriction. For comparison purposes, the diameter of a CHO cell is around 12 - 14 \, \mu m.

For the simulations in FLUENT®, we used the segregated solver model, which performs iterations to sequentially solve the non-linear equations for continuity and momentum. No energy balance was included (isothermal conditions assumed and negligible frictional losses). The implicit formulation was used to solve the equations for steady-state operation assuming laminar flow, viscous fluid model with $P_{\text{absolute}} = 1$ atmosphere at the center of the output face of the simulated channel ($X=30 \, \text{mm}$, $Y=0$, $Z=0$, Figure 3.3) Boundary conditions involved non-slip conditions for walls and constant velocity at the inlet (See Table 3.1). The schemes used for interpolation: for momentum, 1st order upwind; for pressure, the standard FLUENT® interpolation; and for pressure-velocity coupling, the SIMPLE scheme was used. All the simulations were performed using a Pentium®-D CPU running at 3.2 GHz with 1.0 GB RAM and 8.85 GB hard disk.
After FLUENT® converged on a solution for each flow rate, the program provided the velocity vector and pressure for every node point in the mesh. Next, an “execute on demand” user-defined function was implemented which calculated the \( EDR \) at every node point using Equation (3.1). Figure 3.3 contains a number of plots of the magnitude of different components of the velocity vectors for two different y-z planes in the flow-contraction device. The simulated total flow rate for such figure was 90 mL·min\(^{-1}\).

During the course of mesh optimization, a singularity in the \( EDR \) calculation appeared at the “corner” of the constricted region of the device (squared zone in Figure 3.2b). The \( EDR \) value at this point continued to increase indefinitely as the mesh density increased; however, \( EDR \) values more than 10 \( \mu \)m away from this point remained constant.

This observation leads to another problem: as it can be observed from Figure 3.4, a single cell experiences a wide range (4-5 orders of magnitude) of values of \( EDR \) along its path in the microchannel; furthermore, two cells going into the microchannel in different initial positions will experience values of \( EDR \) quite different. It seemed plausible that the most important value of \( EDR \) that every cell experiences is the maximum because most likely it is the more harmful. But if every cell experiences a different maximum, we were faced with the problem of selecting a \textit{SINGLE} value of \( EDR \) that was representative of that particular flow rate for \textit{ALL} the cells in the experiment.

To circumvent this potential problem and to better model the conditions cells experience in the device, a particle tracking technique was employed which is an option in this
version of FLUENT®. Four thousand, 10 μm-diameter virtual particles were released at the inlet of the device and the EDR that each particle encountered at every point throughout the device was recorded for every simulation. As a result of their small size and similar density relative to the medium, cells are expected to follow closely the pathlines of the fluid and so particle-liquid interactions were not considered in the simulations. Also, because of the low cell concentration, particle-particle interactions were negligible in the simulation (uncoupled mode).

The maximum EDR value that each simulated particle experiences was determined by exporting the EDR data and processing them using a program written in Perl®; these 4000 EDR data points were next imported into Excel® to create histograms of the maximum EDR each particle experienced for every flow rate. Figure 3.4 is an example of two particle tracks and a histogram of the EDR each particle experiences. Figure 3.6 is a histogram corresponding to the 4000 values for every flow rate.

3.3.3. Reynolds number

A total of five different volumetric flow rates were simulated: 10, 30, 50, 70, and 90 mL·min⁻¹. The maximum flow rate was selected based on the dimensions of the channel to obtain laminar flow in the throat of the microfluidic device.

Laminar flow is central to our work as it has been shown that velocities measured experimentally agree to within 2% with analytical solutions for laminar flow in
rectangular channels (Meinhart et al., 1999), so the reliability of the results of the simulation could be assured.

In classical fluid mechanics theory for conventional sized circular pipes and for fully developed flow, the experimental limit of the Reynolds number \((Re_{c,cr})\) used to determine if a flow is laminar corresponds to \(Re_{c,cr} \leq 2100\). The Reynolds number is defined as

\[
Re = \frac{L_c \cdot \bar{U} \cdot \rho}{\mu}
\]

(Eq. 3.2)

where \(L_c\) is a characteristic length, \(\bar{U}\) is the average velocity of the fluid, calculated as the flow rate \((Q)\) divided by the flow cross-section area, and \(\rho\) and \(\mu\) represent the density and viscosity of the fluid, respectively. For circular pipes, \(L_c\) is chosen as the pipe diameter \((D)\). However, for rectangular ducts the hydraulic diameter, \(D_h\), is used:

\[
D_h = 4 \cdot \frac{\text{Flow area}}{\text{wetted perimeter}}
\]

(Eq. 3.3)

For a rectangular cross-section duct of dimensions \(w \times b\), Equation (3.3) becomes:

\[
D_h = 4 \cdot \frac{w \cdot b}{2(w+b)} = \frac{2 \cdot w \cdot b}{(w+b)}
\]

(Eq. 3.4)

Replacing Equation (3.4) into (3.2) we get:

\[
Re = \frac{D_h \cdot \bar{U} \cdot \rho}{\mu} = \frac{2 \cdot w \cdot b \cdot Q \cdot \rho}{(w+b) \cdot w \cdot b \cdot \mu} = \frac{2 \cdot Q \cdot \rho}{(w+b) \cdot \mu}
\]

(Eq. 3.5)

Jones (1976) and Obot (1988) have shown data indicating that the use of hydraulic diameter is not the best choice when evaluating the transition from laminar to turbulent
flow regime; to solve the problem they propose a way of “correcting” the Reynolds number calculated for rectangular ducts to fit the same curve used for circular tubes:

\[ \Phi = \frac{Re_{n,cr}}{Re} \]  
(Eq. 3.6)

where \( Re_{n,cr} \) is the critical Reynolds number for any non-circular duct (i.e. rectangular) calculated using the hydraulic diameter and \( \Phi \) is a geometry function. Jones (1976) presented a theoretically derived function and proposed a simpler equation that provides the same results within 2% error:

\[ \Phi = \frac{2}{3} + \frac{11}{24} \frac{w}{b^3} \left( 2 - \frac{w}{b} \right) \]  
(Eq. 3.7)

Because of the converging – diverging nature of the microfluidic device, the value of \( b \) (and therefore of \( \Phi \)) changes along the fluid path; values of \( b, w, \Phi \) and \( Re_{n,cr} \) are presented in Table 3.1 for two zones of interest: the constant cross-section zone at the entrance of the microchannel and the throat (the narrowest point). In Table 3.1 the Reynolds number calculated from Equation (3.5) for both zones at every flow rate tested is also presented.

Inspection of Table 3.1 indicates that the Reynolds number in the throat for every flow rate is significantly higher than the critical and so our assumption of laminar flow would appear to be invalid. However, these calculations apply to a fully developed flow profile;
in contrast, it is highly unlikely that fully developed flow could develop in the short distance in the throat of the channel. Also, inspection of the currently used design indicates that a very short distance after the narrowest portion of the contraction (throat) the channel begins to diverge.

To calculate the position at which the growth of the boundary layer with distance eventually will lead to unstable or turbulent flow at the trailing edge, Gregoriades et al. (2000) argued that at small distances from the inlet, the boundary layer will grow in the same way as it does along a flat plate at zero incidence. They used a modified version of the critical Reynolds number for flow along a plate, given by:

\[
Re_{c,z} = \frac{z \cdot U \cdot \rho}{\mu}
\]

(Eq. 3.8)

where \(z\) is the distance from the beginning of the plate. Transition from laminar flow using this modified Reynolds number, \(Re_{c,z}\), is reported to occur at \(Re_{c,z} > 3 \times 10^5\) to \(3 \times 10^6\) depending on surface roughness and homogeneity of the approaching stream (Bird et al., 2001). At 90 mL·min\(^{-1}\) in the current system, the \(Re_{c,z}\) is calculated to be \(1.75 \times 10^5\), which is just below the critical Reynolds at which the boundary layer undergoes the laminar to turbulent transition.

3.3.4. Flow in entrance and exit of the contraction device

To experimentally verify that the simulated particle tracks accurately represent the flow into and out of the flow contraction device, visualization experiments were conducted
using a human cell line, THP1siP-EGFP, which have been engineered to express green fluorescence protein. (Note: these cells were only used as flow markers in this study due to the fluorescent signal they emit.) The flow contraction device was placed in an Olympus, CK2 inverted scope to which a SCIMAX-M-12 (MVIA, Inc. Monaca, PA) fully digital video camera was placed. Images of the cells flowing through the device at different locations and flow rates were subsequently recorded.

Figure 3.5 presents digital, microscopic photos of the stream lines of the cells flowing into, Figure 3.5A, and out of, Figure 3.5C of the flow contraction. Next to each of the photographs are fluent simulations of the particle traces in the entrance and exit region, Figure 3.5B and D, respectively. The tracks are color coded with respect to velocity, and the rectangular boxes in Figure 3.5B and D correspond to the regions in which the photographs in Figure 3.5A and C correspond.

Visual observations of the video images of the exit region (from which the Figure 3.5C was taken) clearly show a central high speed zone, and recirculation zones (backflows) between the central high speed zone and the walls. The high speed zone is depicted by the red and yellow colors in the simulation and the backflow is depicted by blue lines in Figure 3.5D.

Corresponding to this difference in flow rates, the EDR is orders of magnitude different in the entrance region (region of highest extensional and shear flow) and the exit zone, and in the slow moving recirculation zone. Figure 3.5E is a color plot of the EDR that
simulated particles experience as they flow through the whole contraction channel at the highest flow rate of 90 mL·min⁻¹. The other, lower flow rates produce qualitatively similar EDR distributions.

3.3.5. Cell Culture

Two cell lines were investigated: wild type CHO-K1 (WT-CHO) and CHO-K1 transfected with the human Bcl-2Δ gene (hence referred to as CHO-bcl2). Information regarding the transfection process and selection of the mutants is presented elsewhere (Levine et al., 1993; Mastrangelo et al., 2000a). The Bcl-2 gene product inhibits non-receptor mediated apoptosis by blocking the release of cytochrome c from the mitochondria. Initially, both cell lines were cultured in an anchorage dependent mode in Dulbecco’s Modified Eagle’s Medium (DMEM) with 10% fetal bovine serum (FBS), (both from Hyclone, Logan, Utah). The serum concentration was gradually reduced to 1%, and then the two cell lines were cultured in suspension in 250 mL spinner flasks with Hyclone’s SFM4CHO with 1% FBS. The spinner flasks were placed in a 37°C incubator with 5% CO₂ and the agitation rate was set at 80 rpm. After adaptation of cells to suspended growth was complete, the serum concentration was reduced to 0%. One hundred twenty-five milliliters of the broth from a spinner flask were used to inoculate a 1.25 L working volume stirred tank bioreactor, a Bioflo 3000 from New Brunswick Scientific (Edison, NJ). The temperature was set at 37°C; the pH was maintained at 7.0 by addition of CO₂; the dissolved oxygen was controlled at 70% of air saturation by surface aeration with 1 v.v.m. of a mixture of air and N₂ or O₂ as needed. The bioreactor
was operated in batch mode at an agitation rate of 100 rpm using a pitched blade impeller (PBI). Other geometrical characteristics of the bioreactor and the spinner flask are indicated in Tables 3.2 and 3.3 respectively.

3.3.6. Estimations of mean and maximum \( EDR \) in the Bioflo bioreactor

While absolute values of the mean and distribution of local \( EDR \) levels in the Bioflo bioreactor are not known, a significant number of experimental studies have been conducted, with corresponding correlations created, such that reasonable estimates can be made for the vessels used in this study (Kresta 1998). Specifically, the mean \( EDR \) in the Bioflo bioreactor can be estimated from Zhou and Kresta (1996):

\[
\frac{\rho}{V_T} \tau = \frac{P}{\rho \cdot V_T} = \frac{Np \cdot N^3 \cdot D^5}{V_T}
\]  
(Eq. 3.9)

where \( P \) is the power transferred to the fluid by the impeller, \( \rho \) is the density of the fluid, \( V_T \) is the volume of the fluid in the tank, \( N \) is the stirring speed [rpm], \( D \) is impeller diameter, and \( Np \) is the power number. Tables 3.2 and 3.3 list the values for these variables for the two systems used in this study.

The power number is predominately a function of the Reynolds number, \( Re_{imp} \), and the geometry of the impeller. For the bioreactor used in this study, the closest geometry to the pitch blade impeller (PBI) in Zhou and Kresta’s work is a pitch blade turbine (PBT). For the current system, the \( Re_{imp} \) is approximately \( 1.34 \times 10^4 \); for a PBT at this Reynolds
number, $N_p$ is 1.35. Substituting these values into Equation (3.9), the average $EDR$ is $11.5 \text{ W} \cdot \text{m}^{-3}$.

Also from the work of Zhou and Kresta (1996), a correlation was developed from which the maximum $EDR$ can be estimated based on a specific set of variables and/or parameters in the vessel. This relationship is given by:

$$\psi = \frac{\varepsilon}{\rho N^2 D^2}$$  \hspace{1cm} (Eq. 3.10)

where $\psi$ is a constant dependent on the impeller type. For the PBT the value of $\psi$ is 4.0. Other parameters important in this correlation are the ratio of the off-bottom clearance to the impeller diameter, $C/D$, and the ratio of the liquid height to tank diameter, $H/T$. As the values of these ratios are in the range of the studies by Zhou and Kresta, the maximum $EDR$ for the bioreactor can be calculated through the use of Equation (3.10) to give a value of $1.03 \times 10^2 \text{ W} \cdot \text{m}^{-3}$. While the correlation of Zhou and Kresta (Eq. 3.10) was developed in a system using baffles, Mollet et al. (2004), recently demonstrated that the lack of baffles has only a very minor effect on the maximum $EDR$.

### 3.3.7. Estimation of mean and maximum $EDR$ in spinner vessel

For the spinner flasks, estimates of $\varepsilon$ and $\bar{\varepsilon}$ were based on measurements reported in the literature. Specifically, the results from Venkat (1995) and Venkat et al. (1996) employed a particle tracking velocimetry technique to determine both the maximum and the average $EDR$ in a 250 mL spinner vessel of identical geometric characteristics to the one used in
this study. Venkat et al. (1996) reported specific values at 90, 150 and 210 rpm (Table 3.4): consequently, a quadratic equation was used to estimate values of $\bar{e}$ and $\varepsilon$ of 3.3 W·m$^{-3}$ and 2.0×10$^2$ W·m$^{-3}$ respectively for 80 rpm.

3.3.8. Contractional flow device experiments

Regardless of the cell type and growth conditions, cells were harvested during the mid-exponential portion of their growth phase. Cells cultured in tissue culture flasks were detached using Accutase™ (Innovative Cell Technologies, San Diego, CA) and suspended in serum-free DMEM at a cell concentration of 1.5×10$^5$ cells·mL$^{-1}$. Cells cultured in suspension (spinner or bioreactor) were taken from the reactor and used directly. The cell suspensions were pumped through the contractional flow device at the proper volumetric flow rates using a syringe pump (Harvard Apparatus, Holliston, MA). The effluent from the contractional device was collected in a sterile conical tube of 50 mL; the appropriate volume of FBS was added to bring the concentration to 10% (v/v) FBS, and the cells were placed in a 37°C incubator-shaker. Samples of every tube were taken approximately every 2 hours for analysis. Three separate volumes of cell suspension were pumped through the device for every flow rate, in a random order, and there were three separate control cell suspensions, for statistical analysis purposes.
3.3.9. Cell analysis studies

A number of different analysis techniques were used to examine the effect of acute exposure of various levels of \textit{EDR} on cells. These include total and viable cell counts using either a hemocytometer or a Coulter counter, cell lysis quantification using a lactate dehydrogenase assay (LDH), or a number of fluorescent stains on a FCM.

3.3.10. LDH studies

Four combinations of cells and media were studied with this type of analysis: WT-CHO-K1 cultured in DMEM with 10\% FBS, WT CHO-K1 cultured in serum-free CD CHO-A medium (Invitrogen, Carlsbad, CA), CHO-bcl-2 cultured in DMEM with 10\% FBS, and CHO-bcl-2 cultured in serum-free CD CHO-A medium. For these studies, the cells were cultured in tissue culture flasks (Fisher Scientific, Pittsburgh, PA). They were harvested during late exponential phase using Accutase\textsuperscript{TM} and centrifuging; the pellet was washed with PBS once and the cells subsequently resuspended in a second PBS solution to a final cell concentration of approximately $2\times 10^5$ cells\textcdot mL$^{-1}$. The cell suspension was then divided into three parts. One part (2mL) remained as a control to determine the amount of lactate dehydrogenase (LDH) released in supernatant before the insult ($F_{Ic}$). The second part (2mL) was mixed with 0.2 mL of lysis solution (9\% w/v Triton X-100, Sigma-Aldrich, Milwaukee, WI) to determine the fluorescence intensity of the sample completely lysed ($F_{Id}$). The third part, approximately 30mL, was divided into aliquots for subsequent single passes through the contractional flow device at different flow rates.
The final 2 mL of effluent from each pass through the contraction was collected for analysis. These samples were centrifuged at 250g to remove cells and the supernatant was dispensed into flat-bottom 96-well plates. Following the procedures of the CytoTox-ONE assay kit (Promega, Madison, WI), the fluorescence in the wells was determined using a Cytofluor 4000 fluorescent plate reader (Applied Biosystems, University Park, IL) set to 530/580 nm excitation/emission wavelengths.

The percent cell damage was subsequently determined using the following relationship:

\[
\text{\% cell damage} = \left( \frac{F_{I_c} - F_{I_{cl}}}{F_{I_{cl}} - F_{I_e}} \right) \times 100
\]  

(Eq. 3.11)

where \( F_I \) refers to fluorescence intensity of the given reading, and the subscripts \( e, c, cl \), refer to effluent, control, and completely lysed, respectively.

3.3.11. FCM studies

All flow cytometry analyses were conducted on a BD FACS-Calibur Flow Cytometer; for every sample at least 10,000 gated events were recorded. Following usual FCM convention, the appropriate cell population was first identified on a Forward versus Side Scatter plot. For multicolor analysis, depending on the specific test, appropriate single color controls were conducted.

For viability studies using the FCM we used the dye 7-AAD, which enters cells with a compromised cell membrane, and binds to nucleic acids. The actual staining protocol used was provided by the manufacturer (BD Biosciences, San Jose, CA) and the
fluorescent signal was detected on FL3 (488 nm excitation, >670 nm emission). The appropriate single-color controls were produced by heat-shocking the cells at 42 °C for 45 minutes.

The relative mitochondrial activity was measured using Rhodamine 123, which is a cationic, lipophilic green fluorescent dye. A Rhodamine 123 gradient is produced across the mitochondrial membrane due to the large potential difference between the mitochondria and the cytoplasm (Shapiro, 2003). The more metabolically active the cell’s mitochondria are, the more Rhodamine 123 is localized in the mitochondria, and the higher relative fluorescence can be detected. For these assays, the cells were also stained with DiD oil, a red fluorescent dye which binds to the cell membrane stoichiometrically. During cell division, the dye molecules are distributed evenly among the two daughter cells and the fluorescent intensity halved for the two cells. This dye was used to indicate cell proliferation during the course of the experiment. Both Rhodamine 123 and DiD oil were purchased from Molecular Probes (Eugene, OR). The fluorescent signal for Rhodamine 123 was detected on FL1 (488 nm excitation, 525 nm emission), while DiD fluorescence was detected with FL4 (635 nm excitation, 665 nm emission).

Apoptosis assays on the FCM were conducted using Annexin V and 7-AAD. Due to the transient nature of the expression of PS on the exterior of the cells’ membrane, time course experiments were conducted in which a 5 mL aliquot of cell suspension was taken every two hours after the cell suspension was subjected to a specific level of EDR. Cells (10^5) were stained with 5 μL of AV conjugated to PE and 5 μL of 7-AAD following the
protocol recommended by the supplier (BD Biosciences; San Jose, CA). AV-PE fluorescence was detected with FL2 (488 nm excitation, 585 nm emission).

### 3.3.12. Immunostaining for Bcl-2 over-expressing cells

Bcl-2 over-expressing cells cultured in the bioreactor using serum free media were fixed with a 3.7% formaldehyde solution and then stained with an anti-human bcl-2 antibody conjugated to PE (from BD Biosciences). As a control, a separate sample was stained with an isotype antibody, also conjugated to PE. Bcl-2 over-expressing cells cultured in T-flasks and in serum containing media were also stained in a similar manner. These anti-human bcl-2 antibodies labeled cells were further stained with Hoechst 33342, a DNA stain, and examined both using a Nikon Optiphot-2 fluorescence microscope and by FCM.

### 3.4. RESULTS

#### 3.4.1. Simulation Results of EDR in Flow Contraction Device as a Function of Flow Rate

Figure 3.6 presents histograms of the maximum value that each of the 4,000 simulated particles experienced in the flow contraction rate at the five flow rates simulated. Note that solid vertical line, and numerical value, corresponds to the median of the population
and in each case this median value is close to the maximum frequency of particles that experienced a given EDR.

### 3.4.2. Necrosis/lysis studies

To compare the performance of the contractional flow device presented in this manuscript with the previous device described by Ma et al. (2002), two types of analysis were conducted. The first consisted of measuring the level of necrosis by PI staining and visual count. Figure 3.7a presents results of a cell suspension subjected to a single pass through the microfluidic device at the indicated level of EDR for T-flask grown WT-CHO and CHO-bcl2 cell lines in 10% serum-containing media and subsequently stained with PI and counted in a hemocytometer. Control experiments consisted of pumping the cells through the syringe at the same flow rate, but not through the microfluidic channel (the control test results are plotted at the corresponding EDR level that the abused sample was subject to for comparison purposes). Each data point corresponds to the mean of 3 experiments and the error bars correspond to one standard deviation.

An alternative to visual viability measurements with PI is a change in concentration of LDH. T-flask grown, wild-type CHO-K1 in media containing 10% serum and serum free media, and CHO-bcl-2 in media containing 10% serum were subjected to a single exposure of various levels of EDR, and the level of LDH in the supernatant after the hydrodynamic insult was determined. Figure 3.7b presents the data from this LDH study on WT-CHO grown in the presence of 10% serum and WT-CHO in serum-free medium.
Each data point is the average of three replicates of the same experiment conducted on three different days (different T-flask cultures). In addition, on a given day of an experiment three to five samples were subjected to the each level of $EDR$ and the results averaged. Consequently, each data point on Figure 3.7b represents an average of 9 to 15 independent experiments; again, the error bars correspond to one standard deviation. Figure 3.7c present results of a similar study, conducted on CHO-K1 transfected with the Bcl-2 gene. In this case, each data point corresponds to the average of three to five distinct experiments conducted on the same culture on the same day, and the line connects the mean of the average from different days for every $EDR$. It should be noted that while Figure 3.7a is based on visual observation of cells present, Figures 3.7b and c present the cell damage expressed as the % of the total cell number based on LDH, not visual observations. At this stage, it should be noted that the results presented in Figures 3.7a-c are quite similar to results presented by Ma et al. (2002).

### 3.4.3. Apopotosis/Necrosis studies using FCM

While the LDH studies provide information on the catastrophic effect of $EDR$ on cells, as discussed previously, the focus of this manuscript is to address whether $EDR$ can elicit apoptosis and whether culture history affects this sensitivity, or the lack thereof. To address this apoptosis question, wild type CHO-K1 and CHO-K1 transfected with the Bcl-2 gene were subjected to three different levels of $EDR$ and analyzed with the 7-ADD and Annexin V-conjugated-PE (AV-PE), assays.
Early in the apoptotic pathway, PS, translocates from the interior to the exterior of the cell membrane. AV-PE binds tightly to PS; however, an FCM analysis can not distinguish whether the AV has bound to PS inside of the cells (which could be the result of necrosis) or PS on the outside of the cell as a result of apoptosis. Consequently, in these studies, the cells were simultaneously stained with the viability dye 7-AAD. Viable cells (non-necrotic) will exclude both the 7-ADD and the AV-PE from the interior of the cell. Figure 3.8 is a typical FCM bivariate plot of 7-AAD versus AV-PE; each dot represents fluorescence data collected from a single cell. Debris was excluded in this analysis by previously gating the region in the Forward vs. Side Scatter dot plot believed to be containing cells (typical FCM methodology). Assuming that events in Figure 3.8 correspond to cells, the cells in the lower left quadrant (I) of Figure 3.8 are negative for both dyes and, therefore, are considered healthy. Cells in the lower right quadrant (II) are positive only for AV-PE and are considered to be in the early stages of apoptosis. Cells in the upper right quadrant (III) are positive for both AV-PE and 7-AAD and are either in the late stages of apoptosis or are necrotic (since it is possible that the Annexin V dye can penetrate a necrotic cell). The small amount of events in the quadrant IV stain positive for DNA but are negative for PS: this would correspond to dead cells lacking most if not all of the membrane-based PS; most probably, this corresponds to naked nuclei.

Given the consistency of the data in Figures 3.7a-c with the previous studies of Ma et al. (2002) with respect to the significant cell lysis occurring at $EDR$ values above $1\times10^8$ W·m$^{-3}$, a majority of the 7-AAD Annexin V assays were conducted at EDR levels significantly lower than $1\times10^8$ W·m$^{-3}$. Figure 3.9a presents the results of analysis of early
apoptosis on WT-CHO cells grown in T-flasks in media containing 10% serum (quadrant II in Figure 3.8) while the results on Figure 3.9b correspond to analysis on late apoptosis/necrosis (quadrant III in Figure 3.8). A small increase in the fraction of cells that are apoptotic appears approximately 5 to 6 hours after the exposure to any of the levels of EDR tested. There was also an apparent slight increase in the percent of necrotic cells with increasing EDR, consistent with the data in Figure 3.7a. In contrast, the CHO-bcl2 cell line, cultured in T-flasks and 10% serum, exhibited a lower level of apoptosis (Figure 3.10a) and lower level of necrosis when compared to the WT-CHO. Again, the level of necrosis is consistent with the levels presented in Figure 3.7a.

As stated in the Materials and Methods section, these two cell lines were subjected to a slow removal of serum and subsequent transferal to a suspension culture in a spinner vessel and then a bioreactor. Figure 3.11 is an analysis of the WT-CHO cells soon after transfer to the spinner vessel. Figure 3.11a is a plot of the percent of the cells exhibiting early apoptosis while Figure 3.11b is a plot of the percent of the cells exhibiting late apoptosis/necrosis. An interesting observation is that there is no difference in early apoptosis between the cells subjected to various levels of EDR versus the control; also, note the very high level of late apoptosis/necrosis. This is in contrast to the data in Figures 3.9a and 9b which exhibit a noticeably different, almost opposite effect.

The final stage of scale up consisted of growing both the WT-CHO and CHO-bcl-2 cells in bioreactor. Figures 3.12 (WT-CHO) and 3.13 (CHO-bcl2) present both the early apoptosis (a) and late apoptosis/necrosis (b) data, again as a function of EDR and time.
after insult, in a manner similar to the previous figures. Both the level of early apoptosis and late apoptosis/necrosis was similar for both cell lines and comparable to the spinner vessel studies.

Finally, several studies were performed using Rhodamine 123 to measure mitochondrial activity. Samples chosen for the studies corresponded to conditions most likely to result in apoptotic behavior based on the results presented above. In all of the cases studied, the level of activity was not statistically different between the control and EDR subjected samples; consequently, that data is not presented.

3.5. DISCUSSION

Overall, the results obtained were within the range of expectations when this study was initiated. The following summary conclusions can be made. First, the results obtained with this new flow contraction device and corresponding computer simulations are consistent with previously published studies with respect to the level of EDR required to create significant cell lysis. Catastrophic damage of the cell membrane on the order of 5-15% was detected for CHO cells subjected to $10^8$ W·m$^{-3}$ and those levels of damage appear independent, within a 95% of confidence, of culture medium or the addition of the bcl-2 gene product. Second, while detectable relative to the control in the T-flask experiments (Figure 3.9a), only a fraction (~1-4%) of the cells become apoptotic when exposed to a sub-lysis level of EDR ($< 10^8$ W·m$^{-3}$); unfortunately, the wide scattering of some sets of data does not allow one to draw conclusions with respect to the significance
of this result. It is debatable also whether such a difference exists in cells cultured in spinner flasks (Figure 3.11a) or bioreactor (Figure 3.12a). Third, cells cultured in suspension culture and serum-free media do not exhibit any higher or lower sensitivity (in terms of induction of apoptosis) to various levels of EDR when compared to control cultures grown under the same conditions. Fourth, the addition of the Bcl-2 gene product might have a slight lowering of the occurrence of apoptosis in a T-flask culture; however the baseline response is so low that the difference is insignificant.

Inspection and comparison of Figures 3.7a, 3.9b, and 3.10b seems to indicate that CHO-bcl2 cells are slightly more resistant to lysis (necrosis) than WT-CHO. However, the comparison of the data in Figures 3.12b and 3.13b (bioreactor studies) did not demonstrate that this higher resistance translated to the bioreactor; as a matter of fact necrosis is increased dramatically in bioreactor studies for both cell lines even for control samples. The reason of this trend is not known; we believe is not an artifact due to handling of the sample as this did not occur for cells growing in T-flask and serum-containing media. It may be argued that agitation could be hyper-sensitizing the cells because the cells did not have enough time to adapt to the new growth conditions without serum and more stressing environment.

In bioreactor studies CHO-bcl2 cells appear to be more sensitive in terms of induction of apoptosis than their parental line (WT-CHO-K1), as suggested by the levels of apoptosis up to 13% compared to controls of approximately 2-3%. This fact may seem a contradiction as it is believed that the Bcl-2 gene product blocks apoptosis. Likewise, the
levels of necrosis were significantly higher when compared to T-flasks (although controls again had the same level of necrosis as treatments). The apparent incongruity led us to conduct studies to determine if the Bcl-2 gene product is expressed in all of the cells. Figure 3.14a presents a flow cytometry analysis of CHO-bcl2 cells grown in a T flask and stained with the anti-human bcl-2 antibody conjugated to PE, while Figure 3.14b is a FCM analysis of cells grown in a bioreactor (by way of a spinner vessel). As a control, an isotype antibody, also conjugated to PE, was also used to stain the cells. Clearly, a significant number of the cells had lost their ability to express the Bcl-2 gene product by the time they reached the bioreactor. It is highly likely that this lack of gene product expression in a significant fraction of the cells suspended in the bioreactor reduced the overall resistance of the population to hydrodynamic damage as presented in Figure 3.13. However, a puzzling observation is that one would expect the CHO cells which lost bcl2 gene product should be at least as resistant as the CHO-WT; yet comparison of Figures 3.12 and 1.13 indicate that overall, the cells presented in Figures 3.13a and b are slightly more susceptible to apoptosis than the WT (again, scattering of data makes appear the differences between means as non-significant at 95% confidence). At this time we do not have an explanation for this observation.

