Research for the Design of a Simulated Microgravity environment Bioreactor

Milan, 30 November 2001 *Andrea Guidi*

Index

Index of Figure

Preface

The aim of the present research is to prepare a background knowledge in the field of bioreactors especially those for use in the microgravity environment. The report is addressed to two different audiences, engineers and biotechnologists. The paper deals with two main aspects of the design of such a device. The first part of the paper reviews the biological aspects of a culture of cells, a critical concept in the design of a bioreactor. The report attempts to give a short overview of the different problems arising during cell culturing, with special attention given to mammalian cells. The second part is the technical part. In this part, the ways in which different devices obtain different characteristics of the culturing environment are described and analyzed with special attention to the simulated microgravity environment. Finally the important commercial configuration of "simulated microgravity environment" bioreactors are described. This last part can result in some details being redundant. The intent was to specify some technical and biological characteristics for each configuration, but now with particular attention to the implementation of the system.

1 Introduction

In the past decades, fundamental advances in cellular and molecular biology have improved the ability to culture cells in vitro. Cells and tissue cultures are a major area of research for biotechnology community. Tissue culturing is one of the basic tools of medical research and is key to developing future medical technologies such as therapeutic trial prior to in vivo experimentation and tissue transplantation. Nowadays advances in scientific knowledge and biotechnological capabilities herald an exciting new betrothal between tissue engineering and space biology. In this alliance, microgravity may become a surprising, unconventional, and yet attractive venue for the generation of macroscopic tissue. The physical basis for these effects is thought to be due to the low-shear fluid environment that the cells and tissues encounter in real or simulated microgravity. By eliminating sedimentation, buoyancy and density driven convection, the cells can be grown in a relatively quiescent fluid environment, where interactions between cells can occur with minimal disruption. This would allow cell aggregates and tissues to form as in vivo, but also suggests that methods are required, by which the mechanical environment may be modified to control the development of engineered cell structures. Extensive research on the effect of mechanical stimuli on cell metabolism suggests that tissues may respond to mechanical stimulation via loading-induced flow of the interstitial fluids. During the culture, cells are subject to a flow of culture medium. Flow properties such as flow field, flow regime (e.g. turbulent or laminar), flow pattern (e.g. circular), entity and distribution of the shear stress acting on the cells greatly influence fundamental aspects of cell function, such as regulation and gene expression. This has been demonstrated for endothelial cells and significant research efforts are underway to elucidate these mechanisms in various other biological systems. Local fluid dynamics is also responsible of the mass transfer of nutrients and catabolites as well as oxygenation through the tissue. Most of the attempts to culture tissue-engineered constructs in vitro have utilized either stationary cultures or systems generating relatively small mechanical forces. For example, cartilage constructs have been cultured in spinner flasks under mixed or unmixed conditions, in simulated and in real microgravity. In these mixing studies, however, it is difficult to definitively quantify the effects of mixing-induced mechanical forces from those of convection-enhanced transport of nutrients to and of catabolites away from the cells. At the state of the art, the presence of a more controlled mechanical environment may be the condition required in order to study the biochemical and mechanical response of these biological systems. Such a controlled environment could lead to an advanced fluid dynamic design of the culture chamber that could both enhance the local mass transfer phenomena and match the needs of specific macroscopic mechanical effects in tissue development.

The cell culturist needs to provide an in vitro environment as similar as possible to the natural cell environment. Such an environment is provided by a device known as 'bioreactor'. A bioreactor is generally a tool or device for generating products using a biological system. A current problem in tissue culturing technology is the unavailability of an effective Bioreactor for the in vitro cultivation of cells and explants. It has, in fact, proved extremely difficult to promote the high-density three-dimensional in vitro growth of human tissues that have been removed from the body and deprived of their normal in vivo vascular sources of nutrients and gas exchange. A variety of tissue explants can be maintained for a short period of time on a

supportive collagen matrix surrounded by culture medium. But this system provides only limited mass transfer of nutrients and wastes through the tissue, and gravity-induced sedimentation prevents complete three-dimensional cell-cell and cell-matrix interactions. Several devices presently on the market have been used with only limited success since each has limitations, which restrict usefulness and versatility. Further, no Bioreactor or culture vessel is known that will allow for unimpeded growth of three dimensional cellular aggregates or tissue.

