1. Introduction and Aim of the Work

Industrial biotechnology has grown significantly in the last three decades with an enormous increase in the number of the available licensed products. These products include vaccines, recombinant therapeutic and diagnostic proteins as well as monoclonal antibodies. More than half of these approved products, which are to be applied for human use, are commercially produced by mammalian cell systems. Mammalian cells have the advantage of precisely producing recombinant proteins with the same authentic protein folding structure as those produced in human bodies.

Developments achieved in the different cultivation systems have accelerated the progress of mammalian cell culture. Generally, large-scale commercial mammalian cell cultivations are performed in batch, fed-batch and perfusion processes. Perfusion systems involve cell retention in the bioreactor with continuous exchange of the medium and simultaneous removal of the byproducts. Perfusion cultivations have the advantage of continuously removing toxic metabolites and byproducts from the medium, and thus providing the growing cells with the optimal growth conditions. Additionally, the desired product is continuously removed from the bioreactor preventing product inhibition or instability problems.

The application of perfusion cultivation for commercial mammalian cell production processes depends on the presence of a suitable and an efficient simple-operating cell retention device. Several cell-separating devices are used for retaining cells in the bioreactor during perfusion cultures. These devices include centrifuges, settlers, ultrasonic separators, spin filters and hollow fiber membranes. Hydrocyclones use the same separation principle as centrifuges; however, they are characterized by the absence of movable parts, where the vortex motion is performed by the energy of the fluid itself. Moreover, they do not require high precision components, which are expensive to manufacture and operate. Nevertheless, hydrocyclones have been scarcely applied in mammalian cell perfusion cultures.

The goal of the present work was to investigate the possibility and the potential of hydrocyclone application for the continuous cultivation of mammalian cells in perfusion bioreactors. In order to achieve this goal, the work was designed to focus on the following points:

- General characterization of the physical properties influencing hydrocyclone operation. These properties include the dependency of hydrocyclone performance on

the applied pressure as well as the working conditions and the geometry of the hydrocyclone.

- Investigation of the effect of the pressure on cell viability and the separation efficiency of the hydrocyclone.
- The potential application of hydrocyclone for cell separation in continuous perfusion cultures using different mammalian cell lines.
- Investigation of the effect of different pressures generated by the hydrocyclone as well as different hydrocyclone geometries on the cultivation and the productivity of mammalian cells.
- Finally, the performance of the hydrocyclone as a cell-separating device in mammalian cell perfusion culture will be evaluated in comparison with another conventionally applied membrane perfusion system.

2. Review of Literature

2.1. History of animal cell culture

The history of animal cell culture began in the last few years of the nineteenth century, where many preliminary experiments were done to maintain pieces of tissues in plasma or ascitic fluids over several days or even weeks. This work was challenged by two obstacles, firstly, the requirement of adequate nutritional media and secondly, the lack of high working aseptic conditions. In 1898, Ljunggren showed that he could keep a human skin tissue alive in ascitic fluids so that it might be successfully reimplanted. In 1907, Harrison reported the maintenance and growth of nerve cells over a period of up to 30 days. His experiments showed that normal cell functions could continue *in vitro*. These developments contributed to the first breakthrough in animal cell culture (Spier, 2000; Marx, 2003).

During the next four decades, the progress of animal cell cultivation was limited due to high sterility controls required. The discovery of antibiotics in the late 1940s that was accompanied with the development of sterility techniques was a major breakthrough towards cell cultivation. The addition of antibiotics facilitated handling of complex undefined culture media (Kretzmer, 2002). During the following decade, great progress was made towards mass cultivation of animal cells and production processes.

The great milestone towards animal cell culture was the isolation of HeLa cells. In 1953, Mary Kubicek isolated HeLa cells from the cervical cancer of Henrietta Lacks (Spier, 2000). This cell line provided investigators with a suitable animal cell culture that grew reliably and on which a diverse series of experiments could be effectively performed. At the end of the 1940s, Enders and his coworkers showed that they could grow poliovirus *in vitro* in HeLa cells, which were then cultivated for the production of vaccines (Kretzmer, 2002).

In 1955, Eagle reported a chemically defined medium known as EMEM (Eagle's minimum essential medium) that could replace the biological fluids for cell cultivation. However, the use of this medium was limited by the addition of undefined blood serum (Eagle, 1955).