A common objection/concern of the methodology used in this study, and those of Ma et al. (2002) and Gregoriades et al. (2000) is that the cells were only subjected to a single pass through the flow contraction device. The question can be raised: What if the cells were repeatedly subjected to high levels of EDR yet below those that demonstrated to cause immediate lyses (i.e., on the order of $10^5$ to $10^7$ W·m$^{-3}$)? For this reason, the
spinner and bioreactor studies were conducted and the average and maximum $EDR$ were estimated. As reported previously, the mean and maximum EDR in the spinner and bioreactor were calculated to be $3.3$ and $2\times10^2$ W·m$^{-3}$ and $11.5$ and $10^2$ W·m$^{-3}$, respectively. In addition, it is reported that less than 5 percent of the vessel’s volume has these maximum levels (Zhou and Kresta, 1996a, 1996b; Mollet et al., 2004). Assuming a well mixed vessel, the cells would routinely experience this maximum value, either 200 or $10^2$ W·m$^{-3}$ approximately 5% of the time. Given this information, and the observation in Figure 3.9a that WT-CHO cells grown in a T-flask will exhibit a low level of apoptosis beginning at $2.87\times10^5$ W·m$^{-3}$, it is clear that routine exposure to $2\times10^2$ W·m$^{-3}$ and a subsequent single exposure to an EDR on the order of $10^5$ to $10^7$ W·m$^{-3}$ only has a minor effect on the culture viability.

To further quantify the highest level of EDR that a CHO cell can repeatedly sustain and still perform normally, is non-trivial. Inspection of Figure 3.1 indicates that levels of local $EDR$ in the same range as evaluated in this work ($\geq 10^5$ W·m$^{-3}$) are only achieved in a typical mixing vessel when it is operated in excess of 700 rpm. Alternatively, a loop can be set up in which cells are grown in the spinner vessel or bioreactor (in which the average and maximum EDR can be estimated, as in this study) and the cells are then repeatedly pumped through the flow contraction device. On going studies in our laboratory have developed such a system and studies are currently underway.
3.6. REFERENCES


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<tr>
<td>Hydraulic diameter</td>
<td>$D_h$</td>
<td>$260 , \mu m = 2.60 \times 10^{-4} , m$</td>
</tr>
<tr>
<td>Geometry function</td>
<td>$\Phi$</td>
<td>$1.10$</td>
</tr>
<tr>
<td>Critical Reynolds number</td>
<td>$Re_{n,cr}$</td>
<td>$1.91 \times 10^{5}$</td>
</tr>
<tr>
<td><strong>Fluid</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chemical identity</td>
<td></td>
<td>Water</td>
</tr>
<tr>
<td>Working temperature</td>
<td>$\theta$</td>
<td>$37 ^\circ C$</td>
</tr>
<tr>
<td>Density (at $\theta$)</td>
<td>$\rho$</td>
<td>$997 , Kg/m^3$</td>
</tr>
<tr>
<td>Viscosity (at $\theta$)</td>
<td>$\mu$</td>
<td>$0.6915 , cP = 0.6915 \times 10^{-3} , Pa \cdot s$</td>
</tr>
<tr>
<td><strong>Reynolds number (Re)</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Flow rate, Q [mL · min$^{-1}$]</td>
<td></td>
<td>Average velocity at the inlet [m·s$^{-1}$]</td>
</tr>
<tr>
<td>10</td>
<td>0.105</td>
<td>87.3</td>
</tr>
<tr>
<td>30</td>
<td>0.316</td>
<td>261.9</td>
</tr>
<tr>
<td>50</td>
<td>0.527</td>
<td>436.6</td>
</tr>
<tr>
<td>70</td>
<td>0.738</td>
<td>611.2</td>
</tr>
<tr>
<td>90</td>
<td>0.949</td>
<td>785.9</td>
</tr>
</tbody>
</table>

**Table 3.1.** Table of physical and geometric characteristics used for the calculation of EDR in the microfluidic device.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Symbol</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tank diameter</td>
<td>$T$</td>
<td>$12.1 \text{ cm} = 1.21 \times 10^{-1} \text{ m}$</td>
</tr>
<tr>
<td>Tank height</td>
<td>$H$</td>
<td>$21.9 \text{ cm} = 2.19 \times 10^{-1} \text{ m}$</td>
</tr>
<tr>
<td>Impeller diameter</td>
<td>$D$</td>
<td>$7.46 \text{ cm} = 7.46 \times 10^{-2} \text{ m}$</td>
</tr>
<tr>
<td>Impeller type</td>
<td></td>
<td>Pitch blade turbine 45° (modified) 3 blades</td>
</tr>
<tr>
<td>Off-bottom clearance</td>
<td>$C$</td>
<td>$6.4 \text{ cm} = 6.4 \times 10^{-2} \text{ m}$</td>
</tr>
<tr>
<td>Liquid volume</td>
<td>$V$</td>
<td>$1.25 \text{ L} = 1.25 \times 10^{-3} \text{ m}^3$</td>
</tr>
<tr>
<td>Impeller speed</td>
<td>$N$</td>
<td>$100 \text{ rpm} = 1.667 \text{s}^{-1}$</td>
</tr>
<tr>
<td>Working temperature</td>
<td>$\theta$</td>
<td>$37^\circ \text{C}$</td>
</tr>
<tr>
<td>Fluid</td>
<td></td>
<td>Water</td>
</tr>
<tr>
<td>Density (at $\theta$)</td>
<td>$\rho$</td>
<td>$997 \text{ Kg/m}^3$</td>
</tr>
<tr>
<td>Viscosity (at $\theta$)</td>
<td>$\mu$</td>
<td>$0.6915 \text{ cP} = 0.6915 \times 10^{-3} \text{ Pa.s}$</td>
</tr>
<tr>
<td>Impeller Reynolds number</td>
<td>$Re_{imp}$</td>
<td>$1.34 \times 10^{4}$</td>
</tr>
<tr>
<td>Power number</td>
<td>$Np$</td>
<td>1.35</td>
</tr>
</tbody>
</table>

**Table 3.2.** Physical and geometrical characteristics of the bioreactor used in the calculation of EDR

158
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Symbol</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spinner diameter</td>
<td>T</td>
<td>7.2 cm = 7.2×10^{-2} m</td>
</tr>
<tr>
<td>Impeller diameter</td>
<td>D</td>
<td>5.6 cm = 5.6×10^{-2} m</td>
</tr>
<tr>
<td>Impeller type</td>
<td></td>
<td>Magnetic bar (assimilated to a paddle type impeller)</td>
</tr>
<tr>
<td>Width of the blade of the impeller</td>
<td>w</td>
<td>2.1 cm = 2.1×10^{-2} m</td>
</tr>
<tr>
<td>Liquid volume</td>
<td>V</td>
<td>250 mL = 2.5×10^{-4} m^3</td>
</tr>
<tr>
<td>Impeller speed</td>
<td>N</td>
<td>80 rpm = 1.333 s^{-1}</td>
</tr>
<tr>
<td>Working temperature</td>
<td>θ</td>
<td>37°C</td>
</tr>
<tr>
<td>Fluid</td>
<td></td>
<td>Water</td>
</tr>
<tr>
<td>Density (at θ)</td>
<td>ρ</td>
<td>997 Kg/m^3</td>
</tr>
<tr>
<td>Viscosity (at θ)</td>
<td>μ</td>
<td>0.6915 cP = 0.6915×10^{-3} Pa·s</td>
</tr>
<tr>
<td>Impeller Reynolds number</td>
<td>Re_{imp}</td>
<td>6.0×10^4</td>
</tr>
<tr>
<td>Power number</td>
<td>Np</td>
<td>1.2</td>
</tr>
</tbody>
</table>

Table 3.3. Table of physical and geometric characteristics used for the calculation of EDR in the spinner flask
\[ \text{Re}_{\text{imp}} = \frac{ND^2 \rho}{\mu} \]

<table>
<thead>
<tr>
<th>Impeller speed [RPM]</th>
<th>Re (_{\text{imp}})</th>
<th>EDR(_{\text{av}}) [W (\text{m}^{-3})]</th>
<th>EDR(_{\text{max}}) [W (\text{m}^{-3})]</th>
<th>Bar diameter [cm]</th>
<th>Bar length [cm]</th>
<th>D/T</th>
<th>w/D</th>
</tr>
</thead>
<tbody>
<tr>
<td>90</td>
<td>6.7 (\times) 10(^3)</td>
<td>3.9</td>
<td>285</td>
<td>2.1</td>
<td>5.6</td>
<td>0.778</td>
<td>0.38</td>
</tr>
<tr>
<td>150</td>
<td>1.1 (\times) 10(^3)</td>
<td>6.3</td>
<td>679</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>210</td>
<td>1.6 (\times) 10(^4)</td>
<td>7.05</td>
<td>878</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 3.4.** Average and maximum energy dissipation rate in a 250-mL, 7.2-cm internal diameter spinner flask (Adapted from Venkat, 1995).
Figure 3.1. Summary of the reported energy dissipation rate at which cells are affected as well as the reported levels of energy dissipation rate in various bioprocess environments. Adapted from Ma et al. (2002) and Mollet et al. (2004).
## Hydrodynamic Conditions

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Process</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Agitation</td>
<td>Volume average in typical animal cell bioreactors.</td>
<td>Varley and Birch (1999)</td>
</tr>
<tr>
<td>2</td>
<td>Agitation</td>
<td>Volume average in a 10 L mixing vessel (RT, 700 RPM)</td>
<td>Zhou and Kresta (1996)</td>
</tr>
<tr>
<td>3</td>
<td>Agitation</td>
<td>Maximum in the 10 L mixing vessel (RT, 700 RPM)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Agitation</td>
<td>Volume average in a 22,000 L mixing vessel (RT, 240 RPM)</td>
<td>Wernersson and Tragardh (1999)</td>
</tr>
<tr>
<td>5</td>
<td>Agitation</td>
<td>Maximum in the 22,000 L mixing vessel</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Agitation</td>
<td>Maximum in spinner vessel (200 RPM)</td>
<td>Venkat et al. (1996)</td>
</tr>
<tr>
<td>7</td>
<td>Bubble rupture</td>
<td>Pure water, bubble diameter: 6.32mm</td>
<td>Garcia-Briones et al. (1994)</td>
</tr>
<tr>
<td>8</td>
<td>Bubble rupture</td>
<td>Pure water, bubble diameter: 1.7mm</td>
<td>Boulton-Stone and Blake (1993); Garcia-Briones et al. (1994)</td>
</tr>
<tr>
<td>9</td>
<td>Flow through a pipe</td>
<td>Pure water, 100 mL/min, 1 mm diameter</td>
<td>Mollet et al. (2004)</td>
</tr>
<tr>
<td>10</td>
<td>Flow through a micropipette tip</td>
<td>Flow through a 200 µL micropipette tip in 0.2 sec</td>
<td>Mollet et al. (2004)</td>
</tr>
<tr>
<td>11</td>
<td>Agitation</td>
<td>Volume average in a highly agitated animal cell bioreactor</td>
<td>Oh et al. (1992)</td>
</tr>
</tbody>
</table>

Figure 3.1.(Continued).
Figure 3.2. Views of the microfluidic channel. (a) Photograph of the machined stainless steel sheet. (b) Photomicrograph of the neck region; arrows indicate the surface roughness and actual dimensions.
Figure 3.3. Various plots presenting the simulated velocity profiles in planes in (a) the entrance region \((x=10 \text{ mm})\) and (b) in the beginning of the throat \((x=15 \text{ mm})\). The flow rate of 90 mL·min\(^{-1}\) simulated was the maximum used in this study.
Figure 3.3. (Continued).
Figure 3.4. Simulation of the pathlines of two particles along the microfluidic channel, the range of color corresponding to levels of magnitude of EDR (in W·m⁻³).
Figure 3.5. Photographs ((a) and (c)), of cells flowing through the entrance and exit regions of the flow contraction device at a flow rate of 30 mL·min\(^{-1}\). Parts (b) and (d) present fluent simulations of particle pathlines through the entrance and exit regions of flow contraction device at a flow rate of 30 mL·min\(^{-1}\). Part (e) is a computer simulation of the energy dissipation rate, in W·m\(^{-3}\), that a particle would experience at a flow rate of 90 mL·min\(^{-1}\).
Figure 3.5. (Continued).
Figure 3.5. (Continued).
Figure 3.6. Histogram of the maximum EDR experienced by each simulated particle injection into the simulated flow in the microfluidic channel. The numeric value presented is the median value for each population.
Figure 3.7. Cell damage/lysis as a result of being subjected to a single, high level of hydrodynamic stress (in units of EDR). Cells had been previously cultured in T-flasks. The cell damage/lysis was determined (a) from visual counting, (b, c) from LDH assays.
Figure 3.7. (Continued).
**Figure 3.8.** Bivariate plot distribution of 7-AAD fluorescence vs. Annexin V-PE fluorescence for WT CHO-K1 cells cultured in T-flasks with 10% FBS. Quadrant I: 7-AAD negative, Annexin V negative (healthy cells); quadrant II: 7-AAD negative, AV-PE positive (cells undergoing early apoptosis); quadrant III: 7-AAD positive, AV-PE positive, (necrotic and/or late apoptotic cells); quadrant IV: 7-AAD positive, AV-PE negative, likely naked nuclei/cell debris that bind 7-AAD but lack the membrane-bound PS.
Figure 3.9. Time course experiment of the percent of the cell population exhibiting early (a) and late apoptosis/necrosis (b) as a function of time after exposure to a given level of EDR. The cell population used was WT-CHO cultured in T-flasks with 10% FBS and harvested in late exponential growth.
Figure 3.10. Time course experiment of the percent of the cell population exhibiting (a) early and (b) late apoptosis/necrosis as a function of time after exposure to a given level of EDR. The cell population used was CHO–bcl-2 cultured in T-flasks with 10% FBS and harvested in late exponential growth.
Figure 3.11. Time course experiment of the percent of the cell population exhibiting (a) early apoptosis and (b) late apoptosis/necrosis as a function of time after exposure to given level of EDR. WT CHO-K1 cells were cultured in a spinner flask with 0% FBS and harvested in late exponential growth.
Figure 3.12. Time course experiment of the percent of the cell population exhibiting (a) early apoptosis and (b) late apoptosis/necrosis as a function of time after exposure to given level of EDR. The cell population used was WT CHO cultured in a bioreactor with 0% FBS.
Figure 3.13. Time course experiment of the percent of the cell population exhibiting (a) early apoptosis and (b) late apoptosis/necrosis as a function of time after exposure to given level of EDR. The cell population used was CHO-bcl-2 cultured in a bioreactor with 0% FBS.
**Figure 3.14.** Histogram overlays of FCM analysis of bcl-2 over expressing cells stained with an anti-human bcl-2 antibody conjugated to PE and an isotype control antibody conjugated to PE. (a) is a histogram of a cell sample taken from a spinner vessel and (b) is a histogram of cells taken from the bioreactor.
CHAPTER 4

CELL DAMAGE IN A FLUORESCENCE ACTIVATED CELL SORTER

The content of this chapter is at present in Press: Mollet, M.; Godoy-Silva, R.; Berdugo, C. and Chalmers, J. J. (In Press). Computer Simulations of the Energy Dissipation Rate in a Fluorescence Activated Cell Sorter: Implications to Cells. *Biotechnology and Bioengineering*. This document is founded on the original draft entitled “Cell damage in FACS instruments” written by Dr. Mike Mollet based on the Chapter 5 of his own thesis: “Physiological Effects of Hydrodynamic Forces on Animal Cells”. Claudia Berdugo produced the pictures in Figure 4.2, THP1 data and results shown in Figure 4.7 as well as all THP1 data from cell sorting in FACS included in Tables 4.3 and 4.4. Ruben Godoy’s contribution to this work includes:

1. Participation in all the experiments of the cell sorting in FACS with CHO cells
2. Participation in the flow calibration as a function of pressure presented in Figure 4.4, necessary to perform the simulations.
3. Participation in the generation of the geometry and simulations of the FACS nozzles.
4. Editing of the document, bibliographical review, improvements in the discussion of results and rebuttal to the reviewers.
4.1. ABSTRACT

Fluorescence activated cell sorting, FACS, is a widely used method to sort subpopulations of cells to high purities. To achieve relatively high sorting speeds, FACS instruments operate by forcing suspended cells to flow in a single file line through a laser(s) beam(s). Subsequently, this flow stream breaks up into individual drops which can be charged and deflected into multiple collection streams.

Previous work by Ma et al. (2002) and Mollet et al. (2007) indicates that subjecting cells to hydrodynamic forces consisting of both high extensional and shear components in micro-channels results in significant cell damage. Using the fluid dynamics software FLUENT®, computer simulations of typical fluid flow through the nozzle of a BD FACSVantage indicate that hydrodynamic forces, quantified using the scalar parameter energy dissipation rate, are similar in the FACS nozzle to levels reported to create significant cell damage in micro-channels. Experimental studies in the FACSVantage, operated under the same conditions as the simulations confirmed significant cell damage in two cell lines, Chinese Hamster Ovary cells (CHO) and THP1, a human acute monocytic leukemia cell line.

4.2. INTRODUCTION

Fluorescence activated cell sorting, FACS, is a widely used method to sort small (~10^2–10^7) subpopulations of cells to high purities (~98%). FACS is an integral part of many
biological and medical research projects as well as cell line development. However, there is very little information available in the literature regarding cell death in FACS devices as a result of hydrodynamic forces. One recent report described a significant increase in necrosis and apoptosis in BT474 tumor cells and N1 normal human skin fibroblasts after FACS (Seidl et al., 1999), but the authors clearly state that the damage is not the primary focus of their research and they limit themselves to point out the reduction in viability without offering any additional explanation on the causes or mechanisms of the damage.

When sorting spermatozoa based on their sex chromosomes (X vs. Y) using FACS devices, Maxwell et al. (1997) reported a reduction in sperm viability after sorting; although the reason for this reduction remains unknown, the authors suggested it may be a result of hydrodynamic effects or the exposure of Hoechst-stained spermatozoa to the laser beam. They also indicate that viability seems to increase in spermatozoa passed through the FACS but this is an artifact as only viable cells are gated for recollection.

In a somewhat different area, effects of mechanical flow sorting on phytoplankton physiology have been reported by Jochem (2005); he showed a roughly 40% decrease on the number of metabolically active Emiliania huxleyi cells after sorting as measured by the cleavage of fluorescein diacetate. Although the reason or mechanism causing that reduction was not investigated, they mention that it may be related to the exposure to hydrodynamical forces or the laser beam. With respect to the question of lasers, Rivkin et al. (1986) demonstrated previously that a decrease in carbon uptake rate in several species of dinoflagellate and diatom after sorting is related to the exposure to, and the intensity of, the cytometer’s laser beam rather than to shear in the nozzle; this mechanism
very probably is limited to this type of organisms where growth is intimately related to photosynthesis and, presumably, can be affected by the laser photons.

Williams and Peter (2007) mention that FACS isolation of stem cells is a debatable technique as it may hydrodynamically damage the cells, but again no further discussion is offered. Even a paper commonly mentioned to relate hydrodynamic damage in a flow cytometer (Al-Rubeai et al., 1993) uses flow cytometry to evaluate hydrodynamic damage in conditions of intensive agitation in bottles but it does not address the hydrodynamic damage as a result of passing the cells through a flow cytometer. As it can be seen, although there are reports of hydrodynamic damage to cells in FACS, the reason still remains obscure and it deserves further exploration.

In general, there are two primary pathways for cell death: necrosis and apoptosis. Necrosis is passive cell death or “cell murder”; some external factor causes the cell to die (Darzynkiewicz et al., 1997). Typically, when cells die via necrosis their membrane swells and, eventually, the cell membrane ruptures. Apoptosis, on the other hand, is programmed cell death or “cell suicide”; cells undergo a series of events, which ultimately lead to the cells’ demise (Cotter and Al-Rubeai, 1995). For non-receptor mediated apoptosis, cytochrome c is released from the mitochondria, which initiates the caspase cascade and phosphatidylserine (PS) translocates from the interior of the cell membrane to the exterior. The caspase cascade ultimately initiates endonuclease activity within the nucleus and the enzymatic degradation of the cytoskeleton. Eventually the cell membrane starts to form bulges or “blebs”; these blebs break away from the membrane.
forming apoptotic bodies containing intact organelles and small pieces of DNA, approximately 180 bp long.

Apoptosis can be initiated by events such as radiation, toxins, hypoxia, and nutrient starvation (Al-Rubeai et al., 1995; Fussenegger and Bailey, 1998), and it has been suggested that hydrodynamic forces can initiate apoptosis (Graf et al., 2003). However, a more recent report by Mollet et al. (2007) suggests that an acute exposure to high levels of hydrodynamic forces only caused a minor increase in apoptosis in Chinese Hamster Ovary cells (CHO).

Much has been discussed about the best way to correlate hydrodynamic damage to cells in bioprocesses. One commonly used method is the calculation of the stress tensor, which is composed of shear stress components and extensional components. It has been suggested for many years, dating back to the pioneering work of G.I. Taylor (1934) on oil drop breakup in aqueous solutions, that for the same level of stress, purely extensional flows are more disruptive than purely shear flows (Croughan and Wang, 1989; Garcia-Briones and Chalmers, 1994; McQueen and Bailey, 1989; McQueen et al., 1987). Taylor attributed the difference to the rotation of the drops in pure shear flow, which relaxes the stress drops experience. Motivated by this discussion, Garcia-Briones and Chalmers (1994) proposed a parameter called “amount of rotation” which represents the ratio of the time scale of liquid deformation to the time scale of rotation to supplement energy dissipation in describing the damaging forces. In the case of a cell in a purely shear flow, the vorticity is not zero, cells rotate; therefore no single point along the cell membrane
experiences a continuous, maximum strain (Garcia-Briones and Chalmers, 1994). In contrast, for pure extensional flows it is possible for a relatively large amount of strain to occur at one point on a cell since the vorticity of the flow is zero (i.e., the cell can not rotate).

Another parameter used to represent hydrodynamic force in bioprocesses is the energy dissipation rate (EDR) (Bluestein and Mockros, 1969; Mollet et al., 2004). The EDR represents the rate at which work is done on a fluid element or, in the context of this study, a biological cell. Bird et al. (2001) defined the EDR as the irreversible rate of internal energy increase per unit volume. The EDR appears to be a good candidate to correlate cell damage for a number of reasons: it is a single, scalar parameter that can be obtained from the fundamental equations of fluid dynamics, it only depends on the intrinsic characteristics of the flow (i.e., it can be calculated for both laminar and turbulent flow), it is geometry-independent, it accounts for both shear and extensional components of the flow and it has been used successfully as a parameter to characterize and predict damage in a number of published studies (Aloi and Cherry, 1996; Gregoriades et al., 2000; Ma et al., 2002; Fife et al., 2004; Hu et al., 2007; Mollet et al., 2004, 2007).

For an incompressible, Newtonian fluid, EDR ($\varepsilon$) is a function of both $\mu$, the dynamic viscosity, and $\mathbf{U}$, which is the velocity vector; such function is presented in Eq. 4.1, where $\nabla \mathbf{U}$ is the velocity gradient tensor, and $(\nabla \mathbf{U})^T$ is the transpose of $\nabla \mathbf{U}$.
\[ \varepsilon = \mu \left[ \nabla \mathbf{U} + (\nabla \mathbf{U})^T \right] : \nabla \mathbf{U} \]  

(Eq. 4.1)

Equation 4.1 is valid for any flow regime (i.e., laminar, transient or fully turbulent).

As it can be observed in the above equation, once the velocity vectors are determined, the EDR can be calculated. There are a variety of methods to determine velocity vectors. In laminar flow systems with simple geometries, the velocity vectors can be determined analytically from the momentum balance equation. Examples of such systems are pipe flow and annular flow. For a more complete explanation of how to calculate the EDR on these systems see Mollet et al. (2004). Most systems, however, are geometrically too complex to use the aforementioned analytical methods; therefore the flow must be simulated using computational fluid dynamics software (CFD). The velocity vectors calculated from CFD in a laminar flow system are generally accepted, when properly executed; however, in turbulent systems, the results are not as well received. Fortunately, FACS instruments operate within the laminar flow regime as will be shown below.

The EDR is well suited to describe the hydrodynamic conditions in FACS devices because the cells are in suspension and the cells are forced through a nozzle, which creates nearly pure extensional flow. Also, under normal operating conditions, the suspended cells in a FACS instrument are not in contact with the wall of the nozzle (because of the hydrodynamic focusing), thereby reducing the shear component of the flow the cells experience.
Ma et al (2002) developed a micro-fluidic contractional flow device (subsequently referred to as the “torture chamber”), which was subsequently modified by Mollet et al. (2007) to determine the maximum amount of EDR cells can withstand before the onset of necrosis. The torture chamber resembles a flattened hourglass (4.1). Ma et al. (2002) utilized FLUENT®, a commercial CFD program, to simulate the velocity vectors at every point in the torture chamber for several volumetric flow rates and the results of these simulations were used to determine the local EDR. Based on the results from these simulated volumetric flow rates, and subsequent calculations of EDR, cell suspensions were pumped through the torture chamber and the cell viability was determined using a lactate dehydrogenase (LDH) assay. Ma et al (2002) performed a series of experiments on CHO, HB-24, SF-9 and MCF-7 cell lines, and found that the cells can withstand high levels of energy dissipation, approximately $10^7$-$10^8$ W·m$^{-3}$ before significant cell lysis begins to be detected. More recently Mollet et al. (2007) using flow cytometric (Annexin V-PE/7-AAD) as well as microscopy assays measured the percentage of apoptosis and necrosis induced in CHO cells by passing them through a modified version of the torture chamber; the authors found that anchorage dependent, wild type (WT) CHO cells cultured with 10% FBS displayed low levels (a few percent relative to control) of apoptosis beginning at $3\times10^5$ W·m$^{-3}$, whereas bcl-2-transfected CHO cells displayed no apoptosis.

FACS instruments operate by forcing suspended cells, previously labeled with fluorescent probes to stain some cellular component or function of interest, into a thin stream (or transparent capillary tube depending on the instrument) where cells flow in a
single-file, high-speed line through a focused laser(s) beam(s). An electronic-optic system collects the light scattered or emitted by the cells from the laser beam(s). Subsequently, the flow stream breaks up into individual drops, which can be charged and deflected into multiple collection streams based on an analysis of the characteristics of the light either deflected or emitted from the laser-illuminated cell (Huh et al., 2005; Shapiro, 2003). Figure 4.2a through c provide a number of photos of the fluidics of a BD FACS Vantage. With the Vantage, the cells are squeezed into a single file line through hydrodynamic focusing, where the cell suspension is injected into the core of another stream of sheath fluid (Fig. 4.3a). Changing the air pressure in the sheath tank controls the flow rate of the sheath fluid (Fig. 4.3b).

The sheath pressure reading on the instrument is actually the air gauge pressure in the sheath tank, and is not easily correlated to the sheath fluid pressure at the nozzle due to various pressure drops in the pathway to the nozzle (e.g., sheath fluid filter, compression fittings, valves, etc.). Altering the air pressure within the sample test tube (Fig. 4.3c), via the sample differential, controls the sample flow rate. The sample differential is the amount of pressure above the sheath pressure; for example a sample differential of 0.5 psi and a sheath pressure of 25 psi would mean there is 25.5 psig of pressure over the cell suspension in the tube.

In this study, the hydrodynamic forces in the nozzle of a BD FACS Vantage were simulated using a computational fluid mechanics package (FLUENT®) based on experimentally measured flow rates typically used in sorting experiments. It is suggested,
and will be later demonstrated, that the flow through the nozzle is quantitatively quite similar to that of the torture chamber device described above. In addition, the viability of two cell lines, CHO, and THP-1 (a human monocytic-like cell) after sorting in the FACS Vantage was determined and compared to the results of subjecting the cells to exposure of high hydrodynamic forces in the torture chamber.

4.3. MATERIALS AND METHODS

4.3.1. CFD Simulations

The flow through the nozzle from a Becton-Dickinson (BD) FACS Vantage SE was simulated using the commercial, CFD program, FLUENT®, Ver. 6.2, 2d, dp. The dimensions of the nozzle were determined using a micro-meter as well as diagrams obtained from BD (personal communications with Dr. Diether Recktenwal) and entered into Gambit, the preprocessor included with FLUENT®, to create the corresponding geometry and mesh. An unstructured (pave) mesh consisting of quadrilateral and triangle elements was used to model the flow through the system. Due to the axial symmetry of the nozzle, only half of a two dimensional (2-D) nozzle was simulated. Two different nozzle diameters were considered: 70 and 100 μm. Finally, the geometry and mesh were imported into FLUENT® to start the simulations.

After mesh optimization, the whole 2-D system was modeled with a mesh of approximately 182,000 node points in a grid size of approximately 7 μm at the nozzle
discharge (for the case of 70 μm nozzle). For comparison purposes, the diameter of a CHO cell is around 12–14 μm.

For the simulations in FLUENT, we used the segregated solver model, which performs iterations to sequentially solve the non-linear equations for continuity and momentum. No energy balance was included (isothermal conditions assumed with negligible friction losses). The implicit formulation was used to solve the equations for steady state operation assuming laminar flow using the viscous fluid model with $P_{\text{absolute}}=1.0$ atmosphere at the center of the output face of the simulated channel (Point A, Fig. 4.5a). Boundary conditions involved interaction with the solid walls (non-slip condition) and constant velocity at the inlet of both the sheath fluid and the sample (further descriptions below). No interaction with air was taken into account at the exit of the nozzle (i.e., no surface tension), as the air is outside of the limits of the simulated system. The schemes used for interpolation: for momentum, 1st order upwind; for pressure, the standard FLUENT interpolation; and for pressure-velocity coupling, the SIMPLE scheme was used. The convergence criterion was $\leq1\times10^{-3}$ for $V_x$, $V_y$, and continuity. All the simulations for the present research were performed using a Pentium1-D CPU running at 3.2 GHz with 1.0 GB RAM and 8.85 GB hard-disk.

The sheath inlet velocities were determined for two nozzle sizes by experimentally measuring, in duplicate, the volumetric flow rate, at various sheath pressures (see Fig. 4.3b and c), of the sheath fluid flowing out of the nozzle while the fluidics control knob was set to standby. The average inlet velocity was calculated by dividing the flow rate by
the flow area. The sample inlet velocity was determined using the same methodology by measuring the sample uptake rate, at various sample differential and sheath pressures. These velocities were entered into FLUENT® and after convergence for each set of conditions, a user-defined function was implemented to calculate the EDR at every node point by using Equation (4.1).