The environment created on Earth within a clinostat or rotating vessel is often referred to as "simulated microgravity". Presently, we know that microweight does evoke a number of effects on cells, and a number of well-controlled experiments have shown that the absence of weight can have profound effects on fundamental biological processes active within the cell. All conditions of "weightlessness" results from a net sum of all forces present equalling zero, not from an absence of gravity. Currently two bioreactors for the generation of simulated microgravity have been developed: the rotating wall vessel (RWV) developed by NASA and the random positioning machine (RPM) developed by Fokker Space. Both are based on time averaging of the weight vector acting on the biological particles, which are suspended in the culture medium inside the bioreactor, based on the 'requirement' that the weight vector should act for at least seconds in a constant direction to generate an effect in cells. In the case of the clinostat, while it slowly rotates, the particles are strongly influenced by viscous drag and tend to rotate with fluid medium.

The bioreactor is an excellent example of how the skills and resources of two distinctly different fields can complement each other. Microgravity can be used to enhance the formation of tissue like aggregates in specially designed bioreactors. Theoretical and experimental projects are under way to improve cell culture techniques using microgravity conditions experienced during space flights. Bioreactors usable under space flight conditions impose constructional principles which are different from those intended solely for ground applications. The Columbus Laboratory as part of the International Space Station (ISS) will be an evolving facility in low Earth orbit. Its mission is to support scientific, technological, and commercial activities in space. A goal of this research is to design a unique bioreactor for use sequentially from ground research to space research. One of the particularities of the simulated microgravity obtained through time averaging of the weight vector is that by varying the rotational velocity the same results can be obtained with a different value of g. One of the first applications of this technique in space biology was in fact the Rotating Wall Vessel developed by NASA, and originally designed to protect cell culture from the high shear forces generated during the launch and the landing of the Space Shuttle. A Bioreactor that is used both for ground and flight experiments provides the additional benefit of isolating dependent variable of gravity. This continuity will provide a means to compare results to a control experiment.

2 Cells culture

Aims of *in vitro* cultivation of animal cells can be broadly divided in two categories:

- **Cell Research**

  Mammalian cell and tissue culture is a major area of research for biotechnology community. Tissue culturing in one of the basic tools of medical research and is key to developing future medical technologies such as *ex-vivo* (outside of the body) therapy design and tissue transplantation. In the field of cell research a challenging study is the generation of organs *in Vitro.* An alternative approach to donor organs for transplantation is, in fact, the generation of replacement of organs by means of 3D in vitro cell culture.

- **Manufacture of Biological products** (cell themselves, cell component, metabolites)

  Biological product are proteins, enzymes, hormones, antibodies and antigens. They are often not only for drugs production but even from many other everyday life uses (e.g. agriculture, or production of food). Moreover, in the past decades bioenginered tissue are becoming manufacturable product which provides new opportunity for clinical treatment. Novel techniques allow the creation of defined three-dimensional tissue and scaffolds, and artificial vessel maschwork (hollow-fibre capillary nets) to provide oxygen and nutrients.

Figure 1: Primary human liver culture on PGA scaffolding (14 days) in the Synthecon RCCS™ (Photo Synthecon)

For proper cell propagation, the cell culturist needs to provide an *in vitro* environment as similar as possible to the natural cell environment (e.g. uniform conditions, transport

nutrients, oxygenation, hydrodynamic stresses, etc.). Each tissue in the body is composed of dynamic assemblage of multiple stationary and migrating cell types that are embedded in a complex macromolecular structure.