The period between the late 1940s and 1975 is considered to be the golden age of virus vaccine development that brought about effective cell culture-based vaccines for human diseases such as mumps, measles and rubella, as well as veterinary vaccines, e.g. foot-and-mouth disease. The last three decades of the twentieth century represent the existing phase of

monoclonal antibody production from hybridoma cells. This period was rapidly followed by the cultivation of genetically engineered animal cells to produce specific biopharmaceuticals or growth factors (Spier, 2000).

2.2. Industrial biotechnology and animal cell culture

The approach of animal cell culture technology was first introduced to industrial biotechnology by the production of Salk poliovirus vaccine in primary monkey kidney cells five decades ago (Griffiths, 2000). Since these primary cells are anchorage-dependent, the production was a multiple-unit process. The major breakthrough to industrial application of animal cells occurred in 1962, when Capstick and his coworkers succeeded to culture BHK (baby hamster kidney) cells in suspension as microorganisms. This, together with the establishment of permanent cell lines, drove the development of large-scale processes in industry. Few years later, the use of safer cell lines (WI-38 and MRC-5) instead of the primary monkey kidney cells increased the number of licensed human vaccines: measles (1963), rabies (1964), mumps and rubella (1969). All these processes were batch type since cells were grown to high density and then infected with the virus. After virus propagation, cells were of no further interest and the virus was harvested (Kretzmer, 2002).

In the late 1970s, the progress in gene manipulation and cell fusion technology has enabled the production of large amounts of physiologically active proteins by *in vitro* cell cultivation. In the technology of gene recombination, microorganisms such as *Escherichia coli* have been firstly chosen as a host due to their relative simple well-established cultivation conditions (Tokashiki and Yokoyama, 1997). However, the production of proteins by these microorganisms revealed that some products failed to perform their biological properties satisfactorily, due to the deficiency of carbohydrates and differences in their tertiary or quaternary structures. Thus, the production of recombinant proteins has been rapidly investigated using mammalian cells as a host. The isolation of hybridoma cells by cell fusion and the developments in their energetic and cultivation studies enabled the production of biopharmaceuticals by mammalian cells and their use for therapeutic treatment as well as diagnostic applications, e.g. monoclonal antibodies by hybridoma cells, interferon-α by Namalwa cells and interferon-β by normal human diploid fibroblasts (Kretzmer, 2002).

The ease of production in bacterial systems, e.g. high growth rates, simple and low-cost production processes, was counteracted by the fact that bacteria and yeasts are unable to perform accurate folding of tertiary and quaternary protein structures. Also, they have a

limited capacity to carry out post-translational modifications such as glycosylation, amidation, acetylation and phosphorylation. These modifications are necessary for the solubility, secretion and half-life time of the produced proteins, as well as for protecting them from being attacked by the host immune system *in vivo* (Blohm et al., 1988; Leist et al., 1990). In contrast, mammalian cells can perform complex post-translational modifications of recombinant proteins (Hauser, 1997). Furthermore, the chaperon system of mammalian cells ensures that proteins are secreted in a correctly folded authentic manner (Sandig et al., 2005).

Mammalian cells constitute a demanding system for the production of heterologous proteins. The need for specialized media and sufficient oxygen supply, low cell densities and slow growth kinetics, and high cell sensitivity to mechanical stress are obstacles, which must be overcome in routine fermentation processes. Furthermore, mammalian cells are potential targets for adventitious viral agents, and processes based on such cells must be rigorously monitored. Despite these difficulties, mammalian cells are the preferred production systems for the synthesis of glycoproteins intended for human administration. In 2004, mammalian cell-based therapeutic proteins were expected to reach a market share of 59%, followed by 27% for *E. coli*-based products (Sandig et al., 2005). Table (2.1) gives an example of some of the currently licensed pharmaceuticals produced by animal cell culture.

Table 2.1. Some licensed pharmaceuticals produced by animal cell cultures (Chu and Robinson, 2001).