Employing a methodology similar to that reported in a previous publication (Mollet et al., 2007), a particle tracking technique option in the FLUENT® software was utilized to determine the maximum EDR a simulated particle would encounter while flowing through the simulated nozzle. Two hundred 10-μm-diameter virtual particles were released from the inlet of the sample tube and tracked through the entire geometry. As a result of their small size and similar density relative to the medium, cells are expected to follow closely the pathlines of the fluid; consequently, particle–liquid interactions were not considered in the simulations. Also, because of the low cell concentration, particle–particle interactions were negligible in the simulation (uncoupled mode). The maximum EDR each particle experienced in the nozzle was exported to Excel® to create histograms of the maximum EDR each particle experienced for every condition. Given the fairly narrow distribution for every set of conditions, the peak frequency of each histogram was selected as a representative value for such set.
4.3.2. Cell Culture

Wild type (WT) CHO-K1 cells were adapted to grow in serum free media by gradually reducing the serum concentration in CD CHO-A serum free media (Gibco, Carlsbad, CA). The WT CHO cells were cultured in T-75 flasks and harvested via trypsinization. THP1 is a human acute monocytic leukemia cell line from ATCC routinely used in screening assays to assess cytotoxicity induced by potential agents. THP1 cells were grown in suspension in 75 mL T-flasks in phenol red free RPMI 1640 media containing 2 mM L-glutamine (Gibco) and supplemented with 10% FBS (Hyclone, Logan, UT). Due to an apparent drop in viability of the cells after a high number of passages, cells used in this study were between the third and fourth passages. Both cell lines were grown at 37°C in a humidified atmosphere of 5% CO₂.

4.3.3. Cell Sorting in FACS

Six samples of approximately $2.5 \times 10^6$ CHO cells, suspended in 250 μL of CD CHO-A media (each sample had $\sim 1.0 \times 10^7$ cells·mL$^{-1}$) were sorted using the FACSVantage SE with a 100 μm nozzle and a sheath pressure of 17 psi. Three of the samples were sorted with a sample differential pressure of 0.5 psi, and the remaining three were sorted with a sample differential pressure of 1.5 psi. Two samples of cells at precisely the same concentration were used as controls. After sorting, the cells were collected in polypropylene 12 mm × 75 mm tube with 1 mL of CD CHO-A media. Next, the cells
were centrifuged at 250g for 7 min, and then resuspended in 20 mL of CD CHO-A media.

Similarly to the CHO cells, THP1 cells were suspended in RPMI media at a cellular concentration of ~1.0×10⁷ cells·mL⁻¹ and samples of 250 μL were sorted in the BD FACS Vantage SE under the same conditions as were the CHO cells. After sorting the cells, the recovery was evaluated by visual counting. The cells were centrifuged at 250g for 7 min and the supernatant was used to evaluate cell death based on LDH released.

4.3.4. Micro-Fluidic Contraction Device

The micro-fluidic contraction device, that is, torture chamber, presented in Figure 4.1 and described in detail by Mollet et al. (2007), was used to determine the relationship between EDR and cell damage for the CHO and THP1 cells. Cell suspensions were pumped through the torture chamber at five different flow rates using a syringe pump (Pump 33, Harvard Apparatus, Holliston, MA); the corresponding values of EDR for every flow rate are presented elsewhere (Mollet et al., 2007). The effluent from the torture chamber was collected in a sterile conical tube of 50 mL for damage analysis using LDH.
4.3.5. **Cell Damage Analysis**

Cell damage as a result of being sorted in the FACS Vantage, or being pumped through the torture chamber was analyzed by several methods including a total cell balance based on visual cell counting (cell concentration) and measurements of cell suspension volume, LDH analysis, and apoptosis/necrosis assays using flow cytometry; all three analysis techniques have been described in detail elsewhere (Mollet *et al.*, 2007) and will only be summarized here. Visual determination of cell concentration and viability was made by trypan blue exclusion using a hemocytometer; LDH is an intracellular enzyme that is released into the culture medium when the cell membrane is compromised (necrosis); measurements of LDH were made following the protocol of the CytoTox-ONE assay kit (Promega, Madison, WI): absorbance measurements at 490 nm were made using a SpectraMax 250 spectrophotometer and compared with a standard curve for every cell line to calculate the number of necrotic cells. Fresh medium was used as blank; 690 nm was used as a reference wavelength.

For the apoptosis/necrosis assays in the FACS, after sorting the CHO cell suspensions they were placed in a 37°C incubator shaker. Approximately every 2 h ~5×10^5 cells were removed from each cell suspension, washed twice in PBS and resuspended in 0.5 mL 1X Annexin V binding buffer. A 100 μL aliquot was taken from each of the washed cell suspensions. The cells in this aliquot were stained with 5 μL of Annexin V-PE and 5 μL of 7-AAD; both reagents are from BD Biosciences (San Jose, CA). The cells were then
analyzed using a BD FACSCalibur. The appropriate single color controls were produced using heat-shocked cells—cells exposed to a 42°C water bath for 45 min.

4.4. RESULTS

4.1. FACS Flow Rate Measurements

Figure 4.4a presents the experimentally measured sheath fluid flow rate as function of nozzle diameter and sheath pressure; Figure 4.4b and c presents the experimentally measured sample uptake flow rate as a function of sheath pressure and sample differential pressure for two nozzles of 70 and 100 μm, respectively. As expected, both sheath pressure and nozzle size exerted a significant influence on the sheath flow rate in contrast with sample uptake rate for which these two variables had much less influence. Conversely, the sample differential had a linear correlation with sample flow rate for virtually all conditions tested.

4.2. CFD Simulations

A range of simulations were conducted for many of the operating conditions experienced in the daily use of the BD FACSVantage at the Ohio State University Cell Analysis and Sorting Core. Specifically, for nozzle diameters of 70 and 100 μm, simulations were conducted with sheath pressures ranging from 8 to 45 psi and sample differential pressures of 0.5–2.0 psi.
Figure 4.5a is an example of the unstructured computational grid used and Figure 4.5b is a Fluent® output of one of these simulations showing the simulated particle trajectories which are color coded to correspond to the EDR at the given location. As can be seen in Figure 4.5b, every particle experiences a wide range of EDR along its trajectory, although the maximum EDR value is located close to the entrance of the final section of constant diameter. Figure 4.6 is a histogram presentation of the maximum EDR that the simulated, injected particles would experience flowing through the 70 μm tip using 25 psi sheath pressure, and four differential pressures of: 0.5, 1.0, 1.5, and 2.0 psi. Because of the relatively narrow distribution, the peak value was chosen as representative for every set of conditions. Table 4.1 reports the representative highest level of EDR that a particle would experience for the specific operating conditions in a 100 or a 70 μm nozzles.

Pinkel and Stovel (1985) developed a relationship, based on a mass balance equation, to approximate the core stream diameter:

\[ d = 1130 \times \sqrt[3]{\frac{q}{v}} \]  \hspace{1cm} (Eq. 4.2)

where \( d \) is the core stream diameter (in μm), \( q \) the volumetric flow rate (in mL·s\(^{-1}\)), and \( u \) the exit velocity (in m·s\(^{-1}\)). Table 4.2 presents a comparison of the FLUENT simulation core radius (in μm) and the core radius calculated from Equation (4.2) (also in μm) for the lowest, highest, and middle values of maximum EDR for the 70 and 100 μm nozzles.
Table 4.2 also lists the conditions used as inputs for the specific FLUENT simulation as well as the calculated flow rate and exit velocity. As can be observed, the difference between the calculated core radius and the simulated core radius value averaged 11% which is a reasonably close agreement given all of the assumptions using both methods.

In classical fluid mechanics theory for conventionally sized circular pipes and for fully developed flow, the experimental limit of the Reynolds number ($Re_{c,cr}$) used to determine if a flow is laminar corresponds to $Re_{c,cr} \leq 2,100$. As can be observed in Table 4.2, the Reynolds number is well below this value for all but one case. However, even for this one case, it is highly unlikely that the flow is turbulent since the nozzle distance is too short for fully developed flow to develop. It is generally accept that for a Reynolds number of 2,000, the entry length is approximately 113 pipe diameters, which is well beyond the length of the actual distance of the flow in the solid nozzle (Brodkey, 1995).

### 4.3. Torture Chamber Studies on Cells

Figure 4.7 presents the results of three independent sets of studies of the effect of various levels of EDR created by the torture chamber on the TPH1 cells. The error bars represent the standard deviation of three replicas for a given set of operating conditions. For comparison, the results of a previous published study by Ma et al. (2002) are included. As can be clearly seen, for the same level of damage, the THP1 cells are approximately 1.5 orders of magnitude more sensitive to EDR than the previously studied cell lines.
Table 4.3 presents the results of one of the three studies on the THP1 cells and the results of CHO cells subjected to two levels of EDR, $2.6 \times 10^7$ and $1.09 \times 10^8$ W·m$^{-3}$. Comparison of the CHO data with that presented in Figure 4.7 indicates that these CHO cells, grown in chemically defined, serum free media are more sensitive to hydrodynamic stress in contrast to the CHO cells studied and reported (i.e., compare to Fig. 4.7). It should be noted that in previous studies, the CHO cells were grown in 10% serum.

4.4. FACS Studies

Table 4.4 presents the results of FACS sorting of the CHO and THP1 cells under conditions which FLUENT simulations indicate EDR of $1.14 \times 10^7$ and $1.98 \times 10^8$ W·m$^{-3}$ are created. As can be observed, a significantly high level of damage, both by lowered overall cell recovery, and percent damage based on LDH release is observed.

Figure 4.8 is a typical dot plot of 7-AAD relative fluorescence versus Annexin V-PE relative fluorescence; the events on this plot were gated based on a region of the forward versus side scatter plot that would exclude debris. Events in the lower left quadrant are negative for both 7-AAD and Annexin V-PE and are considered healthy cells. Events in the lower right hand quadrant are positive for AV binding and are considered in the early stages of apoptosis because they are expressing PS, on the exterior. Events in the upper quadrants are positive for 7-AAD and are considered necrotic or late apoptotic; these cells have a compromised cell membrane and are no longer viable. Unfortunately, one cannot determine how these cells died, because cells with a compromised membrane may
also have AV binding the PS on interior of the cell membrane. Due to the time scale of apoptosis (typically hours), events detected in the upper quadrants shortly after sorting are most likely necrotic, not late apoptotic.

Figure 4.9 presents the results of apoptosis/necrosis studies. Figure 4.9a is a plot of the mean percentage of the CHO cells in the lower right quadrant (early apoptosis) versus the amount of time that has past since being sorted. Figure 4.9b is a plot of the mean percentage of the CHO cells in the upper right hand quadrant (late apoptosis/necrosis). The error bars in both figures represent one standard deviation above and below the mean.

4.5. DISCUSSION

The data from the CFD simulations indicate that the FACS Vantage can create levels of EDR that have been shown to damage cells (Ma et al., 2002; Mollet et al., 2007). It is also significant to note, in general, for a given sheath pressure, the maximum EDR goes up as the sample differential pressure goes down.

The design of the nozzle is responsible for the high EDR values; the abrupt constriction at the exit of the nozzle causes large changes in the velocity gradient in the axial direction (high extensional flow) of the core stream which results in a correspondingly higher, EDR values. This design is similar, yet more pronounced, than the torture chamber used to study the effect of hydrodynamic forces on cells (see Fig. 4.1). Also, the CFD
simulation results follow Pinkel and Stovel’s heuristic regarding the relationship between the core stream diameter and the sample volumetric flow rate in that increasing the volumetric flow rate by a factor of four increases the core stream diameter by a factor of 2 (Pinkel and Stovel, 1985).

Consistent with these high levels of EDR created in the FACS, the experimental data presented in Table 4.4 and Figure 4.9a and b indicate that flow through the FACS creates significant cell damage. Given the significant numbers of assumptions and variables involved, reasonable agreement exists between levels of EDR that damage THP1 cells in the torture chamber and in the FACS. A similar observation can be made between the torture chamber studies of CHO cells and the FACS sorter. However, compared to Figure 4.7, at levels of EDR of $1.98 \times 10^8 \, \text{W} \cdot \text{m}^{-3}$ significantly higher levels of damage to CHO cells were observed in this study (on the order of 40% vs. 15%), both in the torture chamber as well as the FACS, relative to previous studies.

A number of possibilities exist for this difference in damage between FACS and the torture chamber. However, care should be taken with respect to strict comparisons of the numbers. First, whether the percentage of cells damaged/ruptured is 15% or 40%, inspection of Figure 4.7 clearly shows a trajectory of a rapid increase in cell destruction as the EDR increases. Given the $x$-axis is logarithmic, and the rapid increase in cell damage once a threshold is reached, it would not take that much of an increase in EDR to significantly increase the percent of damaged cells. Given the significant number of assumption in this work, the authors are quite satisfied with the agreement. In addition, it
is also possible that the use of chemically defined media without any serum and the fact the CHO cells used in this study were grown in T-flask and trypsinized before being subjected to high EDR could also contribute to a slight increase in cell sensitivity (Kunas and Papoutsakis, 1990; Martens et al., 1991). In the previous studies, Ma et al. (2002) had grown the CHO cells in 10% serum and in Mollet et al. (2007) the CHO cells had been grown in Hyclone SFM4CHO with 1% FBS. It is highly likely that the use of chemically defined media, without serum, resulted in the CHO cells slightly more susceptible to hydrodynamic forces.

Difference in the amount of damage between the torture chamber and FACS, for a similar level of EDR could also be related to time of exposure. Visual inspection of Figure 4.5b, and the tabulated values of the core radius in Table 4.2 indicates that the zone of very high EDR in the FACS is only a small cylinder on the order of $4 \times 10^{-8}$ mL; while for the torture chamber, previous reports (Ma et al., 2002; Mollet et al., 2007) indicates that a majority of the damage zone is a rectangle directly in front of the contraction zone and is on the order of $2 \times 10^{-5}$ mL. With a flow rate in the FACS on the order of 0.24 mL·min$^{-1}$, this corresponds to a residence time of $10^{-5}$ s in the high EDR region, while with a flow rate in the torture chamber on the order of 90 mL·min$^{-1}$, this corresponds to a residence time of $1.3 \times 10^{-5}$ s. While similar, it is possible that there is enough of a difference in exposure time to account for difference in the total amount of cell damage. Currently, the relationship between cell damage, the level of EDR exposure and time at that level is not known.
Inspection of Figure 4.6 presents what, at first, can appear to be contradictory results; namely that the data from the 2.0 psi sample differential simulation seems out of place relative to the trend seen with the 1.5, 1.0, and 0.5 psi sample differential simulations. Also, three maximum peaks exist for the 2.0 psi sample differential, where as only one peak exists for the other conditions. However, a general trend of both the data, and Equation (4.2) is that as the sample differential pressure goes up, the core stream gets wider. Computer simulations in this study, and those of the flow contraction device (Torture Chamber) indicates that there are, effectively, only extensional forces on the center core flow, but as one moves out toward the walls, the flow will also experience regions of high shear stress. This is very apparent in some of the plot presented in Mollet et al. (2007). The multiple peaks in Figure 4.6 most likely reflect some of the fluid experiencing only high extensional flow, while other parts of the fluid experience zones of high shear stress. It should be noted that one typically operates the FACS instrument with the core stream as thin as possible so that the cells exit in a “single file”. Consequently, operating at a sample differential of 2.0 or higher is not common. Further studies are ongoing to understand not only this multiple peak phenomena, but also other operating conditions and nozzle designs.

While significant cell damage can be observed, as it can be seen in Figure 4.9b, FACS sorting does not cause significant levels of apoptosis in the cells investigated in this study. This is consistent with the report by Mollet et al. (2007) that showed that while a low level of apoptosis can possibly occur in CHO cells subjected to comparable levels of EDR in the torture chamber, the primary mode of death is complete cell lysis and
necrosis. This is in contrast to the work of Seidl et al. (1999) who found significant levels of both necrosis and apoptosis after FACS in BT474 tumor cells. In addition to being a different cell line, it is also possible that the reduced levels of apoptosis in this study are the result of cells cultured in serum free media, which some have found to make the cells less robust. Therefore, instead of the cells sustaining an injury and then becoming apoptotic, the cells died immediately via necrosis. A common “rule of thumb” practice among FACS operators is to add media containing a large percentage of serum (usually 20%) to the collection tube, in an effort to improve the viability of the cells after sorting.

The results, overall, indicate that the FACSVantage is causing significant levels of cell death to hydrodynamic forces. However, it should also be noted that while significant damage is observed, the fact that FACS sorting is routinely conducted with success indicates that while a significant number of cells can be damaged, enough cells remain viable such that a typical FACS sort is successful. It is also possible that failure to achieve a sort, from the perspective of a customer to a typical FACS facility could be the result of the particular cell system (either a particular cell line or a typical cell line cultured in such a way to make the cells more sensitive) is just more sensitive to hydrodynamic forces than “normal” cells.

Since engineering the cells to become more robust would be impractical to most users of a FACS, a better designed flow nozzle would most likely alleviate the problems associated with cell loss due to hydrodynamic forces. More specifically, an elongated nozzle with a less abrupt constriction would greatly reduce the velocity gradients just
prior to the exit and thereby reduce the EDR in the exit region. Also, an elongated nozzle would reduce the potential onset of turbulence created by small perturbations caused by the sudden change in diameters and translated into a boundary layer separation, which may cause vortices. Retarding the onset of turbulence and vortices would allow for higher, more stable sort rates; since FACS is a slow isolation technique compared with other methods (such as magnetic bead enrichment or depletion), an increase in sorting rates would allow for a wider spectrum of applications.

To the knowledge of the authors, this is the first in depth study of the hydrodynamic phenomena that could be affecting the viability of sorted cells inside the nozzle of currently available flow cytometers. Ongoing studies in our laboratory are focused on further investigating the mechanisms of damage of the cells as well as attempting to reduce cell death by modifying operating conditions.
4.6. REFERENCES


<table>
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<tr>
<th>Nozzle diameter (μm)</th>
<th>Sheath pressure (psi)</th>
<th>Sample differential (psi)</th>
<th>Max. EDR (W·m⁻³)</th>
<th>Nozzle diameter (μm)</th>
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Table 4.1. Highest level of EDR that a particle would experience in a BD FACSVantage for different operating conditions in a 70 and 100 μm nozzle.
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<th>Average exit velocity (m·s⁻¹)</th>
<th>Core radius (μm) FLUENT</th>
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**Table 4.2.** Comparison of the FLUENT simulation core radius (in μm) and the core radius calculated from Equation 4.2 (also in μm) for different nozzle diameters and operating conditions in a BD FACSVantage.
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<td>0.3</td>
</tr>
<tr>
<td>THP1</td>
<td>30</td>
<td>5.9×10⁵</td>
<td>97</td>
<td>2.27×10⁶</td>
<td>6.4</td>
<td>1</td>
</tr>
<tr>
<td>THP1</td>
<td>50</td>
<td>5.9×10⁵</td>
<td>97</td>
<td>6.45×10⁶</td>
<td>27.6</td>
<td>5</td>
</tr>
<tr>
<td>THP1</td>
<td>70</td>
<td>4.15×10⁵</td>
<td>92</td>
<td>2.60×10⁷</td>
<td>43.5</td>
<td>1.8</td>
</tr>
<tr>
<td>THP1</td>
<td>90</td>
<td>5.0×10⁵</td>
<td>94</td>
<td>1.09×10⁸</td>
<td>47.1</td>
<td>6.7</td>
</tr>
<tr>
<td>CHO</td>
<td>70</td>
<td>4.70×10⁵</td>
<td>&gt; 95</td>
<td>2.60×10⁷</td>
<td>22</td>
<td>-</td>
</tr>
<tr>
<td>CHO</td>
<td>90</td>
<td>4.70×10⁵</td>
<td>&gt; 95</td>
<td>1.09×10⁸</td>
<td>27</td>
<td>-</td>
</tr>
</tbody>
</table>

**Table 4.3.**  Cell damage results in the flow contraction device of THP1 and CHO cells subjected to different levels of EDR.
<table>
<thead>
<tr>
<th>Cell type</th>
<th>Sheath pressure (psi)</th>
<th>Sample diff (psi)</th>
<th>EDR (W·m⁻³)</th>
<th>Total cell number before sort</th>
<th>Total cell number after sort</th>
<th>n</th>
<th>% Cells recovered</th>
<th>% Cells damaged based on LDH</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHO</td>
<td>17</td>
<td>1.5</td>
<td>1.14×10⁷</td>
<td>5.50×10⁶</td>
<td>4.00×10⁶</td>
<td>3</td>
<td>73</td>
<td>-</td>
</tr>
<tr>
<td>CHO</td>
<td>17</td>
<td>0.5</td>
<td>1.98×10⁸</td>
<td>5.52×10⁶</td>
<td>3.30×10⁵</td>
<td>3</td>
<td>60</td>
<td>-</td>
</tr>
<tr>
<td>THP1</td>
<td>17</td>
<td>1.5</td>
<td>1.14×10⁷</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>59 ± 24</td>
<td>33 ± 4.3</td>
</tr>
<tr>
<td>THP1</td>
<td>17</td>
<td>0.5</td>
<td>1.98×10⁸</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>81 ± 10</td>
<td>27 ± 7.3</td>
</tr>
</tbody>
</table>

**Table 4.4.** Cell damage results in the FACS Vantage of THP1 and CHO cells subjected to different experimental conditions.
Figure 4.1. Photograph (a), top view (b), and a perspective view (c) of the flow contraction device. All measurements are in millimeters.
Figure 4.2. Photograph of BD FACS Vantage nozzle through drop collection tube (a); an enlarged photograph of the nozzle and reflection of laser beam off the exit stream indicating point of laser interrogation (b); an enlarged photograph of the region in which the stream breaks into individual drops and that are subsequently deflect by the charge on the deflection plates (c).
Figure 4.2. (Continued).
Figure 4.3. Schematic of flow cytometry fluidics: enlargement of the nozzle area (a); locations where pressure measurements were made to determine flow rate: the sheath tank area (b), and the sample injection area (c).
Figure 4.3. (Continued).

\[ P_{\text{total}} = P_{\text{sheath}} + P_{\text{sample diff}} \]
Figure 4.4. Exit sheath flow rate as a function of sheath pressure for the 70 μm and 100 μm nozzles (a), and sample flow rate as a function of sample differential and sheath pressure for a 70 μm (b) and a 100 μm (c) nozzles.
Figure 4.4. (Continued).
Figure 4.5. View of the nozzle geometry and mesh used for the simulation (a), and Fluent output of the simulations of particles flowing through the nozzle (b). For the specific simulated conditions, the sheath pressure was 17 psi and the sample differential pressure was 2 psi. The color coded figures correspond to the levels of EDR, in units of W·m\(^{-3}\).
Figure 4.5. (Continued).
Figure 4.6. Example histograms of maximum EDR that simulated particles would experience flowing through a 70 μm diameter FACS nozzle operating at 25 psi sheath pressure and sample pressures ranging from 0.5 to 2.0 psi.
Figure 4.7. Percent cell damage, based on LDH assay, as a results of a single pass of the specific cell line indicated through the flow contraction device at the given level of EDR. The data for the THP1 cell line is new for this study while the data for the CHO, Sf-9, MCF-7, and hybridoma cell lines are from Ma et al. (2002).
Figure 4.8. Typical dot plot of 7-AAD (FL3-H) versus Annexin V-PE (FL2-H), data taken 7.5 hours after the cells sorted with a sample differential of 0.5 psi.
Figure 4.9. Early apoptosis (a), and late apoptosis/necrosis (b), as a function of time for a CHO cell suspension passed through the FACS Vantage with a 100 μm nozzle at 17 psi sheath pressure and a sample differential of 0.5 psi (dotted) or 1.5 psi (dashed). The solid line is a control.
CHAPTER 5

EXPERIMENTAL EVALUATION OF THE EFFECT OF CHRONIC HYDRODYNAMICAL STRESSES ON CULTURES OF SUSPENDED CHO CELLS

The content of this chapter was submitted to Biotechnology and Bioengineering. Dr. Mike Mollet was included as an author since the idea of the recirculation system was suggested as a recommendation at the end of his doctoral dissertation.

5.1. ABSTRACT

The effect of hydrodynamic forces on animal cell cultures, while extensively studied, still lacks significant, fundamental understanding. A previous manuscript from our lab reported on the acute exposure of CHO cells to hydrodynamic forces in a second generation convergent-divergent microfluidic device (Mollet et al. 2007). In this study, we extend the use of this device to a system in which suspended animal cells, grown in a typical bioreactor, are subjected to chronic exposure of moderately high levels of hydrodynamic forces by way of a continue recycle loop between the bioreactor and the
microfluidic device. CHO6E6 cells were grown in a batch culture under controlled pH, temperature, and dissolved oxygen conditions. At mid exponential stage of growth in the bioreactor the recycle flow was initiated. The cells either stopped growing or started dying at EDR values that were significantly lower (one to two orders of magnitude) than those previously reported to kill cells from a single, acute exposure. These observations allow further refinement in the design of bioprocess equipment since it provides a more accurate threshold, above which one does not want to subject animal cells to continuous exposure to specific levels of hydrodynamic forces.

5.2. INTRODUCTION

While significant progress has been made in the effective design and operation of large scale animal cell culture bioreactors (i.e. over 19 approved monoclonal antibodies as of 2006, with sales of over 15 billion dollars), at the most fundamental level the current knowledge of the relationships between hydrodynamic forces and suspended animal cells is still based on empirical studies. As the number and size of animal cell culture processes continue to grow, increased demand exists to further optimize them, including increased throughput and mass transfer. In addition, beyond the actual culturing of the cells, the increased scale in processing the cells after growth, and concerns with respect to premature cell rupture during this processing, underscores the need to continue characterizing and quantifying the effect of hydrodynamic forces on suspended animal cells.
While a number of parameters can be used to quantify the hydrodynamic forces that can operate on suspended animal cells, Energy Dissipation Rate (EDR), which is a scalar parameter, has been widely used to quantify local mixing performance in stirred tanks (Kresta, 1998) as well as animal cell bioprocesses (Mollet et al. 2004; Mollet et al., 2007). EDR was first suggested by Bluestein and Mockros (1969) to be used to characterize hydrodynamic forces operating on cells as EDR is intrinsic to any moving fluid, it is independent of the flow regime (turbulent/laminar) and accounts for both shear and extensional components of three-dimensional flow. EDR has units of power per unit volume (i.e. W·m⁻³).

In a recent publication, Mollet et al. (2007) reported on a second generation, flow contraction, microfluidic device which allows suspended cells to be subjected to well defined, hydrodynamic forces. As a result of the specific design, manufacture, and analysis of this device, the flow through the device can be simulated to a high level of accuracy, and correspondingly, the EDR can be determined for a range of flow rates. In this latest study, Mollet et al. (2007) subjected Chinese Hamster Ovary (CHO) cells to EDR values sufficient to cause immediate cell membrane rupture, as well as sub-lysis levels in an attempt to determine if these lower values of EDR could initiate apoptosis. While low levels of apoptosis were observed, it was concluded in these studies that the primary mode of cell death from high EDR was necrotic. In addition to these specific studies, Mollet et al (2007) also summarized in a single figure a number of other studies in which the EDR in a number of vessels configuration or the relationship of cell
death/lysis to EDR was estimated. Such figure, with data from that Mollet et al. study and a recent study on algae (Hu et al. 2007) is presented in Figure 5.1.

A common criticism to the use of this flow contraction, microfluidic device is that the studies using it only involve a single passage of the cells through the microfluidic device which does not resemble the typical experiences of suspended animal cells which can undergo short, cyclic exposures to high and low magnitudes of hydrodynamic forces. In this manuscript we have incorporated the flow contraction device into a recycle loop connected to a multiliter bioreactor. Such an arrangement allows cells to be subjected to a chronic exposure of animal cell to high and low levels of hydrodynamic stresses.

5.3. MATERIALS AND METHODS

5.3.1. Cell line

For the experimental data presented in this study, we used the Chinese Hamster Ovary cell line, CHO6E6, purchased from the American Type Culture Collection (ATTC No. CRL-11398). This specific cell line has been modified to produce a humanized immunoglobulin. Information regarding the transfection process and selection of the mutants is presented elsewhere (Rose, 1998; Bowen et al., 1998).
5.3.2. Cell adaptation and maintenance

Upon arrival from ATCC, the content of the frozen vial was thawed and transferred into a 75 cm² tissue culture flask (CORNING) containing 16 mL of a 1:1 mixture of Ham’s F12 medium and Dulbecco’s modified Eagle’s medium with 2.5 mM L-glutamine (JRH BIOSCIENCES, Lenexa, KS.) supplemented with 5% heat-inactivated fetal bovine serum (hi-FBS; GIBCO. INVITROGEN CORP. Carlsbad, CA.). This medium is subsequently referred to as hi-SCM. The T-flask was placed into an incubator at 37°C and 5% CO₂ (IR- autoflow, NUAIRE, Plymouth, MN). The cells were maintained there with medium renewal every 3 days until they reached 100% of confluence. The following sub-culturing procedure was used: first, the culture medium was removed, the cells were rinsed with 10 mL of a 0.0067 M phosphate buffered saline solution (PBS 1X, HYCLONE, Logan, UT), 2.0 mL of Accutase™ (INNOVATIVE CELL TECHNOLOGIES INC. San Diego, CA.) was added to the flask, which was subsequently placed in the incubator for 5 minutes. After that, 10 mL of fresh hi-SCM were added to the flask and the cell suspension was collected into a 50 mL conical tubes and centrifuged (Allegra 6R centrifuge, BECKMAN-COULTER, Fullerton, CA.) at 800 rpm (146×g) for 7 min; these particular conditions of acceleration and time of centrifugation will be referred to as “standard” in following paragraphs. The supernatant was discarded and the pellet was resuspended in fresh hi-SCM. The subcultivation ratio of cell suspension to fresh medium was kept 1:5. The cells were subcultured this way for 5 passes.
Cell adaptation to serum-free media was performed using a sequential approach. Initially, cells were harvested from T-75 flask grown as previously described. They were subcultured at $2 \times 10^5$ viable cell/mL in a 75:25 (v/v) mixture of hi-SCM : SFM4CHO utility medium (HYCLONE, Logan, UT). After reaching confluence (about 4 days), the subculturing procedure was repeated with a mixture 50:50 (v/v); then with a mixture 25:75 and then with 100% serum-free medium (SFM4CHO utility). In this last step, Methotrexate (SIGMA, cell culture tested, St Louis, MO) was added at a concentration 100 nM in order to exert selective pressure on those cells carrying the proper genes. Unfortunately, viability of this last subculture decreased to 40-45%; we attributed the decrease of viability to the combined effect of 0% hi-FBS and the presence of Methotrexate, as it was not used in previous subcultures. However, after 6 passes into SFM4CHO utility + 0% hi-FBS + 100 nM Methotrexate in T-75 flasks with similar low viability results, we decided to transfer the cells to grow suspended in a spinner flask (250 mL, WHEATON) at 37°C, 100 RPM and an atmosphere of 5% CO$_2$. Initial viability was 42% and the initial cell concentration was $3.5 \times 10^5$ viable cell·mL$^{-1}$. At the end of the culture, viability increased to 79.4% and the viable cell concentration was $1.2 \times 10^6$ cell·mL$^{-1}$. Cells from the spinner were harvested, centrifuged at standard conditions and further subcultured into spinners using the same methodology. After obtaining similar growth curves and final IgG concentration for 3 consecutive times, it was assumed the cells had become adapted to this culture medium and we proceeded to create a seed lot.