Most commonly, cell culture is performed in two-dimensions, such as in petri dishes, tissue culture flasks, multy-well plates or any other tool or device for generating products using a biological system. These devices are commonly addressed as 'bioreactor'. In spite of the tremendous amount of information gained in traditional cell culture settings, it is generally acknowledged that conventional tissue culture in two dimensions may be inadequate to model the complex cellular interactions that promote tissue-specific differentiation as they occur, e.g. during organogenesis. A current problem in tissue culturing technology is the unavailability of an effective bioreactor for the in vitro cultivation of cells and explants. A variety of tissue explants can be maintained for a short period of time on a supportive collagen matrix surrounded by culture medium. But this system provides only limited mass transfer of nutrients and wastes through the tissue, and gravity-induced sedimentation prevents complete three-dimensional cell-cell and cell-matrix interactions. Culture longer than 7 week have been difficult to achieve, since crypt cells are unable to survive standard culture regimens. As the scale of cultivation increases, mixing of the culture medium is required to maintain uniform conditions and enhance the transport of limiting nutrients such as oxygen. Since mixing is accomplished by deformation of fluid elements, hydrodynamic stresses are transmitted to cells. Several devices presently on the market have been used with only limited success since each has limitations which restrict usefulness and versatility. Further, no bioreactor or culture vessel is known that will allow for unimpeded growth of three dimensional tissue or organs.

Most of the attempts to culture tissue-engineered constructs in vitro have utilized either stationary cultures or systems generating relatively small mechanical forces. For example, cartilage constructs have been cultured in spinner flasks under mixed or unmixed conditions, in simulated and in real microgravity. In these mixing studies, however, it is difficult to definitively quantify the effects of mixing-induced mechanical forces from those of convection-enhanced transport of nutrients to and of catabolites away from the cells. At the state of the art, the presence of a more controlled mechanical environment may be the condition required in order to study the biochemical and mechanical response of these biological systems. Such a controlled environment could lead to an advanced fluid dynamic design of the culture chamber that could both enhance the local mass transfer phenomena and match the needs of specific macroscopic mechanical effects in tissue development.

2.1 Mammalian Cells Culturing

Mammalian tissue can be grouped into three general categories: organ tissue, structural tissue and blood producing tissue. Mammalian tissue is composed of aggregates of cells that share a functional interrelationship in order to have tissue growth. Mammalian tissue is composed of different types of cells characterised by different morphology and immunochemical properties. Cellular differentiation in mammals involves complex interactions in which cell membrane junctions, extracellular matrices such as basement membrane and ground

substances. Soluble signals produced and shared among the cells play an important role. Cellular differentiation may depend on three simultaneous conditions:

- Three-dimensionality
- Low shear stress and turbulence
- Co-spatial arrangement of different cell types and substrates

The composition of the ECM is variable depending on the type of tissue and his stage of development, and some of the components may undergo a transient change in distribution in response to environmental stimuli or disease states. It mostly consists of polymerised collagens, structural glicoproteins, elastin, glicosaminoglicons, adhesive laminin, fibronectin, arranged in a complex mesh that is constantly bathed by the fluid of the interstitial tissue space. The ECM is also known to be critical for regulating cell morphology, proliferation and differentiation, and is capable of responding to various endocenous and exogenous stimuli.

Mammalian cell culture and tissue generation, is much more complex because such cells are more delicate and have a more complex nutrient requirement for development. Unfortunately, it has proved extremely difficult to promote the high-density three-dimensional in vitro growth of human tissues that have been removed from the body and deprived of their normal in vivo vascular sources of nutrients and gas exchange. High-density, three-dimensional in vitro growth of human cells is problematic due to turbulence and shear effects, or inadequate oxygenation in conventional cell culture systems.

In addition, mammalian cells can have a special requirement because most animal cells must attach themselves to some surface in order to duplicate. This cells are addressed as anchorage-dependent mammalian cells that have thin cell membranes. To provide the necessary attachment surface with a large surface area/volume ratio, small "microcarrier" beads are suspended in fluid medium with fresh nutrients. Anchorage-dependent cells have been widely cultured on microcarriers (or beads). After several division, cells can form a confluent monolayer, with 100 to 200 cells on each microcarrier. Many cell covered microcarrier attach and bridge to form larger cell assemblages. Cell assemblages have been grown up to several millimetre in diameter.