Licensed name	Product type	Cell line	Application field
- Tenecteplase/TNKase™	Recombinant glycoprotein	CHO	Reduction of mortality in acute myocardial infarction patients
- Antihemophilic factor (recombinant)/ReFacto™	Recombinant glycoprotein	CHO	Control of hemorrhagic episodes
- Interferon α-n1, lympho-blastoid/Wellferon™	Endogenous glycoprotein	Human lympho-blastoids	Treatment of chronic hepatitis C patients
- Coagulation factor VIIa (recombinant)/NovoSeven™	Recombinant glycoprotein	BHK	Treatment of bleeding of hemophilia A or B patients
- Infliximab/Remicade™	MAb	Murine myeloma	Treatment of Crohn's disease
- Interferon β-1a/Avonex™	Recombinant glycoprotein	CHO	Treatment of multiple sclerosis

2.3. Bioreactors for animal cell cultivation

The development of bioreactors for animal cell cultivation was based on the properties of different cells. According to their mode of proliferation, mammalian cells can be classified into anchorage-dependent and anchorage-independent cells. The anchorage-dependent cells, to which most of cells belong, attach to solid surfaces and proliferate, while the anchorage-independent cells, e.g. hemocytes and monoclonal antibody-producing cells, proliferate in suspension and need no surfaces to attach on. Table (2.2) summarizes different bioreactors available for animal cell cultivation.

Table 2.2. Classification of bioreactors for mammalian cells (Tokashiki and Yokoyama, 1997).

Cultivation process	Type of bioreactor	Cell-immobilizing carrier
* Suspended cell culture (anchorage-independent)	Stirred tank, airlift (bubble column) and bubble-free	-
* Microcarrier cell culture (anchorage-dependent)		
- Suspension type	Stirred tank and airlift	Microcarriers, porous microcarriers, gel beads, microcapsules, porous glass beads
- Fixed bed type	Packed bed	Glass beads, porous glass beads, polyurethane foam
	Miscellaneous	Ceramic matrix, hollow fiber

2.3.1 Suspended cell bioreactors

Suspension cell cultures have been mainly applied to anchorage-independent cells. However, the possibility of suspension culture even in intrinsically anchorage-dependent cells has been demonstrated by sorting and breeding cells, where optimal conditions are suitably selected, e.g. CHO or BHK cells, which have a great importance in gene manipulation and the production of therapeutics (Leist et al., 1990; Tokashiki and Takamatsu, 1993). This type of bioreactors is in no need of supports for cell immobilization, and allows homogeneous mixing so that nutrients and oxygen are equivalently distributed through the cultivation vessel. These bioreactors include stirred tank, airlift or bubble column and bubble-free bioreactors.

In stirred tank bioreactor, the agitation unit or agitation blades must be arranged, not only to ensure good mixing of cell culture and medium, but also to minimize the mechanical cell damage caused by shear stress during agitation (Prokop and Rosenberg, 1989). Aeration of the culture is achieved by pumping air through a sparge plate or ring near the bottom of the vessel. The use of stirred tank bioreactors has many advantages including uniform culture conditions, ease of sampling, and well-developed systems for controlling process parameters such as pH and dissolved oxygen concentrations, both of which can have profound effects on the proliferation and properties of cells (Carswell and Papoutsakis, 2000).

In airlift bioreactors, in contrast to stirred tank bioreactors, air or oxygen-containing gas is introduced through draft tubes inside the bioreactor into the bottom center of the culture vessel and allowed to bubble up. Since agitation of the culture is performed only by air, no agitator is involved; the effect of mechanical shear stress is further decreased. The system has simple configuration and is easily scalable. Airlift bioreactors have been applied to cultures of freely suspended animal cells (Arathoon and Birch, 1986; Grima et al., 1997). However, cells are subjected to shear stress and turbulence caused by the arising bubbles.

In order to avoid the problems resulting from the arising bubbles, i.e. shear stress and turbulence, Lehmann et al. (1985) developed a bubble-free aeration system for the cultivation of mammalian cells in suspension cultures. They used porous hydrophobic hollow fiber membranes for the aeration, where a gas-liquid interface layer is formed at the outer surface of the membrane. They coiled the membrane onto a carrier, which was slowly moving through the bioreactor to produce a membrane stirrer effect. Through the dual function of the membrane, they achieved effective gas supply to the cells with only very low shear forces. Moreover, they were able to overcome the problem of the undesirable foam formation, which is usually formed in stirred tank and airlift bioreactors, and, if present, results in the floatation of cells, microcarriers and proteins (Wagner and Lehmann, 1988).