To attempt avoiding any problems regarding inoculum differences for subsequent studies, an inoculum lot was developed for the whole series of experiments. 150 mL of broth
containing cells in the late exponential phase were harvested from the fermentor, placed into 50 mL conical tubes, and centrifuged at standard conditions. The supernatant was discarded and the cells were resuspended in 25 mL of fresh SFM4CHO utility media containing 10% hi-FBS and 10% Dimethylsulfoxide (DMSO; ATCC, cell culture tested)) as a cryoprotectant agent. Aliquots of 1.5 mL of this suspension containing about 6x10^6 cells·mL\(^{-1}\) were distributed steriley in 2.0 mL polypropylene cryogenic vials (CORNING, Corning, NY.). The vials were placed in a Cryo-1°C freezing container (NALG NUNC INTERNATIONAL, Rochester, NY) and frozen at –85°C in a Ultralow Freezer (NUAIRE). The maximum time one vial was kept in the freezer was about six months; during this time no difference in viability was observed after thawing (~ 90%)

5.3.3. Chronic exposure studies

The media used for suspended cultures was SFM4CHO utility supplemented with 100 nM Methotrexate. The culture conditions were as follows: Temperature, 37.0° ± 0.2 ºC; dissolved oxygen (DO), 70.0 ± 0.1 % of air saturation in distilled water at 37°C and \(P_{\text{abs}}=1.0\text{ atm} \) (100 % = 6.7 mg·L\(^{-1}\)); pH, 7.0 ± 0.1; the gas flow rate varied between 0.1 and 0.3 standard liter·min\(^{-1}\); this variation was caused because the flow rate was close to the detection level of the control system (designed for a 5 L bioreactor).

Every inoculum for these studies was prepared by choosing randomly one of the vials from the same frozen lot, thawing the cells in a bath at 37°C and transferring the content into a 15 mL sterile conical tube; then, very slowly, 10 mL of culture media were added
drop wise (to avoid osmotic shock) and centrifuged at standard conditions in an attempt to eliminate traces of heat inactivated FBS and DMSO. The supernatant was discarded; the pellet was resuspended in 16 mL of fresh culture media and grown for 24 h in a T-75 flask in the incubator at 37°C and 5% CO₂; subsequently, the broth was transferred into a 50 mL conical flask. Because a part of the cell population remained attached to the T-flask (probably as a result of traces of FBS), 2 mL of Accutase™ were added to the T-flask and placed into the incubator for 5 minutes. Afterward, 10 mL of the cell suspension from the conical tube were transferred back into the T-flask to resuspend attached cells and dilute the enzyme. The suspension then was transferred back into the conical tube and centrifuged at standard conditions. The supernatant was discarded and the cells were resuspended into 25 mL of fresh culture media and transferred into a 25 mL spinner flask (BELLCO GLASS Inc, Vineland, NJ.). The spinner was in the incubator at 80 RPM, 37°C and 5% CO₂ for another 24 h.

One hundred and seventy milliliters (170 mL) of fresh media were charged sterilely into the bioreactor and left there for about 1 h to adjust temperature, pH and DO. Then, an appropriate volume of broth was removed from the spinner and centrifuged at standard conditions; 10 mL of fresh media were extracted from the bioreactor in the laminar flow hood and used to resuspend the cells which were then transferred into the bioreactor. After inoculation, the weight of the bioreactor was registered and the system was connected again to the control system. Cultures were started at a cell concentration ranging between 1.3 to 1.8×10⁵ cell·mL⁻¹.
5.3.4. Cell concentration and viability

Cell concentration was determined by manual cell counting in a Reichert, bright-line hemocytometer (HAUSSER SCIENTIFIC, Horsham, PA.). 16 square zones of 1 mm\(^2\) were counted and then averaged. Viability was determined simultaneously by dye exclusion (0.4% w/v Trypan blue in PBS, ICN BIOMEDICAL INC., Irvine, CA.).

5.3.5. Glucose and lactate

After determining cell concentration, the sample was centrifuged (Eppendorf Centrifuge 5415D) at 13200 rpm for 2 min (16100 \(\times\) g); the supernatant was collected and distributed in two parts: one of them was diluted with distilled water and used for glucose and lactate measurement using a YSI 2700 Select\textsuperscript{TM} system (YELLOW SPRING INSTRUMENTS, Yellow Springs, OH). The other part was used to quantify antibody concentration by using an ELISA protocol.

5.3.6. Antibody production

96-well flat-bottom EIA/RIA high-binding polystyrene plates (COSTAR) were coated with 100 μL/well of a goat anti-human IgG (Jackson Immuno Research Labs Inc., cat. No. 109-005-088) at 10 μg·mL\(^{-1}\) for 2 h at 37°C. After 3 washing cycles with 275 μL of PBS, plates were coated with 275 μL PBS + 2% bovine serum albumin BSA, Fract. V (FISHER SCIENTIFIC, Waltham, MA) for 2 h at 37°C for blocking purposes. The plates were again washed three times with 275 μL of PBS and then 100 μL/well of undiluted,
cell-free sample supernatants were added. Human IgG (SIGMA, Cat. No. I8640) at appropriate dilutions in PBS (9.53×10⁻³ to 0.81 mg L⁻¹) was used as a standard. Plates were again incubated at 37°C for 2 h. After the standard/sample incubation, the plates were washed again with PBS for three times and then 100 μL/well of a dilution 1:5000 of goat anti-human IgG (H+L) conjugated to alkaline phosphatase (secondary antibody, JACKSON IMMUNO RESEARCH LABS INC., West Grove, PA. cat. No. 109-055-088) in PBS were added. Plates were incubated at 37°C for 2 h and then washed three times with PBS. 100 μL pNPP solution (SIGMA, Cat. No. P79998) were dispensed into the wells and incubated at room temperature for 30 min to allow color development. The reaction was stopped by adding 50 μL/well of a solution 1 M of NaOH in distilled water. Absorbance of every well was read in a Spectramax 250 plate reader using SoftMaxPro software (MOLECULAR DEVICES CORP., Sunnyvale, CA) at a wavelength of 405 nm. Concentration was determined by comparison with the standard curve.

5.3.7. Lactate dehydrogenase (LDH)

To determine the concentration of the enzyme, lactate dehydrogenase, LDH, Cytotox 96® assay kit from PROMEGA (Madison, WI.) was used. LDH is a stable, cytosolic enzyme that is released into the culture broth upon cell membrane rupture; therefore, its presence in the medium fluid is indicative of cell lysis by either necrosis and/or late apoptosis.
To establish an appropriate range for the assay where absorbance was linear with cell concentration for this particular cell line, a standard curve was created. Cells in the middle exponential phase were harvested from the bioreactor, centrifuged at standard conditions and then resuspended at a concentration of about \(2 \times 10^6\) cell\cdot mL\(^{-1}\) in SFM4CHO. Eleven successive 1:2 dilutions of the cell suspension in fresh medium were prepared and 1.0 mL of each one placed in triplicate sets of tubes, leaving the 12\(^{th}\) tube of every set for medium only. 10 \(\mu\)L of the lysis solution provided by the kit (9% v/v Triton® X-100 in water), was added to every tube and incubated for 45 min at 37°C and 5% CO\(_2\). Afterward, the tubes were centrifuged at standard conditions to remove cell debris and 50 \(\mu\)L aliquots of the supernatants were transferred to a 96-well plate (same as in ELISA assays).

5.3.8. Bioreactor

For every experiment we used a custom made, 250 mL total volume bioreactor, (working volume of 170 mL). The control of temperature was achieved by circulation of temperature-controlled water (HAAKE circulator, model D1) through a jacket. The bioreactor was equipped with polarographic DO (METTLER-TOLEDO) and pH (INGOLD) probes. Both pH and DO were controlled by changing the composition of a gas mixture of CO\(_2\), O\(_2\), N\(_2\) and air, using the PID control system console of a Bioflo 3000 bioreactor (NEW BRUNSWICK Inc, Edison, NJ). To prevent any hydrodynamic stress from bubble break-up, only surface aeration was used. Because of the small value of \(k_{La}\) experimentally measured (\(\sim 1.4\) h\(^{-1}\)), the agitation speed was set to 200 rpm before...
starting recirculation. When the recirculation was initiated, a reduction in the liquid level inside the bioreactor occurred, reflecting fluid removed to fill the tubing, syringes, and flow contraction device; consequently, to avoid bubble entrainment from mixing, the stirring speed was reduced to 100 rpm. Water evaporation, leading to concentration problems, was detected in the first experiment; it was eliminated by adapting a gas humidifier at the entrance of the bioreactor. Additional characteristics of the bioreactor are summarized in Table 5.1.

While it is not possible to obtain highly accurate mean and maximum values of EDR in the bioreactor, reasonable estimates can be made. Venkat et al. (1996) employed a particle tracking velocimetry technique to experimentally approximate both the maximum and the average EDR in a 250 mL spinner vessel. Table 5.1 lists the relevant geometric factors comparing the spinner vessel and the vessel used in this study. While not identical, these geometric factors are similar enough that we suggest that the maximum EDR calculated in the spinner vessel, 285 to 880 W·m⁻³ for RPM ranging from 90 to 210, respectively, are sufficiently good estimates for the maximum EDR in the current vessel at the same RPM’s.

5.3.9. Microfluidic channel

To exert a well defined and controlled hydrodynamic stress on the cells, a flow contraction, microfluidic device, previously reported by Mollet et al. (2007) was used. We routinely refer to this device as the “torture chamber”, TC, and for the remainder of
this report we will use that name or initials. Figures 5.2a and 5.2b present a photograph of the TC and the dimensions of the flow contraction region.

5.3.10. Recirculation system

To achieve continuous flow of the broth (and cells) through the TC at accurate flow rates, a two-syringe version of the Harvard Apparatus syringe pump (Pump 33, HARVARD APPARATUS. Holliston, MA.) was used. The syringe pump was set in continuous mode, in which one of the syringes is pumping while the other is aspiring the fluid. To prevent reverse flow, a set of two double-solenoid valves (20 Psi, 12VDC; COLE-PARMER, Vernon Hills, Ill.) were connected to an existing on-off voltage output of the pump through a solid state relay. The whole setup of the system is depicted in Figure 5.3. It takes less than 1 second for the pump to automatically stop, change direction and start again. 30 mL polypropylene syringes (BECKTON-DICKINSON, Franklin Lakes, NJ) were used; however, the maximum volume allowed in a syringe at any time was about 5 mL. A number of flow characteristics of the recirculation system are presented in Table 5.2. In contrast to the bioreactor, since the flow in the tubing in the recycle system is laminar, the EDR can be determined analytically and such solutions were presented by Mollet et al. (2004). Table 5.2 lists the EDR in tubing and syringe of the recycle system for the two flow rates used in this study.

During early runs, it was detected that several air bubbles were accumulating at the top of the tubing leading from the bioreactor to the solenoid valves. To prevent further bubbles
in the system, the syringe pump was set up vertically, so any bubble traveling along the liquid path would be trapped in the syringe. Because of continuous use and wear, the plunger’s rubber tip of the syringe had to be replaced approximately every 8 to 12 hours, depending on the pumping speed, to avoid release of rubber particles in the liquid that could affect the fermentation or clog the throat of the torture chamber. The syringe body and the plunger with the tip were autoclaved separately at 121 °C for 30 minutes and assembled together in the laminar flow hood. Also, to avoid any possible contamination because of the periodic vertical displacement of the plunger to zones exposed to the lab’s non-sterile atmosphere, foam was glued to the upper part of the plunger before autoclaving, creating a closed, sterile zone that kept away any possible microorganism present in the air surrounding the syringes.

5.4. RESULTS

5.4.1. Acute exposure to high EDR

To determine if the specific CHO cell line used in this study responded to an acute exposure to specific levels of EDR in the TC in a quantitative similar manner to previous studies using different CHO cell lines, single abuse experiments were conducted, in triplicate using LDH to quantify the damage.

Cells in the middle exponential phase (about 3 days) were harvested from the bioreactor, centrifuged at standard conditions and then resuspended at a concentration of
approximately $5 \times 10^5 \text{ cell/mL}$ in SFM4CHO medium pre-warmed in the incubator at $37^\circ\text{C}$ and $5\% \text{ CO}_2$. All conditions in the bioreactor, from which the cells were obtained for these single abuse studies, were the same as the subsequent chronic exposure studies. The actual acute exposure studies in the TC, and subsequent analysis followed the same protocol as reported by Mollet et al. (2007). Five different flow rates (10, 30, 50, 70 and 90 mL·min$^{-1}$) were tested, in triplicate, in a random order.

Figure 5.4 presents the result of these acute exposure studies to five different flow rates (10, 30, 50, 70, and 90 mL·min$^{-1}$), which corresponds to five different values of EDR ($2.87 \times 10^5$, $2.27 \times 10^6$, $6.45 \times 10^7$, $2.60 \times 10^7$ and $1.09 \times 10^8 \text{ W/m}^3$ respectively, according to Mollet et al. 2007). In addition, data from previously published studies using CHO K1 cells, MCF-7, and THP-1 cells are included for comparison (Mollet et al. 2008; Mollet et al. 2007; Ma et al. 2002). As can be observed by inspection of Figure 5.4, the response of the current CHO cell line is consistent with the response of previously used CHO K1 with respect to the level of EDR at which significant cell lysis occurs. One can also note that the other two cell lines, including the MCF-7 human breast cancer and the THP1 human monocytic-like cell lines, are significantly more sensitive to EDR.

5.4.2. Chronic Exposure to High EDR

Given the similar sensitivity of the CHO cells used in this study to previous studies on CHO cells, chronic exposure studies were conducted which focused on flow rates through the TC which did not result in any detectable damage in the acute studies;
namely flow rates of 10 and 30 mL·min$^{-1}$, which correspond to median maximum EDR values of $2.9 \times 10^5$ and $2.3 \times 10^6$ W·m$^{-3}$, respectively (Mollet et al. 2007).

A total of 10 experiments were conducted with the bioreactor/recycle system, three of which were control experiments. These 10 experiments are summarized in Table 5.3. Two different types of control experiments were conducted. The first was simply a set of batch experiments in which cells were inoculated into the bioreactor and cultured in a batch mode without any recirculation, while the second kind of control experiments involved the use of the recirculation system without the TC. Figure 5.5a are normalized growth curves (cell concentration at a particular time divided by the initial cell concentration) of three of these control studies and Figure 5.5b is the viability of two of the runs presented in 5A. As it can be seen, no significant differences exist.

Figures 5.6 and 5.7 are plots of experimental runs in the bioreactor/recycle system in which the cells were subjected to repeated exposure to $2.9 \times 10^5$ and $2.2 \times 10^6$ W/m$^3$, respectively. The dotted lines correspond to the points at which the recycle flow was initiated (note each dotted line is labeled relative to specific experiment). Despite the fact that each run used cells that had experienced the same seed train (i.e. frozen vial, T-flask) a difference in the initial viable cell concentration existed from run to run. Therefore, we chose to express the results as the ratio $N/No$ vs. time. Each data point is the average of 16 different areas of 1 mm$^2$ in the hemocytometer and the error bars are one standard deviation. As it can be observed at the lower EDR rate (Figure 5.6), and especially at the higher EDR rate (Figure 5.7), a significant, negative effect on cell growth and viability
can be observed after the initiation of the recycle flow. Curves for cultures TE19 and TE21 are somewhat shorter because there was a plug in the TC (as a result of deterioration of the rubber in the syringes) or because contamination (also as a result of changing of syringes).

Figures 5.8a, 5.8B, 5.9 and 5.10 present the effect that repeated exposure to $2.9 \times 10^5$ and $2.2 \times 10^6$ W/m$^3$ had on total glucose consumption and lactate production, specific glucose consumption, and antibody production. No conclusive results can be drawn for these observations except to state the higher, chronic EDR exposure did not have a large effect on the glucose consumption, lactate production, and antibody production rate.

5.5. DISCUSSION

As presented in Figure 5.4, and mentioned previously, the CHO cell line used in this study responded to the single exposure to high levels of EDR in a manner similar to previously published studies. However, as it was also presented previously, this cell line, and potentially all cell lines, are sensitive to lower values of EDR (relative to the values needed for cell lysis in one exposure) if the exposition to such lower levels of EDR is chronic. While this observation is not necessarily surprising, it is significant in that a typical cell in a typical bioprocess will experience a significant range in hydrodynamic forces during the culture, harvesting, and final processing procedures. Therefore, for this work one could conclude that one should not repeatedly expose this cell line to values of EDR of $2.9 \times 10^5$ W/m$^3$ or higher. However, inspection of Figure 5.1 indicates that this
value of EDR, $2.9 \times 10^5 \text{ W/m}^3$ is above the maximum EDR created in a 22,000 L vessel using Rushton turbines at 240 rpm, or a 10 L vessel using Rushton turbines operating at 700 rpm. In other words, the EDR in this study at which inhibitory effects began to be observed is still significantly higher than what an animal cell encounters in a typical large scale, commercial process.

Closer inspection of the data indicates that the average growth of the culture halted after the recirculation was initiated at 10 mL·min$^{-1}$ (Figure 5.6), while a significant drop in viable cell concentration is observed after initiation of the recycle at 30 mL·min$^{-1}$ (Figure 5.7). This phenomena may be explained as follows: at 10 mL·min$^{-1}$ the death rate caused by cells passing through the TC equals the growth rate of the culture, keeping cell concentration steady; on the other hand, at 30 mL·min$^{-1}$ the death rate is higher than the growth rate, consequently, there is an observable reduction in the viable cell concentration in the culture. A quite similar behavior had been observed by Kunas and Papoutsakis (1990), who found growth of hybridoma cells was halted in a completely filled bioreactor at 800 rpm, while cell concentration was reduced when agitation rate was increased to 900 rpm; unfortunately, their experiment involved the presence of bubbles (that went into the bioreactor when samples were taken), so this results can only be qualitatively compared to this study. Nevertheless, in a subsequent experiment they reported that the apparent growth rates obtained in a bubble-free stirred bioreactor were significantly lower at higher agitation speed than in gentle or no agitation at all.
Initial glucose and lactate concentrations were homogeneous given the fact that the same lot of media was used for the whole study. Although trends in Figures 5.8 and 5.9 are quite similar, it is possible to observe slight variations in glucose and lactate concentrations among the cultures, probably as a result of different cell concentrations; an analysis in terms of specific glucose consumption rate (\(q_{\text{gluc}}\)) and specific lactate production rate (\(q_{\text{lac}}\); not shown) seems to support the same conclusion as there is no significant difference among experiments before the onset of recirculation; furthermore, after the onset of recirculation the data seems to be less scattered, suggesting that there is no metabolic effect of the increased EDR in terms of \(q_{\text{gluc}}\) or \(q_{\text{lac}}\). The overall trend for the specific rates of glucose consumption is to start high (~ 80 to 100 pg glucose per cell per hour) and decrease monotonically along time to about 20 pg glucose·cell\(^{-1}\)·h\(^{-1}\); a similar trend is observed for the specific rate of lactate production that goes from 70 pg lactate·cell\(^{-1}\)·h\(^{-1}\) at the beginning of the culture to about 20 pg lactate·cell\(^{-1}\)·h\(^{-1}\) after 5 days of cultivation. This behavior in CHO cells has been reported in other studies in batch and continuous cultures where it has been demonstrated that the higher the glucose concentration, the higher the specific glucose uptake rate and the higher the yield to lactate (Hayter et al., 1991; Hayter et al., 1992; Altamirano et al., 2001). Several hypothesis have been proposed in literature for this phenomena (Gòdia and Cairó, 2006), but no conclusive explanation has been reported yet. It is worth to point out that values reported in literature for of \(q_{\text{gluc}}\) CHO cells goes from a maximum of 54 pg glucose·cell\(^{-1}\)·h\(^{-1}\) in batch culture (Hayter et al., 1991) to minima of 21.2 pg glucose·cell\(^{-1}\)·h\(^{-1}\) (Hayter et al., 1992) or 3.2 pg glucose·cell\(^{-1}\)·h\(^{-1}\)under limiting conditions in chemostat cultures.
(Altamirano et al., 2001); that range corresponds fairly well with values for our particular strain of CHO cells.

The IgG production for representative experiments is presented in Figure 5.10. Time evolution of concentration of IgG is quite similar in every case. As it can be observed, it is not really possible to infer any effect of EDR on IgG production as the concentration of IgG is quite small to really allow for subtle differences to be detected (the variations observed in several cases are a result of the concentration being near the detection limit of the ELISA), and the production of IgG ceased before the onset of recirculation (the time at which recirculation was initiated varied between 60 and 80 hours). This last phenomena results in contradiction with literature reports showing antibody production to be maximum in the G1 and early S phases of the cell cycle (Al-Rubeai and Emery, 1990; Kromenaker and Srienc, 1994; Bi et al., 2004), which are usually connected with lower growth rates or reaching stationary phase of growth; however, maximum production rate of antibodies increasing with increasing growth rate has also been reported (Borth et al., 1982). It may be possible in future work to detect the effect of EDR in antibody production if the culture is allowed to continue longer (industrial cultures are often fed-batch lasting for 10 to 15 days), and achieve a higher cell concentration (i.e. using a different culture mode such as fed-batch). However, since the focus of this study was not optimizing for IgG production, and the levels of productivity are several orders of magnitude lower than is typically obtained in optimized cultures, these productivity results are of secondary interest and only presented to demonstrate that the cell still produced IgG.
While this study begins to address the question of chronic exposure to high levels of hydrodynamic forces, it is only the beginning. Two primary concerns are: a) increasing the flow rate to increase the level of EDR also increases the number of times the cells are exposed to a high level of EDR in a given period of time, and b) the period of time that the cells are exposed to a high level of EDR is still very short.

Quantitatively, estimates can be made on the number of times that an average cell experienced a high level of EDR. Assuming that the cell suspension is well mixed in the bioreactor (a reasonable assumption given the rpm used), a simplistic argument can be made that a cell will experience in average a stressing cycle every residence time defined by:

\[ \tau_1 = \frac{V}{F} \]  

(Eq. 5.1)

With a working volume of 170 mL in the bioreactor, and a flow rate of 10 and 30 mL·min\(^{-1}\), one can assume that any given cell will experience a high level of EDR every 17 or 6 minutes, respectively; with a doubling time close to 24 h for this CHO cell line, it means that in average a typical cell would experience around one or two hundred exposures to high levels of EDR before duplicating, a number that appears high enough to evaluate the effect of chronic exposure on cell metabolism.

Current and future work will focus on two aspect of the use of this chronic EDR exposure system: use of this recycle system on industrially relevant cell lines through collaboration with industrial partners and the development of alternative TC design and/or operator
procedures to attempt to further develop the relationship between both the number of exposures, time of exposures and type of cell line to various levels of EDR.
5.6. REFERENCES


Table 5.1. Characteristics of the bioreactor used in this study, and comparison of specific characteristics of the current vessel to that used by Venkat et al. (1996).

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Vessel used in this study</th>
<th>Spinner vessel used by Venkat et al. (1996)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioreactor material</td>
<td>Borosilicate glass</td>
<td>glass</td>
</tr>
<tr>
<td>Reactor internal diameter, T, (cm)</td>
<td>6.4 cm</td>
<td>7.2</td>
</tr>
<tr>
<td>Impeller type</td>
<td>Magnetic rod, flat end.</td>
<td>Magnetic rod with vertical fin</td>
</tr>
<tr>
<td>Impeller diameter, D, (cm)</td>
<td>4.5</td>
<td>4.5</td>
</tr>
<tr>
<td>D/T ratio</td>
<td>0.71</td>
<td>0.78</td>
</tr>
<tr>
<td>Clearance of impeller’s center line from the bottom (c)</td>
<td>23.7 mm</td>
<td></td>
</tr>
<tr>
<td>Initial liquid height (H_L)</td>
<td>58 mm</td>
<td></td>
</tr>
<tr>
<td>Flow rate (Q) [ml·min⁻¹]</td>
<td>Time between direction switch [s]</td>
<td>Average retention time of cells [s]</td>
</tr>
<tr>
<td>-------------------------</td>
<td>------------------------------------</td>
<td>-----------------------------------</td>
</tr>
<tr>
<td>10</td>
<td>30</td>
<td>82</td>
</tr>
<tr>
<td>30</td>
<td>10</td>
<td>27</td>
</tr>
</tbody>
</table>

¹ Calculated assuming a value of specific oxygen uptake rate (OUR) of 3×10⁻¹³ mol O₂·cell⁻¹·h⁻¹ (Deshpande and Heinzle, 2004; Ducommun et al., 2000), complete impermeability to oxygen diffusion along the tubing wall, ideal plug flow and a maximum concentration of 1×10⁶ cells mL⁻¹

² Calculated at 200 rpm.

³ According to Mollet et al. (2007)

⁴ Calculated using internal diameter for tubing: 3.1 mm and syringe 21.7 mm, according to Eq. (8) in Mollet et al. (2004): \( \varepsilon_{max,pipe} = \frac{\mu}{\pi^2 R^6} \).

**Table 5.2.** Flow characteristics in the recirculation system.
<table>
<thead>
<tr>
<th>Code Name</th>
<th>Condition</th>
<th>Median Maximum EDR (W·m⁻³)</th>
<th>μₘₐₓ [h⁻¹]</th>
</tr>
</thead>
<tbody>
<tr>
<td>TE4</td>
<td>Control without recirculation</td>
<td>8.6×10²</td>
<td>0.0176</td>
</tr>
<tr>
<td>TE5</td>
<td>Control with recirculation, 30 mL·min⁻¹</td>
<td>8.6×10²</td>
<td>0.0157</td>
</tr>
<tr>
<td>TE6</td>
<td>10 mL·min⁻¹</td>
<td>2.87×10⁵</td>
<td>0.0207</td>
</tr>
<tr>
<td>TE7</td>
<td>30 mL·min⁻¹</td>
<td>2.27×10⁶</td>
<td>0.0127</td>
</tr>
<tr>
<td>TE8</td>
<td>Control without recirculation</td>
<td>8.6×10⁴</td>
<td>0.0154</td>
</tr>
<tr>
<td>TE11</td>
<td>30 mL·min⁻¹</td>
<td>2.27×10⁶</td>
<td>0.0218</td>
</tr>
<tr>
<td>TE14</td>
<td>30 mL·min⁻¹</td>
<td>2.27×10⁶</td>
<td>0.0219</td>
</tr>
<tr>
<td>TE18</td>
<td>10 mL·min⁻¹</td>
<td>2.87×10⁵</td>
<td>0.0276</td>
</tr>
<tr>
<td>TE19</td>
<td>30 mL·min⁻¹</td>
<td>2.27×10⁶</td>
<td>0.0265</td>
</tr>
<tr>
<td>TE21</td>
<td>30 mL·min⁻¹</td>
<td>2.27×10⁶</td>
<td>0.0431</td>
</tr>
</tbody>
</table>

**Table 5.3.** List of experiments performed in the recirculation system.
**Cell Response**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Cell</th>
<th>Mode of growth</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>CHO-K1, necrosis</td>
<td>Anchorage</td>
<td>Gregoriades et al. (2000)</td>
</tr>
<tr>
<td>B</td>
<td>Hybridoma, necrosis</td>
<td>Suspended</td>
<td>Thomas et al. (1994); Zhang et al. (1993)</td>
</tr>
<tr>
<td>C</td>
<td>MCF-7, necrosis</td>
<td>Suspended</td>
<td>Ma et al. (2002)</td>
</tr>
<tr>
<td>D</td>
<td>Mouse myeloma, necrosis</td>
<td>Suspended</td>
<td>McQueen and Bailey (1989)</td>
</tr>
<tr>
<td>E</td>
<td>HeLa S3, mouse L929, necrosis</td>
<td>Suspended</td>
<td>Augenstein et al. (1971)</td>
</tr>
<tr>
<td>F</td>
<td>CHO-K1, SF-9, HB-24, necrosis</td>
<td>Suspended</td>
<td>Ma et al. (2002)</td>
</tr>
<tr>
<td>G</td>
<td>Uninfected and viral infected PERC6 cells, necrosis</td>
<td>Suspended</td>
<td>Submitted</td>
</tr>
<tr>
<td>H</td>
<td>Enthomopathogenic nematodes, necrosis</td>
<td>Suspended</td>
<td>Fife et al. (2004)</td>
</tr>
<tr>
<td>I</td>
<td>CHO-K1, apoptosis</td>
<td>Anchorage</td>
<td>Mollet et al. (2007)</td>
</tr>
<tr>
<td>J</td>
<td>THP-1, necrosis</td>
<td>Anchorage</td>
<td>Mollet et al. (In Press)</td>
</tr>
<tr>
<td>K</td>
<td>Algae, loss of flagella</td>
<td>Suspension</td>
<td>Hu et al. (2007)</td>
</tr>
</tbody>
</table>

**Figure 5.1.** Summary of the reported energy dissipation rate at which cells are affected as well as the reported levels of energy dissipation rate in various bioprocess environments. Adapted from Ma et al. (2002) and Mollet et al. (2004).
## Hydrodynamic Conditions

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Process</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Agitation</td>
<td>Volume average in typical animal cell bioreactors.</td>
<td>Varley and Birch (1999)</td>
</tr>
<tr>
<td>2</td>
<td>Agitation</td>
<td>Volume average in a 10 L mixing vessel (RT, 700 RPM)</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>Agitation</td>
<td>Maximum in the 10 L mixing vessel (RT, 700 RPM)</td>
<td>Zhou and Kresta (1996)</td>
</tr>
<tr>
<td>4</td>
<td>Agitation</td>
<td>Volume average in a 22,000 L mixing vessel (RT, 240 RPM)</td>
<td>Wernersson and Tragardh (1999)</td>
</tr>
<tr>
<td>5</td>
<td>Agitation</td>
<td>Maximum in the 22,000 L mixing vessel</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Agitation</td>
<td>Maximum in spinner vessel (200 RPM)</td>
<td>Venkat et al. (1996)</td>
</tr>
<tr>
<td>7</td>
<td>Bubble rupture</td>
<td>Pure water, bubble diameter: 6.32mm</td>
<td>Garcia-Briones et al. (1994)</td>
</tr>
<tr>
<td>8</td>
<td>Bubble rupture</td>
<td>Pure water, bubble diameter: 1.7mm</td>
<td>Boulton-Stone and Blake (1993);</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>Garcia-Briones et al. (1994)</td>
</tr>
<tr>
<td>9</td>
<td>Flow through a pipe</td>
<td>Pure water, 100 mL/min, 1 mm diameter</td>
<td>Mollet et al. (2004)</td>
</tr>
<tr>
<td>10</td>
<td>Flow through a micropipette tip</td>
<td>Flow through a 200 μL micropipette tip in 0.2 sec</td>
<td>Mollet et al. (2004)</td>
</tr>
<tr>
<td>11</td>
<td>Agitation</td>
<td>Volume average in a highly agitated animal cell bioreactor</td>
<td>Oh et al. (1992)</td>
</tr>
</tbody>
</table>

Figure 5.1. (Continued).
Figure 5.2. Torture Chamber (TC). (a) Photograph (b) Scheme detailing the dimensions (lengths in mm).
**Figure 5.3.** Diagram of the experimental setup for continuous, chronic exposure of suspended animal cells to high levels of hydrodynamic forces.
Figure 5.4. Response of CHO cells used in this study to an acute exposure to the hydrodynamic forces created by the TC and characterized by the specific level of EDR. As reference points, data from previously published studies with CHO K1 cells, MCF-7 and THP1 cells are presented (Mollet et al. 2007, 2008, Ma et al. 2002). Dashed lines indicate the flow rate for every EDR.
Figure 5.5. Control experiments of the normalized growth, (a), and viability, (b), for three runs in the bioreactor and/or recycle system. TE4 and TE8 runs are typical growth in the system without recycle, while TE5 is with recirculation without the TC. The dashed line corresponds to the point at which recycling was initiated.
Figure 5.6. Normalized growth, (a), and viability, (b), of two experimental runs in which the cell suspension was subjected to recycle through the TC at 10 mL·min$^{-1}$, corresponding to $2.9 \times 10^5$ W·m$^{-3}$. The point of initiation of recycle is indicated with the dashed lines.
Figure 5.7. Five experimental runs in which the cell suspension was subjected to recycle through the TC at 30 mL·min$^{-1}$, corresponding to $2.27 \times 10^6$ W·m$^{-3}$. The point of initiation of recycle is indicated with the dashed lines.
Figure 5.7. (Continued)
Figure 5.8. The concentration of glucose (g·L⁻¹), and lactate (g·L⁻¹) as a function of time for (a) the controls, and (b) the five experiments run at a recycle of 30 mL·min⁻¹.
Figure 5.9. The specific rate of glucose consumption for the control experiments, (a), and selected experimental runs with recycle, (b).
Figure 5.10. Secreted antibody concentration as a function of time for selected experimental runs.
CHAPTER 6

QUANTIFICATION OF THE SENSITIVITY OF GS-CHO CELLS TO CHRONIC REPETITIVE ENERGY DISSIPATION IN A SCALE-DOWN FED-BATCH BIOREACTOR

The results presented in this chapter were obtained at the Global Research and Development Laboratory, at Pfizer Inc., St. Louis Missouri. Together with some additional results at lower EDR, they will be submitted to the Journal Biotechnology and Bioengineering under the title “Quantitative Study of Physiological Responses of CHO Cells to Repetitive Hydrodynamic Stress” by Ruben Godoy-Silva, Jeffrey J. Chalmers, Susan A. Casnocha and Ningning Ma.