The problem then is to suspend microcarrier beads without inducing turbulence or shear forces which will damage cells. Unless a cell culture is growing in an environment free of gravitational forces, moderate levels of agitation are required to suspend microcarriers that are not neutrally buoyant. Most conventional bioreactor for microcarrier culture have used internal propellers or movable mechanical agitation devices which are motor driven so that the moving parts within a vessel cause agitation in the fluid medium for the suspension of mammalian cells carried on microcarrier beads. However this agitation induce fluid turbulence and shear. Mammalian cells cannot withstand excessive turbulent action without damage to the cells and must be provided with a complex nutrient medium to support growth. Small bioreactor vessels with internal moving parts may damage mammalian cells and also subject the cells to high fluid shearing stresses. If the microcarrier beads collide with one other in the suspension, the attached culture cells can be damaged. The problem then is to suspend microcarrier beads without inducing turbulence or shear forces which will damage cells. Therefore, bioreactors with internal moving parts or obstructions will subject mammalian cells to high fluid shearing forces that will damage the cells. In addition, bioreactors that utilise mechanical parts, air or fluid movement as a lift mechanism to achieve particle suspension will likewise cause damage to growing cells and tissues due to fluid shear.

This problematic will be further explained in the chapter dedicated to the fluid dynamic effects on cells.

Study of normal mammalian tissue, has been limited because of lack of adequate in vitro culture systems that product tissue of sufficient size and functionality. In contrast, tumour cells are by their nature easier to grow in vitro, possess abnormal growth characteristics and do not behave like normal cells in their interaction with neighbouring cells. A variety of different cells and tissues, such as bone marrow, skin, liver, pancreas, mucosal epithelium, adenocarcinoma and melanoma, have been grown in culture systems to provide three dimensional growth in the presence of a preestablished stromal support matrix. Normal mammalian tissue has been grown for limited periods of time as two-dimensional monolayers on gelled substrate or other surface for anchoring the cells.

3 Microgravity and Simulated Microgravity

Normal mammalian tissue and the culturing process has been developed for the three groups of organ, structural and blood tissue. The cells are grown in vitro under microgravity culture conditions and form three dimensional cells aggregates with normal cell function. The microgravity culture conditions may be microgravity or simulated microgravity. Simulation of microweight under normal ground conditions could is usually based on the 'requirement' that the weight vector should act for at least seconds in a constant direction to generate an effect on cells. Conditions in which this requirement is met actually prevent the cell from feeling weight at all; the weight vector escapes its detection machinery. The machines described are based on the hypothesis that sensing no weight would have similar effects as being weightless. One approach is to provide a condition in which the weight vector is randomised in the tridimensional space and never has a constant direction for seconds or longer. This condition is generated by the Random Positioning Machine (RPM). Another approach it can be the Clinostat and Rotating Wall Vessel (RWV) by a vessel rotating horizontally around its axis, mediating the weight vector acting on the cell surface only in the bidimensional space in which act the gravitational force. Both this technique are referred to as "time averaging of the weight vector" acting on the biological particles, which are suspended in the culture medium.

The use of a clinostat for studying how gravity affects plant growth dates back well before the advent of space flight, possibly to early 1700's. Empirical results obtained using simulated microgravity of Clinostat are frequently, though not always, found to be similar to those observed in true microgravity of space flight experiments. Most of the time the result of simulated microgravity are enhanced by true microgravity. This similarity is also present in different non-biological uses of the technique of time averaging of the weight vector as a simulator of microgravity. For example, the industrial production of microscopic spherical particles utilises this technique (Monodisperse Latex Reactor developed by NASA, [Roberts Glyn O., et al, 1991]). This similarity in the results, however, does not support a logical conclusion that all biological mechanisms were identically affected in both environments. It is estimated that the level of microgravity obtained with axial rotation to be around 10^{-2}g against the 10^{-4} , 10^{-6} experienced in today's spacecraft. Theoretical and experimental projects are