2.3.2. Micorcarrier bioreactors

This type of bioreactors is mainly used for anchorage-dependent cells. These systems use microcarrier supports, which are usually small, round beads used to enable and support cell growth (Pringle, 1992). As carriers, different materials have been suggested and investigated, organic materials such as proteins, e.g. collagen or gelatin, polysaccharides as cellulose, dextran, agarose, and synthetic polymers such as polyacrylamide, polystyrene, or inorganic

materials like glass, ceramics and stainless steel. There are some properties, which must be fulfilled by the carrier, for example, carrier material must be innoxious so that cells can easily attach and grow on it, its density and size should enable adequate fluidization by mild mixing to avoid mechanical cell damage, the carrier should have large surface area per unit volume and should be autoclavable (Tokashiki and Yokoyama, 1997).

Microcarrier bioreactors can be generally classified into suspension type and fixed bed type. As in freely suspended cell bioreactors, the suspension type of the microcarrier bioreactors includes stirred tank and airlift bioreactors (Ganzeveld et al., 1995). The carriers on which cells are immobilized are mixed and fluidized in the culture medium instead of the cells themselves. In such case, a faster flow rate is obtained by increasing agitation speed or aeration rate, to keep microcarrier beads in suspension. This, accompanied with the collision and friction of cell-immobilizing carriers, will increase the shear stress exerted on the cells.

Fixed bed bioreactors consist of a reactor vessel packed with microcarrier beads. Oxygenated medium, which is free from bubbles, is pumped from the reactor bottom through the bed. There are several types of fixed bed bioreactors depending on the matrix used, e.g. packed bed bioreactors, hollow fiber and ceramic matrix bioreactors.

2.4. Cultivation processes for animal cell culture

Mammalian cell culture has been employed for the production of therapeutic and diagnostic proteins by using techniques similar to those used in microbial biotechnology. However, there are several distinctions between mammalian cell cultures and microbes that render conventional technologies inadequate (Randerson, 1985; Leist et al., 1990; Tokashiki and Yokoyama, 1997; Sandig et al., 2005). The following table describes the biological characteristics of mammalian cells and their crucial industrial aspects.

Table 2.3. Biological features of mammalian cells and their crucial industrial aspects.

Biological properties	Related industrial aspects
* 10-100 times greater than microorganisms, no cell wall, high degree of differentiation, cytoskeleton involved in metabolism and growth control	* High sensibility to outer stimuli and toxic metabolites, high susceptibility to shear stress
* Complex metabolism and regulation mechanisms, high number of intermediates in metabolism	* Complicated medium composition, complex control of cultivation parameters
* Complex cell cycle, generation time 10-100 times longer than microorganisms	* Lower cell proliferation rate, lower productivity of target products

2.4.1. Batch cultivation

Batch cultivation is the most simple fermentation system. In this cultivation, medium is charged into the bioreactor and then inoculated with cells. During the cultivation, no additive medium is supplied to the vessel and only the cultivation parameters (air, foam, pH, temperature) are controlled. Cells enter the so-called lag phase, in which they do not increase in number, however, by the end of this phase cells increase in size. During this phase, cells synthesize enzymes and transport systems required for their growth. The length of this phase depends on the physiological state of the cells and the concentration of the inoculum. The cells then enter an exponential phase. In this phase, cells grow exponentially following a first order reaction kinetics. When medium components are depleted and the concentration of toxic metabolites is increased, the growth is slowed. Cell growth is finally ceased and cells enter the stationary phase. During this phase, cells continue to metabolize, producing energy required for the maintenance of basic cell functions. When the cell's reserves of energy are consumed, they begin to die, following the same first order kinetics as log phase. This phase is known as decline phase, which continues until all cells are being dead. Batch cultivation is simple with regard to system and cultivation conditions, however cell density is usually low ($1\text{-}2\cdot10^6$ mL^{-1}) and the product concentration is also low (Pringle, 1992; Tokashiki and Yokoyama, 1997).

Until recently, it has been always assumed that cell death in the bioreactor environment proceeds by the so-called necrosis process, mostly resulting from extreme stress parameters affecting cell growth, i.e. physiological and non-physiological. However, it is now clearly