6.1 ABSTRACT

While the effect of hydrodynamic forces on animal cell cultures has been extensively studied, a concern of hydrodynamic sensitivity of animal cells still continues casting a limit on the designing and operating conditions for most large-scale bioreactors around the world. In an attempt to elucidate the effect of hydrodynamics on animal cell’s behavior, a previous manuscript from our research group reported on the chronic
exposure of a research CHO cell line (CHO6E6) to hydrodynamic forces in a second
generation convergent-divergent microfluidic device (Godoy-Silva et al., Submitted). In
this study, we further extended the application of such device (TC) to test the sensitivity
of a monoclonal antibody producing GS-CHO cell line of industrial interest, grown in a
fed-batch culture under controlled pH, temperature, and dissolved oxygen conditions, to
single and repeated exposures of hydrodynamic stress of moderate intensity. In the single
exposure methodology, cells were pumped once through the TC; in the chronic exposure
methodology, cells were continuously pumped through a recycling loop between a 2 L
bioreactor and the TC; this set-up intended to mimic the cyclic passage of cells between a
zone of high stirring intensity around the impeller (modeled by the TC) and zones of low
stirring intensity away from the impeller (modeled by the gently agitated bioreactor)
during normal cultivation in a stirred tank. The mean maximum local energy dissipation
rate (EDR), used as a measure for hydrodynamical stress; was calculated through
simulations in FLUENT®.

Single passage of cells through the microfluidic device revealed that for the tested GS-
CHO cell line, the threshold mean maximum EDR before necrosis became significant
was \(2.3 \times 10^6\) W·m\(^{-3}\). This threshold is very similar to other cell lines tested before,
including CHO-K1, CHO6E6, hybridoma and PER.C6. The single passage test also
showed that the hydrodynamic sensitivity of cells in the exponential, stationary and death
phases of growth was very similar.
Chronic exposure of cells for over ten days revealed that cell growth, viability, productivity, and carbon metabolism, as well as most product quality attributes, including aggregation/fragmentation, charge profile, and methionine oxidation, were not affected at any of the three different levels of EDR evaluated in this study (2.9×10⁵ W·m⁻³, 2.3×10⁶ W·m⁻³, and 6.5×10⁶ W·m⁻³). Similarly, no significant increase in necrosis or apoptosis levels (measured through flow cytometry) was observed; however, a significant increase in the lactate dehydrogenase (LDH) released to the culture medium as well as a significant reduction in the cell diameter compared to controls were detected only at the highest EDR tested (6.5×10⁶ W·m⁻³), concomitantly with the start of the recirculation. Also, a significant shift in the N-glycosylation pattern was detected at every EDR conditions tested, rendering more G1 and G2, but less G0 glycoforms; however, the mechanism behind this shift is unknown.

Overall, this study shows that the EDR generated by impellers in current large-scale bioreactors is safe to GS-CHO and possibly to most CHO cells, indicating that the hydrodynamic sensitivity commonly associated to animal cells might be overestimated, and suggesting that a more intensive agitation should be evaluated in large-scale bioreactors in favor of better mixing. Caution should be exercised, though, if glycosylation shift during scale-up affects undesirably the properties of the product.
6.2 INTRODUCTION

A significant progress has been made in the effective design and operation of large scale animal cell culture bioprocesses (i.e. over 19 approved monoclonal antibodies as of 2006, with sales of over 15 billion dollars and more than 160 molecules in various stages of clinical trails for a projected revenue in 2012 exceeding $40 billion; Datamonitor, 2007). However, as the number and size of animal cell culture processes continue to grow, increased demand exists to further optimize them. In industrial fed-batch processes, peak cell density has increased by one order of magnitude reaching ten(s) of millions of viable cells per mL and product titer has also increased by several orders of magnitude with normal concentration reaching \( \sim 5 \text{ g·L}^{-1} \). This increment in cell and product concentration requires improved mass transfer capabilities, usually reached via more intense mixing. Nevertheless, agitation in many large-scale bioreactors operates at mild, sub-optimal conditions in an effort to avoid “shear damage”. This apparent contradiction is the result of a lack of fundamental knowledge of the relationships between hydrodynamic forces generated by agitation and suspended animal cells and underscores the need to continue characterizing and quantifying such effects.

The intensity of agitation a particular cell line can withstand depends on two parameters. One is the maximum local hydrodynamic force produced by a specific agitation condition and the other is the hydrodynamic sensitivity of the cells. In an agitated bioreactor, mechanical energy is transferred from impellers to the liquid and then dissipated through a series of eddy cascades, which eventually converts all mechanical energy into thermal
energy, heat. Energy dissipation is the driving force for mixing and also the cause of the hydrodynamic stress on the cells. Therefore, while a number of other parameters can be used to quantify the hydrodynamic forces acting on suspended animal cells, energy dissipation rate (EDR), presents several advantages: it is independent of the flow regime (turbulent/laminar); it accounts for both shear and extensional components of three-dimensional flow; it has units of power per unit volume (i.e. \( \text{W} \cdot \text{m}^{-3} \)) so it can be related to engineering parameters; it has been widely used to quantify local mixing performance in stirred tanks (Kresta, 1998) as well as animal cell bioprocesses (Mollet et al. 2004; Mollet et al., 2007); finally, it is a scalar parameter intrinsic to any moving fluid. By using this last characteristic, several authors have used microfluidic channels as a good model to reproduce hydrodynamic forces inside a bioreactor. Ma et al., 2002 designed a contractional microfluidic channel operating in laminar flow regime. As a result of the specific design, manufacture, and analysis of this device, the flow could be simulated to a high level of accuracy, and correspondingly, the EDR could be determined for a range of flow rates. This flow device, dubbed “torture chamber” or TC, was used to quantify the hydrodynamic sensitivity of cell lines relevant to biopharmaceutical production, including wild type CHO-K1, hybridoma, and PER.C6. In general, an energy dissipation rate above \( 2.0 \times 10^6 \text{ W} \cdot \text{m}^{-3} \) was found to elicit detectable cell lysis. This is in agreement with previous results from Zhang and Thomas (1993), who needed average energy dissipation rates of \( 10^8 \text{ – } 10^9 \text{ W} \cdot \text{m}^{-3} \) to completely disrupt TB/C3 hybridoma and NS1 myeloma cells when passing in turbulent flow through capillary tubes. This is far higher than the level that can be generated in bioreactors by agitation, suggesting that agitation in large-scale bioreactor is not responsible for physical death of these cells.
In a recent publication, Mollet et al. (2007) reported on a second generation, autoclavable TC which allows suspended cells to be subjected to well defined, hydrodynamic forces in a sterile manner. In this latest study, Mollet et al. (2007) subjected wild type and genetically engineered Chinese Hamster Ovary (CHO) cells to EDR values sufficient to cause immediate cell membrane rupture, as well as sub-lysis levels in an attempt to determine if these lower values of EDR could initiate apoptosis. While low levels of apoptosis were observed, it was concluded in these studies that the primary mode of cell death from high EDR was necrosis and the levels of EDR necessary to elicit such response were very similar to those found by Ma et al. (2002). In addition to these specific studies, Mollet et al (2007) also summarized in a single figure a number of other studies in which the EDR in a number of vessels configuration or the relationship of cell death/lysis to EDR was estimated. Such figure, with data from that Mollet et al. study and a recent study on algae (Hu et al. 2007) is presented in Figure 5.1.

A common criticism to the use of this flow contraction, microfluidic device is that the studies using it only involve a single passage of the cells through the microfluidic device which neither allows studies for extended periods equivalent to typical culture times nor resemble the typical experience of suspended animal cells which undergo short, cyclic exposures to high and low magnitudes of hydrodynamic forces as they travel from the impeller region to zones away from it, respectively. Impeller region, albeit accounting for only about 10% of tank volume, may account for up to 70% of total energy dissipation; therefore, the local maximum energy dissipation rate in the impeller region might be two orders of magnitude higher than that in the region away from impellers (Wernersson and
The working hypothesis suggests the multiple passages through high intensity EDR zones might weakening progressively the cells. In a recent study, Vickroy et al. (2007) showed that multiple passages of CHO cells through a capillary tube caused cell lysis at lower flow rates than a single passage. They also found that cell death rate was related to culture viability. Cells from high viability culture died more slowly than cells from low cell density culture.

Another limitation on most studies evaluating the effect of hydrodynamical forces on cells is that they concentrate on lethal effects such as cell lysis (necrosis) or apoptosis. Studies that investigated sub-lethal effects of hydrodynamic stress on mammalian cells are limited. Several excellent reviews, including those of Malek and Izumo (1994), Li et al. (2005), Lehoux et al. (2006), Tzima (2006), Li and Xu (2007), Chien (2007) and Haga et al. (2007), document what is known about the response of vascular endothelial cells to sub-lethal hydrodynamic forces. Unfortunately, most of these studies are for medical or cell physiology purposes, so that the cell lines used, the test protocol used, or the sublethal effects monitored are not directly relevant to bioprocess application; nevertheless, these studies did reveal that sub-lethal effects can be triggered by lower level hydrodynamic forces than that lethal effects need.

Experiments with industrial cell lines carried out in stirred tanks have the disadvantage that sub-lethal effects are usually associated to agitation rate instead of a more general parameter such as EDR. Al-Rubeai et al. (1990) found increased glucose consumption and mitochondrial activity under intense agitation and in the absence of air bubbles. Al-
Rubeai et al. (1995) found that in addition to cell death under conditions of intensive agitation (1500 rpm), sub-lethal effects included lost of microvilli on the cell’s surface and changes in cycle distribution of the cell population. Al-Rubeai et al (1993) and Lakhotia et al. (1992) reported changes in DNA synthesis rate at sub-lethal conditions; in contrast, Passini and Gooche (1989) did not find any effect. Mufti and Shuler (1995) observed that human hepatoma cells attached to microcarriers and grown in 50 mL spinners responded to moderate levels of agitation by inducing a cytochrome P450 monooxygenase (CYP1A1) activity; CYP1A1 is involved in the oxidation of arachidonic acid, a substance whose metabolism has been observed to be altered by hydrodynamic stress in endothelial cells cultured in parallel-plate flow chambers (McIntire et al., 1987).

In the case of experiments carried out in flow devices for determination of sub-lethal effects, hydrodynamic forces can be controlled more precisely, but either single-passage has been used or long-term experiments are carried out with attached cells of limited industrial application. As summarized in Ma et al. (2002), shear stress at 1.0-10 N·m⁻², which correlates to 1×10³-1×10⁵ W·m⁻³, could elicit sub-lethal physiological responses. This is significantly lower than the level required to lyse similar cell lines. Of bioprocess relevant cell lines, Ranjan et al. (1996) found induction of transcriptional activator c-fos when CHO cells were subjected to 2.5 N·m⁻² shear force for 1 hour. Ludwig et al. (1992) found that the time for adherent BHK-21 cells to spread after division increased when the cells were exposed to 4.5 N·m⁻². Motobu et al. (1998) found that after 24 hours’ exposure to 0.02 and 0.082 N·m⁻² shear stress, non-confluent CHO cells were arrested at G₀/G₁ phase and mRNA level for the recombinant product was increased, but confluent CHO
cells did not respond to the same levels of shear. Keane et al. (2003) subjected attached CHO cells to shear stress for 32 hours and monitored recombinant human growth hormone production and glucose metabolism. They found that when shear force was increased from 0.005 to 0.80 N·m⁻², recombinant protein product rate was reduced by 51%, glucose uptake rate was increased by 42%, and lactate production was decreased by 50%.

To overcome the previously mentioned limitations, Godoy-Silva et al. (submitted) developed a bioreactor scale-down model that at the same time that simulates the repetitive hydrodynamic exposure that cells experience in large-scale agitated bioreactors, allows for controlled experimental conditions for culture periods as long as desired. In this model, cells continuously circulate between a bench-scale bioreactor and a torture chamber through a recirculation system. The bench-scale bioreactor simulates the low energy dissipation zone away from impellers in large-scale bioreactors and the torture chamber simulates the high energy dissipation zone around impellers in the same large-scale bioreactor. Godoy-Silva et al. (submitted) used this model to test repetitive hydrodynamic stress on the growth and production of a CHO cell line. They found that cell growth was negatively impacted at EDR 1-2 order of magnitude lower than that required to lyse the cells under single transient exposure. At $2.9 \times 10^5$ W·m⁻³, apparent cell growth stopped and at $2.3 \times 10^6$ W·m⁻³, net cell death and viability reduction was observed.
In this study, we used the same scale-down model as in Godoy-Silva et al. (submitted) to test the hydrodynamic sensitivity of a GS-CHO clone in an industrial fed-batch process. Lethal effects were determined through monitoring intracellular LDH release, apoptosis, and drop in culture viability. The focus of this study was to investigate the sub-lethal physiological effects. As the physiological responses that hydrodynamic stress can trigger could be numerous, we focused on those associated with process performance and product quality. The process factors include cell growth rate, recombinant production rate, and metabolic state (exemplified by glucose consumption rate and lactate production rate). The product quality factors include aggregation and fragmentation, charge profile, N-linked glycosylation profile, and methionine oxidation. The ultimate goal of this study was to evaluate lethal and sub-lethal effects of agitation in large-scale bioreactors on CHO cells.

6.3 MATERIALS AND METHODS

6.3.1 Cell line

A clonal GS-CHO cell line encoding a fully human IgG was used in this study. This cell line was derived from transfecting the target protein encoded vector into CHO-SV host cells (Lonza Biologics, Slough, UK) using glutamine synthetase (GS) as the selection marker.
6.3.2 Cell Culture Process

An inoculum train was maintained in 500 mL polycarbonate vented Erlenmeyer flasks (Corning, Acton, MA) where cells were routinely sub-cultured in 100 mL of fresh chemically defined medium (GIBCO™ CD CHO Medium (1X) liquid Cat. No. 10743-029, INVITROGEN, Carlsbad, CA), supplemented with 25 µM of L-methionine sulfoximine (MSX, Cat. No. GSS-1015-F, CHEMICON INTERNATIONAL INC., Temecula, CA) at an initial target concentration of $3 \times 10^5$ viable cell·mL$^{-1}$. Cells were sub-cultured when the viable cell concentration reached 2 to $3 \times 10^6$ cell·mL$^{-1}$. The inoculum train was abandoned when it approached two months and then a new inoculum train was established from a frozen vial from the master cell bank. The Erlenmeyer was kept into a 1 inch eccentric ISF1-X (ADOLF KÜHNER AG, Switzerland) orbital shaker at 36.5°C, 9% CO$_2$ and 130 rpm.

Two 2L bioreactors (APPLIKON INC., Foster City, CA) with 1L working volume were used as production bioreactors. The Applikon bioreactors were equipped with two 3-blade 45° pitched blade impellers of 4.4 cm in diameter for mixing. Temperature was maintained with a 110 W heating blanket connected to the PID control system console (Applikon ADI 1030 Biocontroller). Each bioreactor was equipped with one set of dissolved oxygen (DO) and pH Applisens probes (Applikon).

DO and pH were controlled by bubbling oxygen and CO$_2$ respectively through a straight tube-type sparger with 7 holes of 0.9 mm in diameter placed directly under the lower
impeller. In case pH was below the set point, a solution of sodium hydroxide 0.5 N was pumped. DO and pH were controlled using a PID algorithm applied to on-off gas valves through solid state relays in the control system console (Applikon ADI 1030 Biocontroller).

The gas output was refrigerated through a jacket so water evaporation was not significant. Two 0.22 μm filters were placed at the gas input and output to avoid contamination.

The medium for bioreactor cultures was the same chemically defined GIBCO™ CD CHO medium but supplemented with four amino-acids. The culture conditions were as follows: Temperature, 36.5 ± 0.1 °C; DO, 30.0 ± 0.1 % of air saturation in PBS-1X (Gibco dPBS Cat. No. 14190-136) at 36.5 °C and Pabs=1.0 atm; pH, 7.00± 0.03; agitation speed was set at 150 ± 1 rpm; air at a flow rate of 100 mL·min⁻¹ was pumped into the headspace permanently; oxygen and CO₂ flow rates were set at 200 and 100 mL·min⁻¹ respectively via the needle valve in the rotameters.

The bioreactor was autoclaved with ~500 mL of PBS-1X for 45 min. Then temperature control was initiated. Air was sparged for several hours to reach saturation. The DO probe was calibrated and then the PBS was removed and replaced by 1 L of culture medium. Next, DO, pH and temperature controls were activated; after reaching the set point, an adequate volume from the inoculum was inoculated to target an initial concentration of 3×10⁵ viable cells·mL⁻¹.
The fed-batch cultures started as a batch process; on day 3, two streams consisting one of concentrated glucose (Cat. No. G8920, SAFC, St. Louis, MO) and the other of a custom mixture of amino-acids and supplementary nutrients were fed into the bioreactor continuously until the end of the process. The process was terminated on day 14.

### 6.3.3 Single exposure experiments

A convergent-divergent microchannel, originally devised by Ma et al. (2002) and improved by Mollet et al. (2007), was used to exert a well defined and controlled hydrodynamic stress on the cells. The micro-channel (TC), bored in a stainless steel plate, is in sandwich between two transparent polycarbonate plates to make it autoclavable. The characteristics of the TC are presented in Figure 6.1. Further details of the TC are presented elsewhere (Mollet et al., 2007, corresponding to section 3.3).

A 14 day fed-batch process was carried out in a 2L bioreactor. Approximately 250 million cells from each one of the exponential (day 4), stationary (day 9), and death phases (day 13) were withdrawn for the single exposure test. Lag phase was excluded from this study as there is no lag phase in this fed-batch process. The growth curve of the 2L fed-batch bioreactor and the time points that cells were taken out are shown in Figure 6.2a. Out of the bioreactor, the cells were centrifuged down at 200×g for 5 minutes and resuspended into fresh medium preconditioned by warming it in the Kühner incubator at 9% CO₂ during at least one hour. The final cell density was adjusted to 5×10⁵ viable cells·mL⁻¹. The cell suspension was then immediately pumped through the TC at 5
different flow rates, corresponding to the EDR shown in Table 6.1. These 5 flow rates were chosen based on the sensitivity of other cell lines measured previously (Ma et al., 2002; Mollet et al., 2007). The effluent from the TC was collected and the percentage of cell damage was quantified based on release of intracellular LDH based on a standard curve, as described in section 6.3.5.

6.3.4 Chronic exposure system and experiments

In the repetitive exposure study, a recirculation loop system was utilized to constantly pump cells in a 2L bioreactor through a TC. The system is illustrated in Figure 6.4 and detailed description can be found elsewhere (Godoy-Silva et al.; Submitted, equivalent to section 5.3.10 of the present document). In brief, two syringes were in constant motion, but in the opposite direction. A valve system, synchronized to the syringe, constantly directed the broth from the pushing syringe to the torture chamber to ensure a constant unidirectional flow in the loop. A companion control bioreactor was setup similarly to the test bioreactor, except for the absence the TC in the recirculation system. The control and test bioreactors were always operated side by side to eliminate inoculum cell and raw material variance.

The 14 day fed-batch process was carried out as described previously. Recirculation in both bioreactors was turned on 4 days after inoculation and lasted till the end of the process. The first 4 days were used to confirm similar growth characteristics between the control and test bioreactor before the test started. Therefore, we can rule out batch-to-
batch variation when different performances were observed after recirculation was turned on. Cell density, viability, osmolality, pH, DO and key metabolites, such as glucose and lactate, were monitored daily. Cell and medium samples were also taken daily for various biological assays to be detailed in section 6.3.5. Product in the final process day was purified through a Protein A spin column and used for product quality analysis. Assays for product quality are to be described in section 6.3.5.7.

6.3.5 Analytical Assays

6.3.5.1 pH, dissolved gas, and metabolites

2 mL samples drawn daily from the bioreactor were immediately injected into the Nova Bioprofile 400 (NOVA BIOMEDICAL CORP. Waltham, MA) which automatically analyzes pH, CO2, DO, glucose, lactate, ammonium, glutamine and glutamate among other metabolites. The pH read by the Nova was used to constantly correct the readings from the pH probe connected to the bioreactor. Glucose concentration together with viable cell density were used to calculate the glucose consumption rate and so the rate of feeding of glucose.
6.3.5.2 Cell density and viability

Cell concentration was determined by automated cell counting in a Cedex Analyzer (INNOVATIS AG. Bielefeld, Germany). One milliliter of the sample is mixed by the analyzer with an equal volume of a dye solution (0.4% w/v Trypan blue in PBS-1X) and then the mixture is pumped into a chamber where the system takes 20 photographs. Viable and dead cells in the photographs are digitally recognized and counted by proprietary software and then averaged.

6.3.5.3 Product Titer

Approximately 200 μL of sample were filtered (0.2 μm) and kept at 4°C in sterile conditions until the end of the culture. Then, every sample was injected into an HP 1100 series (Agilent) HPLC equipped with UV detectors and connected with a Protein A POROS ® A 50 μm column (Part No. 1-5552-44; APPLIED BIOSYSTEMS. Foster City, CA). Absorbance was read at 280 nm. The purified antibody was used to build a standard curve to calibrate absorbance vs. concentration.

6.3.5.4 Osmolality

Osmolality was determined using a freezing-point-based 3320 micro-osmometer (ADVANCED INSTRUMENTS INC. Norwood, MA).
6.3.5.5 Necrosis

The enzyme lactate dehydrogenase (LDH) is a stable, cytosolic enzyme that is released into the culture broth upon cell membrane rupture; therefore, its presence in the medium fluid is indicative of cell lysis by either necrosis and/or late apoptosis. To determine LDH we used the Cytotox 96® Non-radioactive cytotoxicity assay kit following the instructions of the manufacturer (PROMEGA CORP. Madison, WI). The assay is based on the reduction of NAD into NADH and the concurrent transformation of lactate into pyruvate by the action of LDH. The NADH is utilized in the stoichiometric conversion of a tetrazolium salt into a red formazan product, whose absorbance can be read at 490 nm. Since lactate is in great excess in the reaction mixture of the kit, its concentration in the broth does not interfere with the results.

Daily samples from the bioreactor were harvested, counted in the Cedex Analyzer and centrifuged at 250×g and 4°C for 10 minutes; the supernatant was membrane-filtered (0.2 μm PES Pall) and then frozen at –80°C for posterior analysis. Because of the reported cold lability of lactate dehydrogenase isoenzymes (Yoshikuni et al., 2001a; 2001b), test were conducted to check for this problem; in the first test no difference (< 3% in average) was found between the LDH activity (measured as the average difference between points in the standard curve) of two identical samples where one was kept frozen for two weeks while the other was kept refrigerated at 4°C for the same period. The second test involved comparing the LDH activity of two identical samples where one was kept at room
temperature while the other one was frozen at –80°C for 10 minutes and thawed immediately. Again the difference in activity was less than 2%

LDH released into the culture medium was measured following the protocol of the manufacturer; shortly, frozen samples from every day of the culture were thawed simultaneously by placing them at 37°C in a shaker for a few minutes and then 50 μL from every sample were placed into a 96-well plate. In the case of samples from recirculated reactors after day 6, dilutions with fresh medium previous to loading into the plate were made so the LDH concentration would be found within the range of the standard curve; the amount of fresh medium was found empirically from initial experiments. After loading the samples, 50 μL of the reaction mixture from the kit were added and then the plate was kept in the dark at room temperature (~22°C) for 30 minutes, moment in which the reaction was stopped by adding 50 μL of a 1M acetic acid solution (also provided in the kit) that changes the pH and deactivate the enzyme. After eliminating air bubbles, the plate was read at 490 nm using a Spectramax 250 plate reader controlled by SoftMaxPro software (MOLECULAR DEVICES CORP., Sunnyvale, CA). A reading at 690 nm was used as reference wavelength.

To establish an appropriate range for the assay where absorbance were linear with cell concentration for this particular cell line, a standard curve was constructed as follows: the cells remaining in the Erlenmeyer after inoculating the bioreactor were collected, counted using the Cedex Analyzer, resuspended in fresh medium at a viable cell concentration of about 2×10^6 cell/ml, counted again and frozen at –80°C. At the moment of sample
processing, the cells were thawed as described before and then vortexed vigorously. In order to ensure rupture of all cells and liberation of LDH into the medium, a 2.5 mL aliquot was mixed with 250 μL of a solution of 9 % (v/v) in water of Triton® X-100 (Cat. 161-0407, BIO-RAD. Hercules, CA) and then placed again in the shaker at 37°C for 45 min. One milliliter of the mixture was then counted again in the Cedex analyzer and the remaining suspension was centrifuged at 400×g and 4°C for 10 minutes. The supernatant was collected and used to prepare eleven 1:2 successive dilutions with fresh medium, by duplicate or triplicate. 50 μL of the 11 dilutions were placed into wells in the same 96-well plates as the samples, leaving the last well in the row for fresh medium (blank). The procedure for LDH determination was the same as described before. A calibration curve was constructed for every experiment. As linear range went up to a viable cell concentration of about 5×10^5 cell·mL^{-1}, samples were diluted to about such cell concentration.

The absorbance of the samples was compared against the standard curve to calculate the concentration of dead cells in every sample. Since along the culture the LDH concentration increases as a result of cell death but, at the same time, decreases as a result of protein degradation and dilution because of the feed and pH control streams, it is very difficult to estimate precisely the amount of dead cells at every moment; furthermore, average LDH content of an individual cell changes along culture time, so breaking 10^4 cells in day 4 produces different amounts of LDH than doing the same to 10^4 cells in day 1. Therefore, a relative cell death was calculated by normalizing the results with the number of dead cells at the moment of the inoculum.
6.3.5.6 Apoptosis

Apoptosis was determined using the Guava Nexin™ assay kit (GUAVA TECHNOLOGIES INC. Hayward, CA). This assay uses the dye phycoerythrin (PE) covalently linked to Annexin V, a protein with a high affinity for phosphatidylserine (PS); PS is a membrane component normally localized in the internal face of the cell membrane that translocates to the external face when the cell undergoes early apoptosis. The Guava Nexin assay also includes the dye 7-AAD as an indicator of membrane integrity. Healthy, non-apoptotic cells are negative for both Annexin V-PE and 7-AAD dyes since they can not cross the membrane. Early apoptotic cells are positive for Annexin V-PE, since PS is outside of the membrane, but negative for 7-AAD because cell membrane integrity is not yet compromised. Late apoptotic/necrotic cells are positive for both dyes.

The procedure for sample preparation was as follows: a sample volume containing $2 \times 10^6$ cells was centrifuged at $400 \times g$ and $4^\circ C$ for 10 min. The supernatant was removed carefully and the pellet was resuspended in 1 mL of ice-cold Nexin 1X buffer (diluted in distilled water from the 10X solution provided in the kit). 64 μL of the previous suspension were mixed with 8 μL of Annexin V-PE and 8 μL of 7-AAD and mixed thoroughly with the micropipette tip. The sample was incubated in ice for 20 minutes; then 720 μL of cold Nexin 1X buffer was added to each tube and mixed softly with the vortex. The sample was read in the Guava EasyCyte Plus System using the Guava Express Plus™ software (GUAVA TECHNOLOGIES INC.). Positive controls were
prepared by heating healthy cells (from the inoculum train) at 42°C during 50 minutes and then incubating the cells at standard conditions (37°C, 130 rpm, 9% CO₂) for about 6 to 8 hours. As negative control we used the cells without staining. Single and dual staining of the positive control was used to adjust the compensation and the gain of the photomultiplier. Negative control was used to set gates for both Annexin V-PE and 7-AAD absorbance.

6.3.5.7 Product quality

6.3.5.7.1 Glycosylation

N-glycosylation profile was determined using a SEC-MS assay. First, the purified product was reduced using DL-Dithiothreitol (SIGMA-ALDRICH CORP., St. Louis, MO). Heavy chains with N-linked glycans were segregated from light chains using a size exclusion column on a HPLC. The heavy chains were subsequently analyzed using a Q-TOF mass spectrometer. At last, the spectrum was deconvoluted and peaks were identified based on theoretical molecular weight of heavy chain with different glycan forms. Percent of a specific glycan is quantified as the ratio of the peak height of this glycan to the combination of all peaks.
6.3.5.7.2 Methionine oxidation

A proteolytic mapping HPLC method was used to quantify oxidized methionyl residues at a specific location in the Fc region of the antibody. The purified mAb was digested enzymatically using Lysyl endoproteinase. The resultant peptide fragments were separated by reversed-phase HPLC. The peptide fragment containing the specific methionine or its respective oxidized form was detected using ultraviolet absorbance. Percent of oxidization was quantified as the percentage of the peak area of oxidized fragment to the total peak area of oxidized and non-oxidized fragment.