currently under way to improve cell culture techniques utilising simulated microgravity conditions. The focus of many of these efforts is to understand if a rotating vessel provides a sufficient "simulated microgravity" environment, or if a complex 3D rotational system is required. To literally experience "Zero g", that is to not experience any gravitational pull, an object would need to be infinitely far from any gravitating body. All other conditions of "weightlessness" result from a net sum of all forces present equalling zero, not from an absence of gravity. In the case of the clinostat or the rotating vessel, while it slowly rotates, the particles are strongly influenced by viscous drag and tend to rotate with fluid medium. Their motion relative to the rotating fluid is determined by a balance of their gravitational and centrifugal forces with forces exerted by the fluid, and the sign of the buoyancy forces depends on the density difference between the particles and the fluid. Moreover, the stress forces exerted by the fluid on the particle have to be separated into pressure force and drag force. Of course, the impracticality of utilising a true 0 g platform drives us towards the first of many constraints in trying to effectively isolate an experimental system from the attraction of gravity while remaining in our solar system. The attracting force drops off non-linearly with increasing distance from the source (i.e. $1/r^2$ from the surface of the Earth), but even in Low Earth Orbit, a spacecraft still experiences approximately 90% of the gravitational acceleration (about 0.9 g) that exists at the surface of the Earth. So technically speaking, an orbiting space shuttle is not in a micro g environment, at least not from the perspective of an observer on Earth. Identifying an appropriate frame of reference (or co-ordinate system) is an important concept for understanding altered inertial environments, whether created by freefall in space or through rotation on Earth. This issue also leads to the practice of using pseudo forces when formulating free body diagram problems in non-inertial reference frames (e.g. an object floating in an orbiting spacecraft).

Acceleration is commonly taken to imply increasing velocity, but is defined as a change in its "magnitude or direction". The weightless state created in a drop tower does arise from a continuous increase in velocity (magnitude) while travelling basically in a straight path along the gravitational vector. The state of freefall experienced onboard a spacecraft in a circular orbit experience constant velocity as governed by orbital mechanics, but the direction of the spacecraft is continually being altered from a straight path into circular orbit normal to the gravitational vector via centripetal acceleration. Parabolic flight likewise induces weightlessness on objects within the aircraft as controlled rate of acceleration (with linear and centripetal component) is maintained that matches the net rate induced by gravity. The weightless state attained in drop towers, parabolic flight and orbital space flight, therefore, may be thought of as a "simulation" of true 0 g, as it is defined above. Gravity is still present, so it is not 0 g, but the sum of the forces acting on object of any density appears to be balanced to zero (or near zero) from within the particular frame of reference. Since strictly speaking, they are not equal to zero, so called pseudo forces are induced to balance the equations. Newton's laws are directly applicable only in the context of a given inertial frame of reference. In the case of orbital space flight, an object may appear to be both at rest and weightless relative to the spacecraft's frame of reference, even though there are actually unbalanced forces acting on it causing it to circle the Earth (hence, it is technically experiencing accelerated motion) rather than flying off into space on a straight trajectory. In this case, the pseudo *centrifugal* force (mv^2/r) is often introduced to balance the system, which is dependent of the acceleration of the reference frame. This force is fictitious in that it is not actually exerted by a real agent, rather it is imposed to make the sum of the forces (F =

ma) = 0 applicable when centripetal acceleration (a_c) is not apparent relative to the non-inertial frame of reference (i.e. inside the spacecraft). Centrifugal force is used to mathematically explain why an object experiences an outward push towards the circumference of a rotating device, when in fact, centripetal force is pulling the object towards the center of rotation to keep it from flying off in a straight path while it "feels" the resistance of the structure supporting it. The Coriolis ($2\mu wv$) pseudo force is also of particular interest when describing rotating environments such as clinostats or Rotating Vessel, as well as for rotating devices in space flight. Coriolis force is used to explain why an object freely moving radially appears to do so in a curved path when observed from inside the rotating frame of reference, when it has in fact travelled in a straight path while the internal observer has moved. Pseudo "forces", therefore, do not have actual physical bases, rather are used to explain apparent forces as observed within non-inertial frames of reference. The resultant relative contributions of centrifugation and Coriolis motion, however, are real.

Perfect weightlessness is difficult to achieve within a spacecraft since gravity-gradients are present as a result of the far end of an orbiting object traversing a larger radius than the end nearer to the Earth. Oscillatory accelerations (vibrations or g-jitter) are also introduced by mechanisms on the spacecraft and by crew movement. Atmospheric drag, solar wind, light pressure, thruster firings, etc. also cause residual forces to be felt which would be non-existent in a perfect weightless environment. Therefore, "near weightlessness" on the order of 10^{-4} to 10^{-6} g is more typically experienced in today's spacecraft and is usually referred to as microgravity (with "micro" defined either literally as 10^{-6}, or figuratively as "very small"). Thus, one can consider the second constraint in studying the effects of gravity on a spacecraft to be the determination of whether or not this less-than-perfect weightless environment results in a sufficiently small force acting on the experimental system so as to be deemed statistically negligible.