6.3.5.7.3 cIEF (Charge heterogeneity)

An imaged capillary isoelectric focusing (cIEF) assay was used to monitor protein charge heterogeneity. Purified mAb’s are focused within a capillary column in an iCE280 analyzer (CONVERGENT BIOSCIENCE, Toronto, Canada) under DC current voltage. Electrophoretic peaks formed by mAb’s with different pI’s are detected using a whole column ultraviolet light absorbance at 280nm. The intensity of UV absorbance along the column is then converted into a spectrum. Peak areas are used to determine the relative abundance of main isoforms, total acidic isoforms, and total basic isoforms.
6.3.5.7.4 SDS PAGE: molecular weight and fragmentation

Monomer, heavy chain, and light chain molecular weight and fragmentation were monitored using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). Protein-A purified mAb’s were first treated with SDS (BIO-RAD LABORATORIES, Hercules, CA). For non-reducing SDS-PAGE samples, iodoacetamide (IAM) (SIGMA-ALDRICH CORP., St. Louis, MO) was added to minimize disulfide shuffling. For reducing SDS-PAGE, NuPAGE reducing agent (INVITROGEN CORP., Carlsbad, CA) was added to break disulfide bonds. The treated samples were then analyzed on a Mini-Gel Apparatus (INVITROGEN CORP., Carlsbad, CA) using NuPAGE 4-12% Bis-Tris Gels (INVITROGEN CORP., Carlsbad, CA). Proteins were stained with a silver stain kit (OWL SEPARATION SYSTEMS, NH) where the images were captured by a densitometer (BIO-RAD LABORATORIES, Hercules, CA).

6.4 RESULTS

6.4.1 Single Exposure Test of Cells in Different Growth Phases

The sensitivity of the GS-CHO cell line to energy dissipation rate was first measured in a single exposure study. The purposes to start with a single exposure experiment before the more representative chronic exposure experiment were to compare the sensitivity of GS-CHO cells to cell lines tested before in single passage mode. test the sensitivity of GS-
CHO cells in different growth phases during a fed-batch process and to help determine the EDR range to be used in chronic exposure studies.

Cell damage breakthrough curves are shown in Figure 6.3b. Cells in the three tested growth phases showed similar hydrodynamic strength. At energy dissipation rates between $2.9 \times 10^5 \text{ W m}^{-3}$ and $2.3 \times 10^6 \text{ W m}^{-3}$, there was a flat baseline for cells in all three stages. However, when energy dissipation went above $2.3 \times 10^6 \text{ W m}^{-3}$, there was a gradual increase in cell death. Although cells in the death phase showed the same breakthrough point, their damage was always slightly higher (1 to 2 %) than the other two healthier populations. This is most likely due to the presence of dead cells in the pre-tortured suspension where dead cells would slowly release out LDH; although the centrifugation - resuspension in fresh medium procedure precisely pointed towards reducing this effect to maximum, the leakage might have increased when the dead cells were subjected to hydrodynamic forces.

The comparison of cells in different growth phases indicated that their sensitivity to hydrodynamic force is similar. Cells in the late stage of the fed-batch process showed no higher susceptibility to energy dissipation (as indicated by a smaller breakthrough EDR value). A comparison of Figures 6.3b and 4.7 shows that the sensitivity of GS-CHO cells is also similar to other mammalian cell lines tested previously. Based on the single exposure results, it was decided that EDR dissipation lower than $6.5 \times 10^6 \text{ W m}^{-3}$ should be the focus for chronic exposure test.
6.4.2 Repetitive Exposure of Cells in the Scaled-down Fed-Batch Process

It seems logical to assume that the energy dissipation rate needed to trigger sublethal responses of CHO cells to chronic exposure to hydrodynamic stress would be lower than that causing acute cell damage (i.e. intracellular LDH release) under single exposure. Hence, based on single exposure results as shown in Figure 6.3b, energy dissipation rate of $2.9 \times 10^5$ W·m$^{-3}$ and $2.3 \times 10^6$ W·m$^{-3}$ were chosen as the starting levels for the chronic exposure test. One higher level, $6.5 \times 10^6$ W·m$^{-3}$, was added later to extend the range.

6.4.2.1 Cell growth, viability, and productivity

Viable cell density, viability, and product titer of each pair of 3 test and control bioreactors are shown in Figure 6.5. Viable cell density and product titer were normalized to the highest peak cell density and highest harvest titer respectively achieved in the total 6 bioreactor runs. The starting working volume in the 3 sets of experiments was 1.1 L. In all 3 sets of experiments, cell growth, viability, and product titer were comparable between the test bioreactors and their corresponding control bioreactors, which indicates that repetitive exposure to energy dissipation rate up to $6.5 \times 10^6$ W·m$^{-3}$ does not affect cell growth, death, and production. At $6.5 \times 10^6$ W·m$^{-3}$, peak cell density was about 15% higher in the test bioreactor than that in the control bioreactor (Figure 6.5c). This could be attributed to run-to-run variation as just before recirculation was started on day 4, cell density in the test bioreactor was already 11% higher than the control bioreactor. Cell growth in the $2.3 \times 10^6$ W·m$^{-3}$ test and control bioreactors (Figure 6.5b) was slower than
all other bioreactors and lower peak cell densities were reached. As a result, product titer was lower in this pair of bioreactors.

6.4.2.2 LDH release, cell size, and apoptosis

The cell growth and viability results under repetitive exposure to $6.5 \times 10^6$ W·m$^{-3}$ (Figure 6.5c) were not expected. The single exposure study showed that at the same energy dissipation level, there was a roughly 0.5% increase in LDH release compared to the baseline, as shown in Figure 6.3b. If LDH release resulted purely from cell lysis, a 0.5% cell lysis per passage would represent a killing rate of 0.43 day$^{-1}$ in the repetitive exposure bioreactor based on an average exposure frequency of 72 day$^{-1}$ (calculated assuming plug flow in the reactor, the volume of medium and the flow rate). This killing rate is significant compared to the cell growth rate of 0.69 day$^{-1}$ during the exponential growth phase. However, Figure 6.5c shows that after exposure was started on day 4, neither cell growth rate nor viability was affected in the test bioreactor. To reconcile single exposure and chronic exposure results, supernatant LDH concentration in each pair of chronic exposure test and control bioreactors was compared, which is shown in Figure 6.6. Figure 6.6a and 6.6b demonstrate that at $2.9 \times 10^5$ W·m$^{-3}$ and $2.3 \times 10^6$ W·m$^{-3}$, supernatant LDH concentration was similar between the test and control bioreactors both before and after repetitive exposure started on day 4. However, at $6.4 \times 10^6$ W·m$^{-3}$, there was an immediate increase in supernatant LDH after exposure started (Figure 6.6c). This is in good agreement with the single exposure experiment which showed elevated LDH at $6.5 \times 10^6$ W·m$^{-3}$, but not at $2.9 \times 10^5$ W·m$^{-3}$ and $2.3 \times 10^6$ W·m$^{-3}$. At $6.5 \times 10^6$ W·m$^{-3}$ the
average cell diameter in the test bioreactor was smaller compared to the control bioreactor (Figure 6.6c). In contrast, cell size in the two lower levels of energy dissipation rates was similar between the test and control bioreactors (Figure 6.6a and 6.6b).

A summary of the results shown in Figures 6.5c and 6.6c indicates that under a chronic repetitive exposure to $6.5 \times 10^6$ W·m$^{-3}$,

1) Cell growth rate was not affected,
2) Viability was not affected,
3) LDH released into medium increases, and
4) Cell size was reduced.

These observations suggest that at $6.5 \times 10^6$ W·m$^{-3}$, cell membrane of bigger cells might be disrupted which led to release of intracellular components into the medium; nevertheless, smaller cells stayed viable and maintained the same growth and production rate. This underscores the strong resistance of CHO cells to hydrodynamic abuse.

Early apoptotic population during the fed-batch process was monitored. The comparison of each test bioreactor to its control is shown in Figure 6.7. In general, there was no significant difference between test and control bioreactors in either necrotic or apoptotic populations. It was also generally observed that the timing that apoptotic and necrotic cells started to increase coincided with the timing that viable cell density started to drop based on trypan blue quantification. Figure 6.7b ($2.3 \times 10^6$ W·m$^{-3}$) shows significantly
lower apoptotic population in both test and control bioreactors compared to Figure 6.7a (2.9×10⁵ W·m⁻³) and Figure 6.7c (6.4×10⁶ W·m⁻³). This correlates to the slower cell growth rate and reaching peak cell density later in this pair of bioreactors than the other two pairs (Figure 6.5), but the exact reason is unknown. Although immediate LDH release was observed in the test bioreactor after 6.5×10⁶ W·m⁻³ exposure started (Figure 6.6c), neither apoptotic nor necrotic population (as determined by flow cytometry) in the test bioreactor differentiated from the control bioreactor (Figure 6.7c). One possible explanation of this phenomena involves a quick lysis of the larger cells immediately after the onset of recirculation, so the remains of broken cells would be degraded enough to be separated together with the supernatant in the first mild centrifugation during the sample processing for the apoptosis assay. That would explain the reduction in size and LDH release. Such explanation, however, fails to explain why there was no change in growth rate or viable cell concentration. Another explanation proposes that initial release of LDH may not be the result from cell lysis, but from cell shrinkage; in this case, larger GS-CHO cells would be able to maintain their main functions of growth and production while their plasma membrane is slightly disrupted and part of intracellular components are lost. Disruptions of cell membrane, as well as rapid reseal are common in vivo and in vitro (McNeal and Steinhardt, 1997), and may very well explain the size reduction and LDH release while viability and cell growth rate would not be noticeably affected.
6.4.2.3 Glucose metabolism

Glucose usage and lactic acid production were used as indicators of overall carbon metabolism. Glucose is a critical carbon source for both energy and biosynthetic intermediates while lactate is a major byproduct of glucose metabolism. The cumulative glucose consumption and lactic acid concentration in the three sets of tests are shown in Figure 6.8. All sets of data were normalized to the highest glucose consumption and highest lactate peak concentration respectively achieved in the total 6 bioreactor runs. In general, cumulative glucose consumption is very similar between the test and control bioreactors at all 3 EDR levels. Lactate profile was similar between each pair of test and control bioreactors as well. In all bioreactor runs, lactate concentration increased during the exponential growth phase. One day before reaching stationary phase, cells stopped producing lactate and switched to consuming lactate instead until all lactate was completely consumed. As the 2.3×10^6 W·m^{-3} test and control bioreactors entered stationary phase later than other bioreactors, the shift of lactate metabolism in these two bioreactors was also later. Based on the glucose and lactate results, we can infer that the amount of glucose entering the Krebs cycle through pyruvate was similar between the test and control bioreactors. Results shown in Figure 6.8 indicated that energy dissipation as high as 6.5×10^6 W·m^{-3} did not significantly affect glucose metabolism.
6.4.2.4 Product quality

Four assays were used to assess the effects of repetitive hydrodynamic force on product heterogeneity. These assays were SDS-PAGE (reduced and non-reduced) for protein size, aggregation, and fragmentation, cIEF for protein charge profile, a peptide Mass Spectrometry for methionine oxidation, and glycan mass spectrometry for N-linked glycosylation.

Neither reduced nor non-reduced SDS-PAGE results revealed any difference between test and control bioreactor products at all 3 levels of energy dissipation rates. Gel images comparing products from the $6.5 \times 10^6 \text{ W} \cdot \text{m}^{-3}$ exposure test are shown in Figure 6.9. The band pattern and band intensity between the test and control bioreactor samples were very comparable in both non-reduced and reduced assay.

cIEF is a capillary isoelectric focusing assay for the quantification of charge profiles. Acidic and basic species refer to products with either lower or higher isoelectric focusing point than the main product. As shown in Table 6.2, the percentage of acidic or basic species was similar between control and test bioreactors at $2.9 \times 10^5 \text{ W} \cdot \text{m}^{-3}$, and $2.3 \times 10^6 \text{ W} \cdot \text{m}^{-3}$. However, at $6.5 \times 10^6 \text{ W} \cdot \text{m}^{-3}$, acidic species increased by ~30% while basic species decreased by ~35%. In monoclonal antibodies, terminal sialiation on the glycan and asparagine/glutamine deamidation are the two major contributors to the formation of acidic species. As terminal sialic acid is negligible in all products from this study (results not shown), deamidation is believed to be the major contributor to the formation of acidic
species. Basic species were primarily C-terminal lysine variants. Existence of C-terminal lysine renders a mAb basic. Overall, cIEF results indicate that at the highest energy dissipation rate tested, $6.5 \times 10^6 \text{ W} \cdot \text{m}^{-3}$, deamidation rate increased while more C-terminal lysine was cleaved.

A peptide Mass Spectrometry assay monitored the percentage of a specific methionine being oxidized in the mAb. The results, listed in Table 6.2, showed variations among different sets of experiments. At $6.5 \times 10^6 \text{ W} \cdot \text{m}^{-3}$, the oxidation level of mAb’s from either the test or the control bioreactors is around 6% while that of mAb’s from $2.9 \times 10^5 \text{ W} \cdot \text{m}^{-3}$ and $2.3 \times 10^6$ test and control bioreactors is around 3.5%. The variation is likely resulted from different storage durations at 2-8°C before the broth samples were purified. Nevertheless, within each set of experiment, the results between the test and control bioreactors are very similar. This indicates that methionine oxidation was not affected by energy dissipation rate up to $6.4 \times 10^6 \text{ W} \cdot \text{m}^{-3}$.

Three glycoforms, G0, G1, and G2, accounted for the majority of all glycoforms quantified by glycan Mass Spectrometry. Of these three glycoforms, G0 is the most dominant one while G2 is the least abundant. As it can be seen in Figure 6.10, while cells were under hydrodynamic stress, the distribution of these three glycoforms was significantly skewed toward G1 and G2 direction. In general, G0 percentage was reduced from 80% to 55% while that of G1 and G2 was increased from 10% to 30% and from 1% to 5% respectively. The extent of glycan population shift is similar among all test conditions, suggesting that glycosylation change is not proportional to EDR or exposure.
frequency within the test range. It is surprising to observe that hydrodynamic stress actually increase CHO cells’ glycosylation capability. The link between cause and effect is unknown, but one possible mechanism is through integrin activation. Integrin is a cell surface receptor for cells to attach to extra cellular matrix. It is also a major mechanical force sensor for cells. The activation of integrin could trigger multiple signal transductions, where one leads to actin cytoskeleton polymerization (DeMali et al., 2003). It had been shown that actin dynamics can change Golgi apparatus’s structural organization (Egea et al., 2006). When mammalian cells were treated with actin toxin to depolymerize actin cytoskeleton, swelling of Golgi cisternae had been observed. This indicates the criticality of actin cytoskeleton in maintaining the flat morphology of Golgi cisternae (DeMali et al., 2003). Because the spatial distribution of glycosylation enzymes in Golgi relies heavily it morphology, actin polymerization may be able to improve Golgi apparatus’s glycosylation capability.

This study indicates that EDR as low as 2.9×10^5 W·m⁻³ could trigger glycosylation shift. The threshold EDR could be even lower. In order to continue this study, modification in the torture chamber (i.e. a larger throat) are recommended to exert lower EDR avoiding, at the same time, cell sedimentation in the recirculation system

6.5 DISCUSSION

The scale-down model use in this study mimics the physical and nutritional environment that suspended cells experience in large-scale agitated bioreactors. First of all, the fed-
batch process in the bench-top bioreactor is the same as that to be executed in large-scale
bioreactors in respect to nutritional environment and pH, dissolve oxygen, and
temperature setpoints. Secondly, the repetitive high-low fluid dynamic stress
environment that cells were subjected to in large-scale bioreactor is reproduced using the
bench-scale bioreactor and torture chamber loop in tandem. The energy dissipation rate in
the bench-scale bioreactor is low where the volumetric average rate is calculated to be 12
W·m\(^{-3}\) and the maximum local rate around the impellers won’t exceed \(1\times10^3\) W·m\(^{-3}\).
Conversely, the energy dissipation rate generated in the torture chamber is intense at
\(2.9\times10^5\) W·m\(^{-3}\) to \(6.4\times10^6\) W·m\(^{-3}\). The only major difference between this scale-down
model and large-scale bioreactors is exposure frequency. The flow rates tested in this
study were 10 mL·min\(^{-1}\), 30 mL·min\(^{-1}\), and 50 mL·min\(^{-1}\), which correspond to average
exposure intervals of 100 min, 33 min, and 20 min. These intervals are much longer than
those in large-scale bioreactors (average circulation time < 1 minute). However, we
believe the possibility that the frequency difference would change the results is low.
Firstly, recirculation was continuous for an extended period of time (10 days), so that
even with a low exposure frequency, the overall exposure time was high. Therefore, a
small change through a single passage should be propagated over time. Secondly,
glycosylation results showed no correlation between exposure frequency to the extent of
cellular response. 10 mL·min\(^{-1}\) yielded the same level of glycosylation shift as 50
mL·min\(^{-1}\) did. This indicates at least some physiological responses are not sensitive to
exposure frequency.
In the single exposure test, where LDH release was used to assess cell damage, the threshold energy dissipation rate for lethal cell damage was determined to be $2.3 \times 10^6$ W·m$^{-3}$. This is similar to the sensitivity of most other mammalian cell lines in suspension. In the repetitive exposure study, the same hydrodynamic sensitivity was obtained when LDH release is used as cell damage indicator. This clearly demonstrates the high sensitivity of single exposure model, while using LDH release to detect cell damage, to study the effects of hydrodynamic stress on mammalian cells.

LDH release under repetitive $6.5 \times 10^6$ W·m$^{-3}$ exposure could result from cell death or cell leakage. Should LDH release be from cell death, either a decrease in viability or a slow down in apparent cell growth rate should be observed. However, neither was observed. Instead, correlating to the medium LDH increase, there was a decrease in average cell diameter. Therefore, initial release of LDH may not result from cell lysis, but from cell shrinkage. This is a quite surprising observation as it indicates that GS-CHO cells can maintain their main functions of growth and production while the plasma membrane is slightly disrupted and part of intracellular components are lost.

Besides LDH, an array of process and product quality attributes was monitored. These attributes were used to assess physiological effects of hydrodynamic stress on the cells. For process attributes, which include cell growth rate, productivity, glucose utilization, and lactate production, no significant effect was observed even under the highest EDR, $6.4 \times 10^6$ W·m$^{-3}$. Prior reports on effects of hydrodynamic force on protein production and glucose metabolism of suspended cells are rare. Keane et al. (2003) studied an
anchorage-dependent CHO clone producing recombinant hGH. They found that under chronic 0.8 N·m⁻² (6.4×10² W·m⁻³) exposure, hGH production rate decreased by 51% compared to the control. It is well known that attached cells are more sensitive to hydrodynamic force, hence the results from this study and Keane et al.’s study are not directly comparable. Keane et al. (2003) also observed that at 0.8 N·m⁻² (6.4×10² W·m⁻³), glucose uptake increased by 42%. This is consistent with the observation of McDowell and Papoutsakis (1998) where they found that by increasing the agitation of a 2L bioreactor from 80 rpm to 300-400 rpm, glucose consumption of HL60 cells increased by 40%. However, our finding indicated that glucose consumption of suspended GS-CHO cells was not significantly affected.

The threshold EDR required for product quality change was much lower. Four representative assays were used in this study to characterize product quality. Methionine oxidation and product aggregation and fragmentation were not affected by hydrodynamic force within the test range. But mAb charge profile determined by cIEF showed that at 6.5×10⁶ W·m⁻³, deamidated molecules increased while the level of C-terminal lysine decreased. Deamidation is predominantly non-enzymatic. Major factors that affect deamidation rates are protein primary sequence and three-dimensional structure, pH, temperature, and ionic strength. Between test and control bioreactors, protein sequence and three dimensional structure, temperature, extra cellular pH, and extra-cellular ionic strength were all identical or similar. What factor(s) contributed to the increase of deamidation level and C-terminal lysine cleavage is unclear. Ziegelstein et al. (1992) had
observed that hydrodynamic force could make cytosol of vascular endothelial cells acidic, but they did not measure pH change in the organelles where mAbs reside.

Glycosylation, the addition of an oligosaccharide, is one of several posttranslational modifications that proteins undergo inside eukaryotic cells and that may result in a series of changes of the physiochemical as well as biological properties. Recombinant glycoproteins require “correct” glycosylation for therapeutic efficacy; such “correct” pattern must correspond to the profile (fingerprint) licensed by the FDA for a specific drug and, therefore, should be conserved along the whole research-development-manufacture pathway (Harcum, 2006). In the present study, the profile of Asn297 linked glycoforms was changed at even the lowest EDR tested, $2.9 \times 10^5 \text{ W} \cdot \text{m}^{-3}$. Although it is not unexpected to find that hydrodynamic stress could change glycosylation pattern, it is surprising that the change is actually toward the more complete glycosylated direction. Compared to the materials generated in the control bioreactors, G0 population was reduced by about 30% while those of G1 and G2 were increased by roughly 200% and 300% respectively. This suggests that hydrodynamic stress actually improve CHO cell’s glycosylation capability. The exact reason is unknown. However, the activation of integrin by hydrodynamic force and subsequent enhanced actin cytoskeleton polymerization could play an important role to improve Golgi apparatus’s glycosylation function. Although changes in glycosylation profiles as a function of culturing conditions (feeding strategy, nutrient limitation, pH, ammonium concentration, CO$_2$ and oxygen concentration and osmolality) have been reported (Harcum, 2006), there is no prior report on the effect of hydrodynamic forces on glycosylation pattern shift. Senger and Karim
Ma et al. (2002) and Mollet et al. (2007) previously compared the hydrodynamic sensitivity of several mammalian cell lines, characterized by cell lysis or LDH release, to the hydrodynamic forces generated in agitated tanks. Such comparisons have shown that although bubble rupture could cause immediate cell lysis, energy dissipation from agitation is far from causing cell death. The present study adds a new dimension to the comparison. The energy dissipation levels that cause physiological responses were added, as shown in Figure 6.11. Figure 6.11 clearly demonstrates that at least one physiological response, glycosylation shift, can be triggered at a much lower EDR than that required to physically damage the cells. Although the lowest EDR to cause glycosylation shift has not been identified, the currently identified EDR is already two orders of magnitude lower than that to cause intracellular LDH release. Figure 6.11 also indicates that the lethal sensitivity of GS-CHO cells is similar to other previously tested cell types and cell lines. Even the maximum EDR generated in a 22000L fermentor is far from physically killing the cells. However, the energy dissipation from agitation in large-scale bioreactors could be approaching the level to trigger physiological responses of the cells. It should be noted that the 22000L fermentor listed in Figure 6.11 was equipped with “high shear” Rushton Turbine impellers, which, at the same agitation rate, produce much higher
energy dissipation rate than “low shear” impellers. Meanwhile, the agitation rate used (140 rpm) was quite intense compared to typical mammalian cell bioreactors. Therefore, although such fermentor produced sufficient high EDR to trigger glycosylation pattern shift, a mammalian cell bioreactor of similar size may not. A rough estimation of a similar size bioreactor equipped with hydrofoil impellers operated at 70 rpm showed that it would generate a maximum EDR 50 times lower than the 22000L fermentor would. From the previous discussion, the criticality to determine the threshold EDR to cause glycosylation change in a future work becomes obvious in order to guide large-scale bioreactor design and operation.

Although the observed variations in charge and glycosylation profiles may not result in unfavorable product quality, shifting of product characteristics during process scale-up or operational changes is undesired. Therefore, caution still need to be exerted during design and operation of large-scale bioreactors for CHO cells. The medium used in this study is chemically defined. We had grown the same cells in a process containing non-animal derived peptones and compared them to cells from the chemically defined process in a single exposure test. The cell damage breakthrough curves were almost identical (not shown), which indicates that existing of peptones may not change cells’ mechanical properties.
6.6 CONCLUSIONS

The sensitivity of a monoclonal antibody producing GS-CHO cell line to energy dissipation was quantified with a novel microfluidic test device. The quantification was achieved in two models of hydrodynamic stress. The first model is a simple single passage where cells are forced through the torture chamber once and the effluent was used to quantify lethal cell damage (acute cell lysis). The second model is a repetitive exposure model that simulates the fluid dynamic environment cells are subjected to in large-scale bioreactors. In the second model, cells continuously circulate between a 2 Liter fed-batch bioreactor and a torture chamber where the 2 Liter bioreactor simulates the low energy dissipation zone in the bulk of a large-scale bioreactor and the torture chamber simulates the high energy dissipation zones around impellers of a large-scale bioreactor; an intensive array of lethal and sublethal effects was monitored to assess and quantify cell damage. These effects include cell growth rate, viability, necrosis, apoptosis, carbon metabolism, productivity, and product quality.

In the single passage test, it was shown that the threshold energy dissipation rate for GS-CHO cell death is 2.3×10^6 W·m^3. This threshold is similar to that of CHO-K1, hybridoma, PER.C6 and other cell lines tested before (Ma et. al, 2002, 2007; Mollet et. al, 2007). The single passage test also showed that the sensitivity of cells in different growth phases of the fed-batch process is very similar. While dying, cells in death phase are not more susceptible to energy dissipation than cells in either exponential growth phase or stationary phases.
In the repetitive exposure tests, CHO cells in a fed-batch process were subjected to energy dissipation spanning a little more than one order of magnitude, $2.9 \times 10^5 \text{ W} \cdot \text{m}^{-3}$ to $6.5 \times 10^6 \text{ W} \cdot \text{m}^{-3}$. The threshold for physical cell damage, $2.3 \times 10^6 \text{ W} \cdot \text{m}^{-3}$, is the same as that found in the single passage test. Although being repetitively exposed to acute EDR for 10 days, cells maintained their physical integrity at EDR level of $2.3 \times 10^6 \text{ W} \cdot \text{m}^{-3}$ or lower. At $6.5 \times 10^6 \text{ W} \cdot \text{m}^{-3}$, an increase on intracellular LDH release was observed, but surprisingly, cell growth and productivity was not significantly affected. This underlines the tremendous resistance of CHO cells to hydrodynamic stress.

Of all sublethal effects monitored, cell growth, viability, recombinant protein production, glucose metabolism were not affected across the whole range of EDR tested. Most product quality attributes, including aggregation, fragmentation, and methionine oxidation, were not affected. Two sublethal effects, alteration of protein charge and glycosylation, were observed at different levels of energy dissipation rates. At $6.5 \times 10^6 \text{ W} \cdot \text{m}^{-3}$ deamidation and C-terminal lysine cleavage increased which result in an increase of acidic species and a decrease of basic species. Glycosylation is the most sensitive product quality attribute to hydrodynamic forces. The EDR level required to trigger glycosylation shift is at least one order of magnitude lower than that to cause detectable LDH release or charge profile shift. At all EDR levels tested, G1 and G2 glycan populations increased significantly while that of G0 glycan decreased. This indicates that hydrodynamic stress can actually increase CHO cells’ glycosylation capability. The mechanism(s) for the glycosylation capability increase is unknown, but integrin
activation and subsequent actin cytoskeleton polymerization could be a cause of improvement of Golgi apparatus’s function through improving Golgi cisternae structure.

Results from this scale-down study indicate that CHO cells are very resistant to hydrodynamic forces in respect to cell growth and carbon metabolism, and recombinant protein productivity. Hydrodynamic force resulting from agitation in large-scale bioreactor is very unlikely to cause either cell death or productivity drop. However, physiological responses of CHO cells can be triggered by much lower levels of hydrodynamic forces. We observed in this study that glycosylation shift occurred at an energy dissipation rate more than one order of magnitude lower than that causing physical cell damage. Unfortunately we could not determine the threshold EDR that affects glycosylation using the current Torture Chamber which was designed to study high EDR. In the next step, we will use a Torture Chamber with a wider flow channel to study the glycosylation effect in the lower EDR range. This will allow us to quantify the threshold EDR for glycosylation shift and to define large-scale bioreactor operation in order to avoid this shift.

6.7 ACKNOWLEDGEMENTS

The authors appreciate Ellen McCormick and Dave Brunner for the discussion and feedbacks during the study and for the critical review of the manuscript, Laura Bass and members of Process Development Analytics group for conducting all analytical assays, and Michael Dupuis for purifying the products.
6.8 REFERENCES


Ma, N.; Koelling, K. and Chalmers, J. J. (2002). The fabrication and use of a transient contraction flow device to quantify the sensitivity of mammalian and insect cells to hydrodynamic forces. *Biotechnology and Bioengineering.* **80:** 428-437.


Figure 6.1. Characteristics of the microfluidic device (Torture chamber). (a) Photograph. (b) Scheme of the stainless steel plate (dimensions in mm).
Figure 6.2. Equipment setup for single pass-experiments.
Figure 6.3. (a) Viable cell concentration in a fed-batch process and the time points at which cells were taken for energy dissipation sensitivity test. Arrows indicate the sampling time (b) Sensitivity of GS-CHO cells at different growth stages to EDR.
Figure 6.4. Equipment setup for chronic exposure.
Figure 6.5. Comparison of cell growth and antibody production curves for both chronically exposed to EDR and corresponding control bioreactors. (a) $10 \text{ mL} \cdot \text{min}^{-1} - 2.9 \times 10^5 \text{ W} \cdot \text{m}^{-3}$, (b) $30 \text{ mL} \cdot \text{min}^{-1} - 2.3 \times 10^6 \text{ W} \cdot \text{m}^{-3}$, (c) $50 \text{ mL} \cdot \text{min}^{-1} - 6.5 \times 10^6 \text{ W} \cdot \text{m}^{-3}$. Product titer was normalized to the highest titer of all bioreactor runs.
Figure 6.5. (Continued).
Figure 6.5. (Continued).
Figure 6.6. Comparison of LDH concentration in the medium and cell size between control and test bioreactors. (a) 10 mL·min⁻¹ – 2.9×10⁵ W·m⁻³, (b) 30 mL·min⁻¹ – 2.3×10⁶ W·m⁻³, (c) 50 mL·min⁻¹ – 6.5×10⁶ W·m⁻³.
Figure 6.6. (Continued).
Figure 6.6. (Continued).
Figure 6.7. Comparison of glucose metabolism in control and test bioreactors. (a) 10 mL·min\(^{-1}\) – 2.9×10\(^{5}\) W·m\(^{-3}\), (b) 30 mL·min\(^{-1}\) – 2.3×10\(^{6}\) W·m\(^{-3}\), (c) 50 mL·min\(^{-1}\) – 6.5×10\(^{6}\) W·m\(^{-3}\). Glucose metabolism was based on cumulative volumetric glucose consumption and lactate concentration. All data are normalized to the highest lactate concentration and glucose consumption of all bioreactors.
Figure 6.7. (Continued).
Figure 6.7. (Continued).
Figure 6.8. Comparison of apoptotic and necrotic population (measured by flow cytometry) between test and control bioreactors. (a) 10 mL·min$^{-1}$ – 2.9×10$^{5}$ W·m$^{-3}$, (b) 30 mL·min$^{-1}$ – 2.3×10$^{6}$ W·m$^{-3}$, (c) 50 mL·min$^{-1}$ – 6.5×10$^{6}$ W·m$^{-3}$.
Figure 6.8. (Continued)
Figure 6.8. (Continued)
Figure 6.9. Comparison of product aggregation and fragmentation in control and test bioreactor exposed at chronic energy dissipation of $6.5 \times 10^6$ W·m$^{-3}$. Lanes 3 and 7: molecular weight markers; Lane 4: standard antibody. Lane 5: Test bioreactor material. Lane 6: Control bioreactor material. (a): Reducing conditions. (b): Non-reducing conditions.
Figure 6.10. Effect of chronic exposure to energy dissipation in glycosylation. (a): \(2.3 \times 10^6 \text{ W·m}^{-3}\); (b) \(6.5 \times 10^6 \text{ W·m}^{-3}\)
**Lethal Cell Response**

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Cell</th>
<th>Mode of growth</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>CHO-K1, necrosis</td>
<td>Anchored</td>
<td>Gregoriades et al. (2000)</td>
</tr>
<tr>
<td>B</td>
<td>Hybridoma, necrosis</td>
<td>Suspended</td>
<td>Thomas et al. (1994); Zhang et al. (1993)</td>
</tr>
<tr>
<td>C</td>
<td>MCF-7, necrosis</td>
<td>Suspended</td>
<td>Ma et al. (2002)</td>
</tr>
<tr>
<td>D</td>
<td>Mouse myeloma, necrosis</td>
<td>Suspended</td>
<td>McQueen and Bailey (1989)</td>
</tr>
<tr>
<td>E</td>
<td>HeLa S3, mouse L929, necrosis</td>
<td>Suspended</td>
<td>Augenstein et al. (1971)</td>
</tr>
<tr>
<td>F</td>
<td>CHO-K1, SF-9, HB-24, necrosis</td>
<td>Suspended</td>
<td>Ma et al. (2002)</td>
</tr>
<tr>
<td>G</td>
<td>Uninfected and viral infected PERC6 cells, necrosis</td>
<td>Suspended</td>
<td>Submitted</td>
</tr>
<tr>
<td>H</td>
<td>Enthomopathogenic nematodes, necrosis</td>
<td>Suspended</td>
<td>Fife et al. (2004)</td>
</tr>
<tr>
<td>I</td>
<td>CHO-K1, apoptosis</td>
<td>Anchorage</td>
<td>Mollet et al. (2007)</td>
</tr>
<tr>
<td>J</td>
<td>THP-1, necrosis</td>
<td>Anchorage</td>
<td>Mollet et al. (In Press)</td>
</tr>
<tr>
<td>K</td>
<td>GS-CHO</td>
<td>Suspension</td>
<td>This work</td>
</tr>
</tbody>
</table>

**Figure 6.11.** Summary of the reported energy dissipation rate at which GS-CHO and other cell lines are affected as well as the reported levels of energy dissipation rate in various bioprocess environments. Adapted from Ma et al. (2002) and Mollet et al. (2004).
Non-lethal physiological response

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Cell</th>
<th>Mode of growth</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>Algae, loss of flagella</td>
<td>Suspension</td>
<td>Hu et al. (2007)</td>
</tr>
<tr>
<td>β</td>
<td>GS-CHO Glycosylation shift</td>
<td>Suspension</td>
<td>This work</td>
</tr>
<tr>
<td>γ</td>
<td>GS-CHO charge shift</td>
<td>Suspension</td>
<td>This work</td>
</tr>
</tbody>
</table>

Hydrodynamic Conditions

<table>
<thead>
<tr>
<th>Symbol</th>
<th>Process</th>
<th>Description</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Agitation</td>
<td>Volume average in typical animal cell bioreactors.</td>
<td>Varley and Birch (1999)</td>
</tr>
<tr>
<td>2</td>
<td>Agitation</td>
<td>Volume average in a 10 L mixing vessel (RT, 700 RPM)</td>
<td>Zhou and Kresta (1996)</td>
</tr>
<tr>
<td>3</td>
<td>Agitation</td>
<td>Maximum in the 10 L mixing vessel (RT, 700 RPM)</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>Agitation</td>
<td>Volume average in a 22,000 L mixing vessel (RT, 240 RPM)</td>
<td>Wernersson and Tragardh (1999)</td>
</tr>
<tr>
<td>5</td>
<td>Agitation</td>
<td>Maximum in the 22,000 L mixing vessel</td>
<td></td>
</tr>
<tr>
<td>6</td>
<td>Agitation</td>
<td>Maximum in spinner vessel (200 RPM)</td>
<td>Venkat et al. (1996)</td>
</tr>
<tr>
<td>7</td>
<td>Bubble rupture</td>
<td>Pure water, bubble diameter: 6.32mm</td>
<td>Garcia-Briones et al. (1994)</td>
</tr>
<tr>
<td>8</td>
<td>Bubble rupture</td>
<td>Pure water, bubble diameter: 1.7mm</td>
<td>Boulton-Stone and Blake (1993); Garcia-Briones et al. (1994)</td>
</tr>
<tr>
<td>9</td>
<td>Flow through a pipe</td>
<td>Pure water, 100 mL/min, 1 mm diameter</td>
<td>Mollet et al. (2004)</td>
</tr>
<tr>
<td>10</td>
<td>Flow through a micropipette tip</td>
<td>Flow through a 200 μL micropipette tip in 0.2 sec</td>
<td>Mollet et al. (2004)</td>
</tr>
<tr>
<td>11</td>
<td>Agitation</td>
<td>Volume average in a highly agitated animal cell bioreactor</td>
<td>Oh et al. (1992)</td>
</tr>
<tr>
<td>12</td>
<td>Agitation</td>
<td>Max. (hypothetical 20,000 L reactor, A310, 50 rpm) by power number calculation</td>
<td>This work</td>
</tr>
</tbody>
</table>

Figure 6.11. (Continued).
Table 6.1. Experimental flow rates and corresponding calculated energy dissipation rates.

<table>
<thead>
<tr>
<th>Flow Rate (mL·min⁻¹)</th>
<th>10</th>
<th>30</th>
<th>50</th>
<th>70</th>
<th>90</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Energy dissipation rate (W·m⁻³)</strong></td>
<td>2.87×10⁵</td>
<td>2.27×10⁶</td>
<td>6.45×10⁶</td>
<td>2.60×10⁷</td>
<td>1.09×10⁸</td>
</tr>
</tbody>
</table>
### Table 6.2

Effect of chronic exposure to hydrodynamic force on product charge profile and methionine oxidation. Acidic and basic species are normalized to the corresponding control bioreactor. Data on the percentage of mAb with oxidized methionine for both the test and control experiments are shown.

<table>
<thead>
<tr>
<th>Energy Dissipation Rate (W·m⁻³)</th>
<th>Acidic Species (% control)</th>
<th>Basic Species (% control)</th>
<th>% oxidized methionine in the mAb</th>
<th>% oxidized methionine in the mAb in the control</th>
</tr>
</thead>
<tbody>
<tr>
<td>2.9×10³</td>
<td>100.1</td>
<td>88.4</td>
<td>3.7</td>
<td>3.3</td>
</tr>
<tr>
<td>2.3×10⁶</td>
<td>101.1</td>
<td>109.5</td>
<td>3.8</td>
<td>3.5</td>
</tr>
<tr>
<td>6.5×10⁶</td>
<td>129.5</td>
<td>66.4</td>
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CHAPTER 7

AN IMPROVED METHODOLOGY FOR THE MODELING AND SIMULATION OF THE EFFECT OF ENERGY DISSIPATION RATE ON ANIMAL CELLS IN MICROFLUIDIC DEVICES

7.1. ABSTRACT

The effect of hydrodynamic forces on animal cell cultures, while extensively studied, still lacks significant, fundamental understanding. A series of previous manuscripts from our lab have reported on the acute and chronic exposure of CHO cells to hydrodynamic forces in a second generation convergent-divergent microfluidic device (Mollet et al. 2007; Godoy-Silva et al., Submitted), as well as in a Fluorescence activated Cell Sorter (Mollet et. al., In Press). In this study, we present an improved methodology for the analysis of the experimental effect of hydrodynamic forces on animal cells in such devices. Improvements are based on considerations of momentum balance, specifically in non-slip flow inside channels, and the assumption of a physical limit of stress beyond which the cell membrane loses its integrity and cell dies. This model allows the theoretical prediction of the trend of observed breakthrough curves and could lead to further refinement in the design of bioprocess equipment since it provides a methodology
to establish a more accurate threshold, above which animal cells subjected to hydrodynamic forces could be destroyed.

7.2. INTRODUCTION

Animal cells have an innate ability for proper folding and post-translational processing of proteins that makes them preferable as host for producing biological components of therapeutic and diagnostic interest; as a result, the culture of mammalian cells has been used extensively for the production of substances with biological activity. There has been an exponential increase on the number of FDA’s approved products generated through mammalian cell culture (i.e. 24 approved monoclonal antibodies as of 2006, with sales of over 15 billion dollars) driving the industry to expand the size of bioreactors and GMP production facilities; in spite of that, a current shortage in installed capacity has been reported (Molowa, 2001), forcing companies to optimize their resources and processes in order to maximize productivities and economical profits with existing facilities.

While significant progress has been made in the effective design and operation of large scale animal cell culture bioreactors and downstream equipment, concerns about premature cell rupture during processing underscores the need to continue characterizing and quantifying the effect of hydrodynamic forces on suspended animal cells.

A large amount of studies using a wide variety of methodologies have focused on the effects of hydrodynamic damage to eucaryotic cells (the interested reader is referred to
several excellent reviews by Papoutsakis, 1991; Chisti, 1999; Kieran et al, 2000; Chisti, 2001; Ma et al, 2006; Lehoux et al., 2006; Haga et al., 2007). As a result, several different parameters have been suggested in literature to correlate the effects that hydrodynamic forces exert on animal cells; among them, the scalar parameter Energy Dissipation Rate (EDR), has been widely used to quantify local mixing performance in stirred tanks (Kresta, 1998) as well as animal cell bioprocesses (Mollet et al. 2004; Mollet et al., 2007). EDR was first suggested by Bluestein and Mockros (1969) to characterize hydrodynamic forces operating on cells; as EDR is intrinsic to any moving fluid, it is independent of the flow regime (turbulent/laminar) and accounts for both shear and extensional components of three-dimensional flow. EDR has units of power per unit volume (i.e. W·m$^{-3}$); although it can not be measured directly, it can be easily calculated from Equation (7.1), provided that the broth behaves like an incompressible, Newtonian fluid (common situation in cell culture where the medium has water-like properties) and that the velocity vector has been accurately determined:

$$\varepsilon = \tau : \nabla \vec{V} = \mu \left[ \nabla \vec{V} + (\nabla \vec{V})^T \right] : \nabla \vec{V} = \mu \sum_i \sum_j \left[ \nabla V_i + (\nabla V_i)^T \right] \nabla V_j$$  \hspace{1cm} (Eq. 7.1)

In Eq. (6.1), $\mu$ is the viscosity of the medium, $\nabla \vec{V}$ is the gradient of the velocity vector and $\nabla \vec{V}^T$ is the transpose of the gradient of the velocity vector.

A series of reports from our laboratory have been using the EDR concept to explain cell damage; Ma et al. (2002), designed and manufactured a microfluidic device in which suspended cells could be exposed to acute, high levels of EDR in a laminar, very well...
controlled environment; using such device, they subjected an insect cell line (SF-9), a Chinese Hamster Ovary cell line (CHO), a mouse hybridoma cell line (HB-24) and a human breast cancer cell line (MCF-7) to single exposures of EDR and measured the release of the cytoplasmic enzyme lactate dehydrogenase (LDH) as an indication of cell membrane rupture. Every one of the cell lines studied showed significant cell lysis beginning around an EDR of $10^7$ W·m$^{-3}$. Mollet et al. (2007) developed an improved, autoclavable second-generation microfluidic device to further investigate if besides the evident levels of necrosis already reported, the effect of single exposures to high levels of EDR could lead to apoptosis; their results were consistent with the previous work by Ma et al. (2002) in the levels of necrosis obtained after passing the cells once through the micro-device for two strains of Chinese Hamster Ovary cells (CHO-K1 and CHO-bcl2). In addition, Mollet et al. (2007) discovered that while detectable relative to a control, only a very small fraction of cells become apoptotic when exposed to sublysis levels of EDR. In a related subject, Mollet et al. (In Press) used the improved microfluidic device as well as computer fluid dynamics (CFD) commercial software to simulate the single passage of cells through the nozzle of a commercial fluorescence activated flow sorter (FACS); they discovered that in the FACS the cells experience high levels of EDR as a result of hydrodynamic focusing in the nozzle, and levels of cell necrosis on the order of 20 to 35% could be detected using CHO cells under typical operating conditions. Finally, to resemble the real behavior inside a reactor where cells moving with the fluid undergo cyclic transitions from zones away from the impeller (low hydrodynamic stress) to a zone of high hydrodynamic stress in the neighborhood of the impeller, Godoy-Silva et al. (submitted) performed studies to quantify lethal and non-lethal effects of chronic
exposition of animal cell to hydrodynamical stresses in a series of suspended cultures that simulate the real environment inside an industrial bioreactor by taking the broth from a gently agitated reactor (low stress zone), and pumping it through the aforementioned contractional device (high stress zone) back into the bioreactor. Godoy-Silva et al. (submitted) addressed the question on how sensitive really are animal cells to repeated, chronic hydrodynamical stress from the standpoint of cell growth, finding that for a particular CHO cell line (CHO6E6), cells would stop growing at EDR values much lower than those needed to kill them with a single exposure to hydrodynamic stress.

On all of the previous reports, EDR values were obtained through simulation using commercial CFD software. A crucial aspect of the methodology focuses in the definition of a single value of EDR representative for every condition (flow rate) tested. This was done by injecting and tracing a set of virtual particles at the entrance of the simulated device and recording the maximum value every particle experienced in its flowpath; at the end, the result was a set of maximum values which were grouped into arbitrary ranges to create a distribution. The median of the distribution, that corresponded fairly well with the modal peak, was used as representative EDR for every flow rate.

The main weak point of this approach resides in the fact that there is a long tail of EDR values larger than the median value chosen (i.e. see Figure 6 from Mollet et al., 2007, corresponding to Figure 3.6 of this dissertation); actually, by definition, 50% of values are above the median. Conceptually, it seems logic to think that the larger EDR values are the more damaging. Therefore, choosing the median may not be a realistic
representation of the phenomena happening in the flow devices. In this paper, we present our more recent work on the treatment of data from simulations that attempts to remedy some of the just mentioned limitations.

7.3. MATERIALS AND METHODS

7.3.1. Microfluidic channel

The convergent-divergent micro-channel, originally devised by Ma et al. (2002) and improved by Mollet et al. (2007) was used to create the geometry for the simulations. Such micro-channel, designed to exert a well defined and controlled hydrodynamic stress on the cells, is made of a stainless steel plate bored using wire electrical discharge machining, sandwiched between two transparent polycarbonate plates to make it autoclavable. The contraction-expansion design of this device, which will be routinely referred to as the “Torture Chamber” (TC), emphasizes the potentially more lethal extensional flow (Taylor, 1930) over shear forces. The geometrical characteristics of the TC are presented in Figure 7.1. Further details of the TC are presented elsewhere (Mollet et al., 2007).
7.3.2. Laminarity of fluid flow inside the TC

CFD simulations were conducted to model the flow inside the TC at the conditions experimentally considered in previous work (Ma et al., 2002; Mollet et al., 2007). Laminar flow was crucial to get reliable results from the simulations since it has been shown that velocities measured experimentally agree to within 2% with analytical solution for laminar flow in rectangular channels (Meinhart et al., 1999).

The nature of flow in a conduit (laminar, transition or turbulent) depends on the Reynolds number (Re); the Reynolds number is defined as:

$$Re = \frac{L_c \cdot |v| \cdot \rho}{\mu}$$

(Eq. 7.2)

Where $L_c$ is a characteristic length, $|v|$ is the magnitude of the velocity of the fluid, and $\rho$ and $\mu$ represent the density and viscosity of the fluid, respectively. Eq. (7.2) is troublesome as the velocity in every point of the TC is likely to have a different magnitude because of the geometry and the non-slip condition at the solid walls of the channel (see Figure 7.5). Therefore, to calculate a representative value of $Re$, it is a common practice to use an average velocity magnitude, defined by Eq. (7.3).

$$Q = \overline{|v|} \cdot A$$

(Eq. 7.3)
where \( Q \) is the flow rate and \( A \) is the cross-sectional area of the TC, which happens to be a rectangle (rectangle R in Figure 7.1b); the area of such rectangle can be easily calculated from Equation 7.4, since \( b \) (the distance across the opening in the metallic sheet of the TC, which is variable) and \( w \) (the thickness of the stainless steel sheet, which is constant) are perfectly known, as indicated in Figure 7.1b.

\[
A = b \cdot w \quad \text{(Eq. 7.4)}
\]

For rectangular conducts, the characteristic length generally accepted for Eq. (7.2) is the hydraulic diameter, \( D_h \), defined as:

\[
D_h = 4 \cdot \frac{\text{Flow area}}{\text{wetted perimeter}} = 4 \cdot \frac{b \cdot w}{2 \cdot (b + w)} = \frac{2 \cdot b \cdot w}{(b + w)} \quad \text{(Eq. 7.5)}
\]

Replacing equations (7.3), (7.4) and (7.5) into Eq. (7.2), a new expression for the Reynolds number is obtained, given by:

\[
\text{Re} = \frac{L_v \cdot |v| \cdot \rho \cdot \mu}{D_h \cdot \frac{Q}{A}} = \frac{2 \cdot b \cdot w \cdot Q}{(b + w) \cdot b \cdot w \cdot \rho \cdot \mu} = \frac{2 \cdot Q \cdot \rho}{(b + w) \cdot \mu} \quad \text{(Eq. 7.6)}
\]

Every component on the right side of Eq. (7.6) is constant with the exception of \( b \) that decreases as the constriction gets smaller.
In classical fluid mechanics theory, the experimental upper limit of the Reynolds number generally used to determine if a flow is laminar in conventional size pipes with circular transversal section and for fully developed flow, correspond to $Re_{c,cr} = 2100$, where the sub index $c$ indicates circular pipe and $cr$ indicates critical. However, several reports suggest such number can not be applied directly to conducts of different transversal section. Jones (1976) and Obot (1988) have shown data indicating that the use of hydraulic diameter is not the best choice to calculate $Re$ when evaluating the transition from laminar to turbulent flow regime in rectangular channels, because in laminar flow $Re$ also depends on the aspect ratio ($w/b$); instead, they propose a way of “correcting” the Reynolds number calculated for rectangular ducts to fit the same curves as for circular tubes:

$$Re_{n,cr} = \frac{Re_{c,cr}}{\Phi}$$  \hspace{1cm} (Eq. 7.7)

Where $Re_{n,cr}$ is the critical Reynolds number for any non-circular duct (i.e. rectangular) calculated using the hydraulic diameter (i.e., Eq. (7.5)) and $\Phi$ is a geometry function. Jones (1976) presented a theoretically derived function and offered a simpler equation that provides the same results within 2% error:

$$\Phi = \frac{2}{3} + \frac{11}{24} \frac{w}{b} \left(2 - \frac{w}{b}\right)$$  \hspace{1cm} (Eq. 7.8)
Therefore, the critical Reynolds number for a rectangular conduct can be calculated from Equation 7.9 as:

\[
\frac{Re_{n,cr}}{\Phi} = \frac{2100}{\frac{2}{3} + \frac{11}{24} \frac{w}{b} \left(2 - \frac{w}{b}\right)}
\]  

(Eq. 7.9)

The term in the denominator of the right side of Eq. (7.9) is not constant since \( b \) changes in the TC along the fluid path; representative values of \( b \), \( \Phi \) and \( Re_{n,cr} \) are presented in Table 7.1 for two zones of interest: the constant cross-section at the entrance of the microchannel and the throat. Table 7.1 also presents the Reynolds number for the same zones calculated from Eq. (7.6) for the different flow rates tested in this work.

Inspection of Table 7.1 indicates that the Reynolds number in the constant cross-section of the TC at any flow rate is below the critical number and therefore the flow is laminar; however, in the throat \( Re \leq Re_{n,cr} \) only at a flow rate of 10 mL·min\(^{-1}\). To make matters worse, recent studies indicate that the critical Reynolds number is not only dependent on the geometry but also in the channel size: most published reports indicate a reduction in the \( Re_{n,cr} \) necessary for the onset of transitional flow in microchannels. Choi et al. (1991) found \( Re_{n,cr} = 2000 \) for microtubes with \( D_h \) of 53 and 81.2 μm, while \( Re_{n,cr} = 500 \) for \( D_h = 9.7 \) μm. Wang and Peng (1994) evaluated microchannels micromachined on steel plates with hydraulic diameters ranging from 200 to 700 μm and found a monotonic increase in \( Re_{n,cr} \) from ~200 to ~1600 respectively. Sabry (2000) analyzed published data and
proposed a model to explain the reduction in the Re_{n,cr} in microchannels as a result of an increase in the relative influence of the walls’ surface roughness (δ); the model offered by Sabry (2000) takes the form of:

\[ \text{Re}_{n,cr} = \left( \frac{K}{\delta} \right)^2 \frac{D_h^2}{8} \]  
\[
\text{Eq. 7.10}
\]

where K is an experimental constant; Sabry used \((K/\delta) = 0.148 \text{ μm}^{-1}\) to correlate the data from Wang and Peng (1994). Since the conditions for the TC are quite similar to those assayed by the aforementioned authors, we can use Sabry’s model to get:

\[
\left[ \text{Re}_{n,cr} \right]_{\text{constant cross-section}} = \left(0.148 \text{μm}^{-1}\right)^2 \frac{\left(574 \text{ μm}\right)^2}{8} = 902
\]  
\[
\text{Eq. 7.11}
\]

\[
\left[ \text{Re}_{n,cr} \right]_{\text{throat}} = \left(0.148 \text{μm}^{-1}\right)^2 \frac{\left(260 \text{ μm}\right)^2}{8} = 185
\]  
\[
\text{Eq. 7.12}
\]

Based on results from different authors, Sabry (2000) suggests that K should be around 4 to 7 if one assumes δ in the order of 27 μm. In our case, δ was measured in the edge of the stainless steel plate at several points and it was averaged to a value of about 10 μm (see Figure 2b in Mollet et al., 2007, for further details); therefore, it is expected that Re_{n,cr} at the throat shall be somewhat bigger than 185 as the roughness of the microchannel in the TC is significantly lower than the one proposed by the Sabry. Yet, the Reynolds number in the throat for every flow rate is significantly higher than the new
critical values given by Eq. (7.11) and (7.12) and so our assumption of laminar would seem invalid.

At this point, it is important to point out that all previous information regarding critical Reynolds number has been obtained in ducts where fully developed flow profile exists; by contrast, in our case the fluid goes from a completely developed, laminar flow in the constant cross-section zone into the contracting zone where the fluid will need to go through a certain length before obtaining a fully developed profile; because the area changes, so it does the velocity profile; therefore, the whole length of the contracting zone will have an undeveloped profile. Liquid near the wall will remain in laminar regime (boundary layer) but an increasing portion of the downstream flow (measured from the start of the contracting zone) may become turbulent as it is suggested by the Reynolds number. To calculate the distance at which the growth of the boundary layer ($\lambda$ in Figure 7.2) eventually will lead to unstable or turbulent flow at the trailing edge, Gregoriades et al. (2000) argued that at small distances from the entrance to the contracting zone, the boundary layer will grow in the same way as it does along a flat plate at zero incidence. The reasoning behind this supposition lies in the fact that the entrance to the contracting zone resembles closely the flow of a fluid passing a wedge (actually, half of it), as it is sketched in Figure 7.2; in the neighborhood of the leading edge the stream lines of the fluid bend following the shape of the wall and after a short distance are parallel to it, becoming very similar to a flat plate at zero incidence. Actually, the mathematical description of flow over wedges ($0 < \beta < \pi$) is part of a
general class of mathematical solutions named Falkner-Skan flow, of which the flat-plate ($\beta = 0$) is just another particular case (Churchill, 1988).

Gregoriades et al. (2000) used a modified version of the critical Reynolds number for flow along a plate given by:

$$Re_{c,x'} = \frac{x' \cdot \mid \vec{P}_\infty \mid \cdot \rho}{\mu}$$  \hspace{1cm} (Eq. 7.13)

Where $\mid \vec{P}_\infty \mid$ is the magnitude of the velocity of the fluid far away from the plate and $x'$ is the distance from the beginning of the plate in the direction of the $x'$-axis, as indicated in Figure 7.2. Transition from laminar flow using this modified Reynolds number is reported to occur at $Re_{c,x'} > 3.5 \times 10^5$ to $10^6$ depending on the surface roughness and homogeneity of the approaching stream (Schlichting and Gersten; 2000). Since in the contracting zone the velocity of the fluid is different in every point, it results difficult to chose a proper value for $\mid \vec{P}_\infty \mid$. Gregoriades et al. (2000) argued that in the worst case, $\mid \vec{P}_\infty \mid$ could be replaced according to Eq. (7.3) for $Q/A$ at the constriction to obtain:

$$Re_{c,x'} = \frac{x' \cdot \mid \vec{P}_\infty \mid \cdot \rho}{\mu} = \frac{x' \cdot \mid \vec{P} \mid \cdot \rho}{\mu} = \frac{x' \cdot Q \cdot \rho}{\mu \cdot b \cdot w}$$  \hspace{1cm} (Eq. 7.14)

At 90 mL·min$^{-1}$ in the current system, $Re_{c,x'}$ is calculated to be $1.75 \times 10^5$ for $x' = 5.584$ mm (throat) which not only is below the critical Reynolds at which the boundary layer
undergoes the laminar to turbulent transition, but also is quite probably overestimated because the velocity is assumed to be the highest from the beginning of the contraction and because not angle correction was used for $|\vec{V}_\infty|$ as indicated in Figure 7.2. As a result of the previous discussion, the original assumption of laminar flow for all experiments will be regarded as valid henceforth.

7.3.3. Quantification of fluid’s velocity and energy dissipation rates inside the TC by computer fluid dynamics (CFD)

Simulations of the flow through the torture chamber were performed using the commercially available software FLUENT® ver. 6.2 ((FLUENT INC.), in mode 3d (three-dimensional), dp (double-precision), to obtain the velocity vector in every point along the TC. The whole microchannel was simulated. The geometry of the TC and its mesh was developed in GAMBIT® from data obtained from microscopic images and presented elsewhere (Mollet et al., 2007). The same authors developed a mapped grid with about 690,000 nodes (~5 μm at the throat, equivalent to the radius of a single cell); when their work was reproduced, it was found that the highest values of EDR were concentrated in the single row of grid-cells next to the walls of the throat. Since for modeling purposes we needed a much better sharpness of the EDR profile (as it will be explained later on), an ever finer mapped grid of about 934,000 nodes was attempted for the present work; further details of the mesh are presented in Figure 7.3. It must be pointed out that because the system was close to the limit of its computational resources, the meshing had to be improved by several different methods. First, in Table 7.2 it can be
observed that even though every part of the torture chamber increased the grid-cell density, volumes 2 and 3 were the ones with a higher increase in grid density since it is there where the highest EDR was found. Second, the meshing of the edges directly involved in the throat was made with a double successive ratio of 1.11 instead of making it with a ratio 1 (i.e. grid cell size constant); this caused the grid to be more concentrated in the region close to the walls, as it can be observed in the lower right panel of Figure 7.3.

Simulations with FLUENT® were performed using the segregated solver method, which performs iterations to sequentially solve the non-linear equations for continuity and momentum. No energy balance was included as the system is supposed to be isothermal and friction losses are estimated to be very small. The implicit formulation was used to solve the equations for steady state operation. As deducted from previous discussion, laminar flow was used as viscous model. Operating conditions were set as $P_{\text{absolute}} = 1$ atmosphere at the center of the output face of the simulated channel ($x = 30 \text{ mm}, y = 0, z = 1.52 \times 10^{-4} \text{ m}$; see origin of coordinates at Figure 7.1b). Boundary conditions involved non-slip flow for walls and constant velocity at the inlet (i.e. $0.316 \text{ m s}^{-1}$ for $30 \text{ mL min}^{-1}$ as presented in Table 7.1). The schemes for interpolation were as follows: 1st order upwind interpolation scheme for momentum; the standard FLUENT interpolation for pressure and the SIMPLE scheme was used for pressure-velocity coupling. Properties used for the fluid are those for water presented in Table 7.1. Values of the residuals of $10^{-3}$ for continuity and $10^{-4}$ for the $x, y$ and $z$ components of the velocity were used as convergence criteria; in every case, the hardest variable to meet the convergence criterion
was the continuity, being the others usually one to three orders of magnitude lower at the
time of convergence. All the simulations for the present research were performed in a
Pentium®-D CPU running at 3.2 GHz with 2.0 GB RAM and 8.85 GB hard-disk.

FLUENT® solves the equations and provide values for the pressure and velocity vector
at every position inside the TC. With the velocities obtained in the simulation in
FLUENT, EDR can be easily calculated by using Eq. (7.1). A subroutine, created in C
language by Mollet (2004), allows calculating the EDR in every point of the TC from the
velocity data.

Since every single cell experiences a vast range of values of EDR as it moves along the
TC (see Figure 4, Mollet et al., 2007), it is necessary to choose a single EDR value
representative of a particular flow rate for all the cells; one initial way of facing the
problem, proposed by Mollet et al. (2007), involves simulating in FLUENT a group-type
injection of 4000 virtual particles non-interacting with the fluid; the maximum value of
EDR experienced by every particle is then recorded (the maximum is always located at
the throat). The resultant 4000 values are then grouped arbitrarily into ranges to create a
frequency distribution; because of the wide distribution of values of maximum EDR
experienced by the different particles, the median value was chosen as the representative
of every flow rate; the median value corresponds fairly well with the value of EDR with
the highest frequency in the distribution. A graphic representation and a more detailed
discussion about this issue can be found elsewhere (see Figure 6 in Mollet et al., 2007).
In an attempt to improve the statistical management of the EDR data, a refined methodology for choosing a particular value of EDR as representative of a flow rate is presented next. Because of the non-slip condition imposed on the fluid by the walls (both stainless steel and acrylic), the $x$-velocity distribution of the fluid is not flat in a plane perpendicular to the $x$-direction, but it goes from zero at the walls to a maximum in two areas very close to the stainless steel walls and then there is a valley in the middle of the maximums with a somewhat lower velocity as is shown in the left panel of Figure 7.5. It is expected that fluid will flow faster in the zones with higher velocity; therefore, on any specific time period, much more fluid will flow through the central zone and the peaks than through the zones adjacent to the walls. Because the cells are simulated as following the fluid pathway (which they do very closely because their small size and the very small difference in density), more cells will cross the peaks and the central valley per unit time than the zones close to walls; however, it is in the zones near to the walls where higher EDR are located, as is shown in the right panel of Figure 7.5 (observe the logarithmic scale in such panel). Consequently, a relative lower amount of cells will “see” higher EDR compared to a homogeneous, single-shot distribution like the 4000 particle injection method proposed by Mollet et al. (2007). Furthermore, the just mentioned authors show in their Figure 4 (corresponding to the Figure 3.4 of the present dissertation) that it is in the throat where the highest EDR are found. Therefore, attempting any correction in the frequency cells experience EDR must be performed at the throat where the cells are more likely to suffer any effect as a result of hydrodynamic conditions.
To perform a proper weighing of the distribution of EDR the cells actually perceive, an “equation” for both the velocity distribution and the EDR distribution in every point of the particular plane of interest (throat) must be found. Because of the difficulty in finding an analytical expression (as it can be seen in Figure 7.5, EDR and velocity are a very complex function of both $y$ and $z$), the results from FLUENT were used instead. FLUENT is able to either assign a value to the center or to the nodes of every grid-cell when performing the simulation; cell-centered values were exported to Excel® and used to perform further mathematical analysis. This procedure converted the apparently continuous “analogous” distributions of EDR and velocity into “digital” ones where there is a discontinuity between the values from one cell to the other, as it can be appreciated in Figure 7.6.

Because of the grid size and conformation, 3600 rectangular cells and corresponding 3600 sets of values ($x$-, $y$- and $z$-coordinates, $V_x$ and EDR) were obtained from FLUENT® for the throat; with these sets, a mass balance in each grid-cell can be performed; as the density is constant along the plane, the mass balance reduces to:

$$q_i = V_x \times Area_{\perp i} \quad \text{(Eq. 7.15)}$$

Where $Area_{\perp i}$ is the area perpendicular to the $x$-axis and $q_i$ is the volumetric flow rate of fluid that will cross the $i$-th grid-cell located at coordinates $x_i, y_i, z_i$ (with $i = 1 \rightarrow 3600$).
In a homogeneous distribution of cells (which is assumed based on the fact cells are coming from a fairly well stirred bioreactor, small enough to be modeled as perfectly mixed), the number of cells crossing a certain area in the liquid \( N_i \) is directly proportional to the fluid flow rate \( q_i \) and the cell concentration \( C \) in cell·mL\(^{-1}\):

\[
N_i = q_i \cdot C
\]  
(Eq. 7.16)

As it was said before, for every position in the throat there is a certain value of EDR that is assumed to be constant in the whole area of each grid-cell (that is the reason of the “digitization” procedure). Therefore the fraction of cells \( \phi_i \) crossing the \( i \)-th grid-cell at steady state and experiencing a particular \( EDR_i \) is given by:

\[
\phi_i = \frac{N_i}{\sum N_i} = \frac{q_i \times C}{\sum q_i \times C} = \frac{q_i}{q_i} = \frac{V_{xi} \times Area_{zi}}{\sum V_{xi} \times Area_{zi}}
\]  
(Eq. 7.17)

Weighing of data was performed as follows: first, \( x \), \( y \)- and \( z \)-coordinates, \( V_x \) and EDR data from FLUENT for the throat plane was exported as columns into EXCEL® and then area of every cell was calculated based on the coordinates and the geometric characteristics of the mesh. Next, data in all columns were ordered from lower to higher EDR; after that, the value of \( x \)-velocity for every row was multiplied by the correspondent area of the grid cell to calculate \( q_i \) according to Eq. (7.15).
Next, the value of $\phi$ was calculated for every row (grid-cell) based on Eq. (7.17) and a new function, named (% damage), was calculated as follows:

$$
(\% \text{ damage})_j = \left(1 - \sum_{k=1}^{j} \phi_k\right) \times 100\% \tag{Eq. 7.18}
$$

where $j$ (1→3600) corresponds to the row under analysis. The meaning of (% damage) should be explained: for every row ($j$), its particular value of EDR$_j$ is higher than the previous row and lower than the next one because the rows were ordered by EDR value; then $\sum_{k=1}^{j} \phi_k$ is the fraction of cells passing through the throat of the torture chamber that are experiencing values from zero to EDR$_j$. (i.e., values $\leq$ EDR$_j$). Therefore, $\left(1 - \sum_{k=1}^{j} \phi_k\right)$ is the fraction of cells that will experience values of EDR $\geq$ EDR$_j$. Now, if one assumes that all cells are identically resistant to hydrodynamic stress (a somewhat gross assumption that will be discussed later on) and that resistance is precisely equal to EDR$_j$, then the fraction of cells experiencing values of EDR $\geq$ EDR$_j$ will die by necrosis. Such fraction, expressed as percentage is precisely what is presented in Equation 7.18. The (% damage) for the five flow rates used in this work are presented in Figure 7.7.

As mentioned previously, a finer meshing compared with the work of Mollet et al. (2007) was employed in this work; such finer mesh was necessary to be able to fit adequately a regression model for the (% damage). The fitting was performed using the commercial
software Tablecurve® (Jandel Scientific). The equation employed for fitting and the coefficients of the equation for the different flow rates tested are presented in Table 7.3.

As it can be observed from Table 7.3 ($r^2$) and Figure 7.7 (fitted model for 10 mL·min$^{-1}$), the fitting for the equation is satisfactory. Once an equation is obtained for the (% damage), the next step is quite simple: a value of EDR at which cells are sensitive (EDR$_s$) is arbitrarily chosen and the (% damage) is calculated for every flow rate; as an example, in Figure 7.7 the line EDR$_s$ = $1\times10^9$ W·m$^{-3}$ was plotted and the calculated values are circled for every flow rate.

7.4. RESULTS AND DISCUSSION

7.4.1. Grid independence and comparison with previous results from simulation

For comparison purposes, examples of the velocity profiles obtained in both studies are presented in Figure 7.4; as it can be appreciated there, the velocity profiles are quite similar and an increase in the number of elements at the throat (volume 2) of 125% (from 1600 to 3600) resulted in a difference of only 2 to 3% on the maximum velocity at the center of the planes shown.

However, although the results in terms of velocity from the FLUENT simulation in the present study matched well the results obtained by Mollet et al. (2007), the results were quite different in terms of the profiles of EDR, specially at the throat. Figure 7.10
presents a comparison of such profiles from a simulation in the same conditions for both the same mesh as Mollet et al. (2007) and the finer mesh employed in this work. Observe that not only the profile is different, but also the values for the present study are one order of magnitude higher than theirs. Although they performed a grid-independence analysis, it was based on the velocity profiles that, as it was mentioned before, it is very similar to ours; however, the EDR calculation is based on velocity gradients, that were importantly affected by considering a finer mesh.

Mollet (2004) mentioned that reducing the size of the grid-cells would create higher and higher values of EDR in the corner at the throat of the torture chamber and he disregarded this phenomena as an artifact of the program because at the corner the derivatives are not continuous; he argued that for finer meshes no cell would be able to go that close to the wall. However, observe that the levels of EDR go higher and higher not only close to the wall but also in the center of the throat. To establish a comparison with his results, a surface injection of virtual particles was simulated according with his methodology. The results are shown in Table 7.4. Not only the median maximum EDR (EDR_{median,max}) is displaced towards higher values but this displacement is not homogeneous and for flow rates equal or higher than 30 mL·min\(^{-1}\), the EDR_{median,max} becomes essentially a constant, practically eliminating our ability to predict any damage in the torture chamber using this parameter.

The logical question is: how can be proven that grid-independence has finally been reached so the same phenomena will not happen again? Since for this work we are
already very close to the limit of the resources of our computer, trying to increase the mesh even further was not feasible. Given the fast development of technology, the validity of our results will be probably investigated in a posterior work.

7.4.2. New methodology for assessing the results of EDR from Fluent results

The calculated (\% damage) for several different values of EDR$_s$ is presented in the left panel of Figure 7.8, while experimental results for several different cell types obtained in our laboratory is presented in the right panel of the same Figure. Observe that the $x$-axis in such Figure is the median maximum EDR as suggested by Mollet et al. (2007); this is done for comparison purposes with previous results. As it can be seen, the theoretical model predicts reasonably well the observed trend of the breakthrough curves for a wide variety of cells; it also offers an explanation why it seems to exist a “saturation” of the curve for cells highly sensitive (i.e., THP1). However, the agreement is not perfect. Figure 7.9 presents combined from previous reports (Ma et al. 2002; Mollet et al., Submitted); as it can be seen, while the curve for THP1, 1$^{st}$ assay, is fairly well modeled by the theoretical development just presented, some experimental curves like those for THP1, 2$^{nd}$ assay and MCF-7 cross the lines of different “iso-EDR$_s$”; this behavior should not be considered unsatisfactory as it was assumed that all cells have the same resistance to hydrodynamic forces and this is hardly true: since there are cells in different stages of the cell cycle and with different ages and history, it is very likely the resistance will be within a range instead of being a single value. As an example, consider the case for THP1 cells; in the 1$^{st}$ assay cells behave well inside the curves for a resistance to EDR$_s$ in the
order of $1.0 \text{ to } 1.5 \times 10^9 \text{ W} \cdot \text{m}^{-3}$. In the second assay, a range as narrow as the previous example cannot be assigned, but a range within $2.5 \times 10^8 \text{ W} \cdot \text{m}^{-3}$ and $2.5 \times 10^9 \text{ W} \cdot \text{m}^{-3}$ can be proposed, as shown in the right panel of Figure 7.9.

One particular aspect that should not be overlooked is that the numbers obtained for cell’s sensitivity are one and two orders of magnitude higher than in the previous methodology proposed by Mollet et al. (2007); although they still are around the same range as those found for bubble bursting, they are now in the higher end of such range. It also should be mentioned that the ranges of EDR proposed in the literature for bubble break-up are based on simulations performed several years ago with computing power significantly smaller than what can be used at present; therefore, it would be worth to check those results as well.

Besides the ability to predict the overall behavior of cells inside the torture chamber from a standpoint of just theoretical balances and considerations, the proposed model has the advantage over the previous methodology from Mollet et al. (2007) of allowing us to assign a single value (or at least a narrow range) to the cells’ sensitivity instead of using an arbitrarily chosen statistic (median).

7.5. CONCLUSIONS

The proposed methodology for analyzing the data from FLUENT seems to predict reasonably well the general trend of the break-through curves of animal cell damage
observed in the TC. However, the threshold value of EDR above which cells undergo necrosis seems to be much higher than previously reported which would support the use of a much higher agitation intensity in industrial bioreactors and downstream devices.

7.6. FUTURE WORK

Work is under development to apply the proposed methodology to other flow devices such as FACS in order to evaluate its ability to predict the cell damage experimentally determined in our lab. Similarly, previous results from simulations of bubble-break up (i.e., Boulton-Stone and Blake, 1993; García Briones et al., 1994) will be checked to see the validity of their computational results.
7.7. REFERENCES


Figure 7.1. Torture Chamber (TC). (a) Photograph (b) Scheme detailing the dimensions (lengths in mm).
Figure 7.2. Sketch of the boundary layer growth modeling along the edges of the constriction in the Torture Chamber (TC).
Figure 7.3. Torture Chamber Mesh details

366
Figure 7.4. Results of velocity profiles for the region $x = 15$ mm (throat) and $y = 0$ (centerline of the plane) after simulation of the torture chamber at two different flow rates using a standard (Mollet et al., in press) and a finer (present study) meshing.
Figure 7.5. Velocity and EDR profile in plane $x = 15$ mm (Throat) for a flow rate $= 90$ mL·min$^{-1}$. Position of the plane (gray-color top or bottom plane in $y$-$z$ plane) is indicated by the red zone in the small TC sketch.
Figure 7.6. Velocity “digitization” of the grid in plane $x = 15$ mm (Throat). Flow rate = 90 mL·min$^{-1}$. 
Figure 7.7. % Damage as a function of EDR in the throat calculated for every flow rate tested in this study.
Figure 7.8. Comparison between the predictions of the model and the experimental results for single abuse experiments
Figure 7.9. Comparison between the predictions of the model and the experimental results for single abuse experiments
Figure 7.10. Comparison of the profiles of EDR in plane $x = 15$ mm (Throat) for a flow rate $= 90$ mL·min$^{-1}$. Position of the plane (gray-color top or bottom plane in y-z plane) is indicated by the red zone in the small TC sketch. Left panel: Mollet et al. (2007). Right panel: this study.
<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Symbol</th>
<th>Value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thickness of the stainless steel sheet</td>
<td>$w$</td>
<td>$304 , \mu m = 3.04 \times 10^{-4} , m$</td>
</tr>
<tr>
<td>Critical Reynolds number for circular pipes</td>
<td>$Re_{e,cr}$</td>
<td>$2.1 \times 10^3$</td>
</tr>
</tbody>
</table>

**Constant cross-section**

| Channel spacing                                  | $b$    | $5.2 \, mm = 5.2 \times 10^{-3} \, m$           |
| Aspect ratio                                      | $w/b$  | $5.85 \times 10^{-2}$                           |
| Hydraulic diameter                                | $D_h$  | $574 \, \mu m = 5.74 \times 10^{-4} \, m$       |
| Geometry function                                 | $\Phi$ | $0.72$                                          |
| Classic critical Reynolds number                  | $Re_{n,cr}$ | $2.92 \times 10^3$                            |
| Microfluidic critical Reynolds number             | $Re_{n,cr}$ | $9.02 \times 10^2$                            |

**Throat**

| Throat spacing                                    | $b$    | $227 \, \mu m = 2.27 \times 10^{-4} \, m$       |
| Aspect ratio                                      | $w/b$  | $0.747$                                         |
| Hydraulic diameter                                | $D_h$  | $260 \, \mu m = 2.60 \times 10^{-4} \, m$       |
| Geometry function                                 | $\Phi$ | $1.10$                                          |
| Classic critical Reynolds number                  | $Re_{n,cr}$ | $1.96 \times 10^3$                            |
| Microfluidic critical Reynolds number             | $Re_{n,cr}$ | $1.85 \times 10^2$                            |

**Fluid**

| Chemical identity                                 | Water                                          |
| Working temperature                               | $\theta$ | $37^\circ C$                                    |
| Density (at $\theta$)                            | $\rho$  | $993.3 \, Kg/m^3$                               |
| Viscosity (at $\theta$)                          | $\mu$   | $0.6915 \, cP = 0.6915 \times 10^{-3} \, Pa\cdot s$ |

<table>
<thead>
<tr>
<th>Reynolds number (Re)</th>
<th>Flow rate, Q $[\text{mL \cdot min}^{-1}]$</th>
<th>Average velocity at the inlet $[\text{m} \cdot \text{s}^{-1}]$</th>
<th>Re at inlet [-]</th>
<th>Average velocity at the throat $[\text{m} \cdot \text{s}^{-1}]$</th>
<th>Re at the throat [-]</th>
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<tr>
<td></td>
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<td>0.105</td>
<td>87.0</td>
<td>2.42</td>
<td>901.7</td>
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<td></td>
<td>30</td>
<td>0.316</td>
<td>261.0</td>
<td>7.25</td>
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<td></td>
<td>50</td>
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<td>12.08</td>
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<td></td>
<td>90</td>
<td>0.949</td>
<td>782.9</td>
<td>21.73</td>
<td>8115</td>
</tr>
</tbody>
</table>

**Table 7.1.** Table of physical and geometric characteristics used for the calculation of EDR in the microfluidic device.
<table>
<thead>
<tr>
<th>Geometric element (1)</th>
<th>Standard mesh (Mollet et al., 2007)</th>
<th>Finer mesh (this study)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Total nodes</td>
<td>Total elements</td>
</tr>
<tr>
<td>Volume 1</td>
<td>169,781</td>
<td>160,000</td>
</tr>
<tr>
<td>Volume 2</td>
<td>169,781</td>
<td>160,000</td>
</tr>
<tr>
<td>Volume 3</td>
<td>169,781</td>
<td>160,000</td>
</tr>
<tr>
<td>Volume 4</td>
<td>102,541</td>
<td>96,000</td>
</tr>
</tbody>
</table>

(1) see Figure 6.3 for description of volumes.

**Table 7.2.** Mesh summary for the FLUENT® simulations of the torture chamber.
% Damage = \( a + b \cdot x + c \cdot x^{2.5} + d \cdot x^3 + e \cdot (\text{Ln}(x))^2 \)

\( x = \text{EDR [W/m}^3] \)

<table>
<thead>
<tr>
<th>Flow rate (mL·min(^{-1}))</th>
<th>( a )</th>
<th>( b )</th>
<th>( c )</th>
<th>( d )</th>
<th>( e )</th>
<th>( r^2 )</th>
</tr>
</thead>
<tbody>
<tr>
<td>10</td>
<td>1.742E+02</td>
<td>-6.292E-08</td>
<td>8.640E-20</td>
<td>-5.465E-24</td>
<td>-4.253E-01</td>
<td>0.99825</td>
</tr>
<tr>
<td>30</td>
<td>1.898E+02</td>
<td>-5.204E-09</td>
<td>2.570E-22</td>
<td>-4.566E-27</td>
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<tr>
<td>50</td>
<td>2.018E+02</td>
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<td>1.260E-23</td>
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<tr>
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<td>90</td>
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<td>-3.774E-01</td>
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**Table 7.3.** Parameters of the fitted model for (% Damage) in the torture chamber.
<table>
<thead>
<tr>
<th>Flow rate [mL·min⁻¹]</th>
<th>Median maximum EDR [W·m⁻³]</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mollet et al. (2007)</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td>2.87×10⁵</td>
<td></td>
</tr>
<tr>
<td>30</td>
<td>2.27×10⁶</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td>6.45×10⁶</td>
<td></td>
</tr>
<tr>
<td>70</td>
<td>2.06×10⁷</td>
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</tr>
<tr>
<td>90</td>
<td>1.09×10⁸</td>
<td></td>
</tr>
<tr>
<td></td>
<td>This study</td>
<td></td>
</tr>
<tr>
<td>1.27×10⁸</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.06×10⁹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.21×10⁹</td>
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<td></td>
</tr>
<tr>
<td>1.42×10⁹</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1.48×10⁹</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

**Table 7.4.** Comparison between the median maximum EDR from Mollet *et al.* (2007) and this study.
CHAPTER 8

CONCLUSIONS AND RECOMMENDATIONS

8.1. CONCLUSIONS

The overall objective of this research was to study the effect of hydrodynamic forces on animal cells of research, medical and industrial interest. To accomplish such goal, this study targeted the development and use of a methodology capable of subjecting cells to single and multiple exposures of controlled hydrodynamic stress in a simple, accurate, reproducible, and aseptic manner. A convergent-divergent micro-channel, routinely named as torture chamber (TC), developed by Ma et al. (2002) and improved by Mollet (2004) has proven to be reliable, relatively easy to build and simple to operate, clean and set-up. Using this device, Ma et al. (2002) generated reproducible curves of the percentage of necrotic (lysed) cells as a function of EDR for a wide variety of cells. Such curves, named as breakthrough curves, suggest that the level of EDR at which significant cell lysis can be detected is within a narrow range independent of the cell line.

By using the TC, we (Dr. Mike Mollet and I) determined the amount of hydrodynamic abuse cells could withstand before undergoing necrosis or apoptosis. To do that, we used
two Chinese Hamster Ovary (CHO) cell lines: wild-type (WT) CHO-K1 and CHO-K1 transfectected with the anti-apoptotic gene Bcl2. Both cell lines were cultured in two different sets of conditions: in suspension with serum-free media and attached to tissue culture flasks in media containing 10% fetal bovine serum (FBS). WT-CHO-K1 cells growing attached to T-flasks and in serum-containing media, displayed average apoptosis levels slightly higher (~ 5–10%) than the control when pumped once through the TC at the highest EDR (flow rate) tested, compared to cells growing suspended in serum-free medium (~2%) under the same treatment. However, the variability of different replicates did not allow concluding there is a significant difference in the levels of apoptosis as a result of culture conditions. An opposite behavior was observed in terms of necrosis, where at the highest level of EDR tested, up to 30% of cell lysis, measured as LDH released into the culture medium, was observed for WT-CHO-K1 growing suspended in serum-free media compared to a maximum of 10% of cell necrosis for cell attached to T-flasks and grown in serum-containing media at the same conditions. No significant differences between the levels of apoptosis or necrosis were observed for CHO-bcl2 cells growing either suspended or attached. As a conclusion, single insults, even at high levels of EDR, are not effective to elicit apoptosis on the evaluated cell lines, independently of the culture conditions.

Fluorescent-activated cell sorters (FACS) are analytical devices with a wide application in scientific and industrial research as well as for clinical testing. In FACS devices, a laser beam of a precise wavelength is directed onto a hydrodynamically focused stream of fluid; hydrofocusing of the fluid generates strong hydrodynamic stresses that might
compromise cell’s viability. In this study we (Dr. Mike Mollet, Mrs. Claudia Berdugo and I) simulated the hydrodynamic forces in the nozzle of a BD FACS Vantage using a computational fluid mechanics package (FLUENT®) based on experimentally measured flow rates typically used in sorting experiments. Assuming that the flow through the nozzle is quantitatively similar to that of the torture chamber, the viability of two cell lines, CHO-K1 and THP-1 (a human monocytic-like cell) after sorting in the FACS Vantage was determined and compared to the results of subjecting the same cell lines to a single exposure of high hydrodynamic forces in the TC. The results, overall, indicate that the FACS Vantage causes significant levels of cell death through necrosis, but no significant levels of apoptosis could be detected in the cells investigated in this study. This is consistent with the report by Mollet et al. (2007) that showed that while a low level of apoptosis can possibly occur in CHO cells subjected to comparable levels of EDR in the torture chamber, the primary mode of death is complete cell lysis and necrosis.

Following the suggestion by Dr. Mollet of evaluating the effect of multiple exposures of the same population of cells through the TC to ascertain the possible cumulative effect of hydrodynamic forces on cell’s behavior, I undertook the task of building a system allowing to keep cells flowing continuously through a tubing line, throughout the TC and then back into the bioreactor. This was achieved thanks to an electronically controlled valve system of my own design, connected to a commercially available syringe pump, it was possible to accomplish the objective of keeping the cells flowing continuously through a tubing line throughout the TC and then back into the bioreactor. By changing
the plates from poly-methyl methacrylate (PMMA) to polycarbonate I avoided the deformation and degradation commonly found in previous designs at the moment of autoclaving the system; allowing for repeated sterilization and prolonged use. This setup allowed to measure cell’s susceptibility to cyclic, moderately high levels of hydrodynamic forces for as long as 11 days without getting contamination.

The hydrodynamic sensitivity of an industrial, monoclonal antibody producing, GS-CHO cell line to energy dissipation was quantified using the TC. Single passage test of samples taken at different ages of the fed-batch process (i.e. exponential growth, stationary phase and death phase) revealed that the threshold energy dissipation rate (calculated as median maximum EDR) for cell necrosis at different stages of growth is very similar, around $2.87 \times 10^6 \text{ W} \cdot \text{m}^{-3}$, and does not increase in the death phase as one may expect. This threshold is quite similar to those of other industrial and research cell lines tested before (i.e., CHO-K1, hybridoma, PER.C6, etc.).

By contrast, the overall results from the chronic exposition of industrial cell lines to moderate levels of EDR indicate that those cells seem to be much more robust than research or medical cell lines. Three different levels of EDR ($2.87 \times 10^5$, $2.27 \times 10^6$ and $6.45 \times 10^6 \text{ W} \cdot \text{m}^{-3}$), were tested in the TC in order to determine the lowest energy dissipation rate triggering any cell metabolism change or death. Although repetitive exposure was applied for 11 days, an increase in necrosis compared to control only could be detected at the highest tested EDR level ($6.45 \times 10^6 \text{ W} \cdot \text{m}^{-3}$). However, this fact did not significantly affect the apparent cell growth rate and productivity; actually, compared to
the control bioreactor, cells exposed to $6.45 \times 10^6$ W·m$^{-3}$ reached a slightly higher peak cell density and gave a similar product titer.

Most of sub-lethal effects monitored were not affected by all three level of energy dissipation. Cell growth, viability, productivity, and carbon metabolism were similar between all three sets of test bioreactors and control bioreactors. Most product quality attributes, including aggregation, clipping, charge profile, and methionine oxidation, were similar too. The only significant differences were observed for apoptosis and glycosylation. In the same way as necrosis, an apoptosis increase compared to the control reactor was observed only for $6.45 \times 10^6$ W·m$^{-3}$. Interestingly, a shift in the antibody’s glycosylation pattern was detected at both $2.27 \times 10^6$ and $6.45 \times 10^6$ W·m$^{-3}$, EDR values at which cells produced more G1 and G2, but less G0 glycoform. Considering that the glycosylation pattern may affect the biological activity and effectiveness as a drug of an antibody, this effect results important and deserves further exploration.

A comparison of the levels of EDR affecting cells in the TC and the energy dissipation rate that could be generated by impellers in large-scale bioreactors suggests that the operation of current bioreactors is safe to GS-CHO and possibly to most CHO cells. Moreover, agitation in most large-scale bioreactors might be too conservative in fear of “shear damage” to achieve optimal mixing. This study clearly indicates that more intensive agitation should be evaluated in large-scale bioreactors in favor of better mixing.
Finally, in this work I also developed an improved methodology for the analysis of the data delivered by FLUENT, that describes the behavior of the cells and the fluid inside the TC more realistically than the previous methodology, developed by Dr. Mollet, of injecting a set of virtual particles during the simulations. Such improvement was based on considerations of momentum balance and the sound assumption of a physical limit of stress beyond which the cell membrane loses its integrity and cell dies. The proposed methodology allowed predicting the overall trend of observed breakthrough curves through the TC; while the agreement with the experimental results is not perfect, recognizing that the main assumption of every cell behaving exactly the same way and having precisely the same sensitivity to hydrodynamical damage is far from realistic, might explain the difference. The new methodology proposes a range of lethal EDR independent of the statistical treatment given to the data, an improvement that solves the *a priori* selection of a particular statistic to describe the EDR intensity.

In solving the computational challenges to finish off the just mentioned improved methodology for data treatment, a comparison of the results obtained by Mollet (2004) and those given by FLUENT for a finer mesh allows to conclude that although the difference in the magnitude of the velocities computed by FLUENT in both cases was less than ~3%, the differences in the levels of EDR calculated by the software were as large as one order of magnitude. Therefore, I must conclude grid-independence was not satisfactorily achieved in the work by Mollet (2004).
8.2. RECOMMENDATIONS

Even though the torture chamber design and manufacture have been improved in both the work by Dr. Mike Mollet and the present one, attention to several details could enhance the reliability and easiness of use of the TC. Although the plastic degradation with repeated use and, specially, autoclaving of the TC has been practically eliminated by use of polycarbonate plates, it is suggested the use of ½ inches polycarbonate plates instead of the 1”. The reason lies in that 1” plates are made of a series of at least 3 plates of lower thickness, bonded together; at the moment of autoclaving the system, the plates remain intact, but the adhesive tends to flow and create bubbles, not only making the visual aspect of the TC very unattractive but also creating risk of contamination. Machining of a ½ inches plate is more difficult but solves this problem.

For the present work the syringe pump was used vertically. In doing that, one can solve several problems: first, any bubble in the tubing is trapped before reaching the throat of the TC, interfering with the flow pattern; second, the broth keeps constantly lubricated the rubber plunger head, extending its working life; third, leaving some air between the rubber plunger head and the liquid level in the syringe ensures no cell damage by shearing by the syringe movement; fourth, since cells tend to settle, they settle on the bottom of the barrel, being pulled by the broth again into the liquid as opposed to remaining (and accumulating) in the syringe. Unfortunately, the pump used for this study is designed to work horizontally; when operated vertically for long periods, it tends to stall, stopping the flow and creating problems. A new, vertical syringe pump is
recommended to avoid this problem, or the use of syringes with the tip off-centered, so
the bottom of the barrel is at the same height as the tip, reducing cell accumulation in the
syringe.

Especial attention should be paid to finding or making a syringe made of Teflon or some
other autoclavable material that does not deteriorate by friction as quickly as rubber and
that can be autoclaved repeatedly in a simple way. Rubber plunger heads wear out after a
few hours of continuous operation, forcing to constant replacement and creating the risk
of TC plugging by a fragment of rubber at the throat; this last problem was solved
through the use of stainless steel 100 μm mesh filters before and after the TC, but the
constant need of replacement creates a risk of contamination and requires constant
attention, even during the night. Syringes with Teflon-plunger heads were evaluated but
they required independent autoclaving of the barrel (glass) and the plunger, causing
plunger-head deformation and limited performance. Stainless steel syringes with o-rings
were tested and discarded as their movement required a lubricant that could affect cells
behavior in an unexpected way.

Although the TC seems to be able to predict the necrotic effects found in pass-through
devices such as FACS, it would be interesting to check if the results obtained with the
chronic exposure setup can predict the behavior obtained through increasing EDR by
means of increasing stirring intensity in a bioreactor. Stirring intensity can be achieved
via increasing stirring speed, increasing impeller diameter or changing the geometry of
the impeller. EDR values for several impeller geometries as a function of diameter and
stirring speed has been measured, correlated and reported in literature; therefore, having
the precaution of avoiding superficial gas entrance (by means of, for example, having a
taller reactor, a lower placed impeller, a surface baffle system or a combination of them
all), in principle should be possible to evaluate if at similar EDR values, the turbulent,
agitated reactor produce the same responses on cells as the laminar, highly extensional
TC.

Even though chronic exposure to several different levels of EDR was evaluated, changing
flow rate changed simultaneously the intensity of EDR and the frequency cells were
exposed to it. A series of modified versions of the TC with wider throat dimensions could
be used to easily keep constant the EDR intensity but changing the frequency cells are
exposed to it. An alternative to simulate the circulation time cells experience in real
reactors would involve to use a set of two reactors, pumping the whole broth through the
TC from one reactor to the next one; in such a way, it would be possible to know
precisely the number of times cells have been exposed to a particular level of EDR and
the frequency could be changed at will by just changing the flow direction of the pump.

Since it has been shown that antibody’s post-translational modifications could result from
continuous exposition to moderate levels of EDR, it would be interesting to further
explore such effects. If it is desired to avoid any antibody modification because of safety
concerns, determinations could be made at lower values of EDR to determine what is the
minimum threshold level effecting such responses; since most large-scale bioreactors
seem to be under-mixed as a result of fear to “shear damage”, finding the minimum level
at which product modification can be detected could be useful to propose a much better mixing strategy, resulting in improved productivity, product quality.

If higher levels of post-translational modifications (particularly higher levels of glycosylation) are desired, it is recommended to explore higher levels of EDR to determine if the effect is monotonic (i.e. the higher the EDR the higher the glycosylation shift) and what is the maximum threshold before compromising cell’s viability.

Finally, the use of the new methodology proposed for data analysis of the results from FLUENT is suggested to analyze necrosis results from FACS; it might produce a better agreement with the experimental results from the TC for the same cell lines tested.
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