4 Microgravity Biology

As already mentioned in a previous paragraph, the similarity in the results obtained in microgravity and simulated microgravity, do not support a logical conclusion that all underlying mechanism were identically affected in both environments. Biological effects attributed to gravity must ultimately be established as a cascade of events beginning with the altered inertial environment (physics), which potentially leads to altered structural loads (engineering) and/or electrochemical gradients (chemistry) being generated, and concludes with the observed response of the organism (biology).

Research conducted in biological sciences support human space exploration by using the environment of space as a unique laboratory for study of biological processes. Fluid dynamics is an area of major interest in Biological science and great interest is devoted to its study in microgravity.

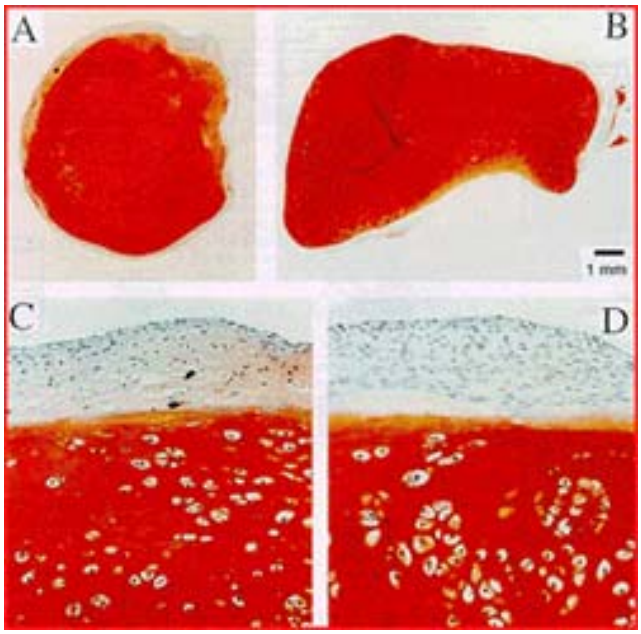

Figure 2: Comparison of construct structure cross sections A & C = Mir Space Station and B & D = ground-based in RCCS as reported in: Freed L, at all 1997.

Fluid flows and transport of mass and heat occur in almost every biological process, from molecular and subcellular scale to whole system. Human exposure to long-duration low-gravity environments is known to produce many undesirable physiological effects. Change in vascular fluid distribution result quickly from the loss of hydrostatic pressure, and, on a longer time scale, from the shift of intercellular flows. Reduction of weight-bearing stress in microgravity induces bone loss and remodelling behaviour. An adequate understanding of the underlying fluid physics and transport phenomena can provide new insight needed to develop effective countermeasures.

Before Spacelab flew for the first time in 1983, those scientist who had thought about possible effects of weightlessness on biological cells had expectation that weight in the microworld of cells would be a negligible force, and that therefore weightlessness would make no difference in the function or architecture of a biological cell. Preliminary results proving the contrary in the pre-Spacelab period, had been reasoned away as being the consequence of other spaceflight effects such as radiation, mechanical stress during launch and landing, or suboptimal life support conditions for the organisms flown. Presently, we know that microweight does evoke a number of effects on cells, and a number of well-controlled experiments have shown that the absence of weight can have profound effects on fundamental biological processes active within the cell (e.g. gene expressions). In trying to understand possible mechanisms that contribute to these effects, it was helpful not to think of cell in transition from normal weight to microweight conditions, but to think of it in transition from microweight to a normal weight condition. How can it perceive weight? Weight would somehow cause a situation inside the cell of differential mass displacement, due to differences in the mass of cell-internal organelles and supramolecular structures. Such a condition of

displacement should be of a sufficient magnitude to trigger the adoption by the cell of a new internal equilibrium. This very condition could be called the 'weight signal', the energy level of which will have to be above the local thermal noise inside the cell. The concept of weight signal, and its relation to mass displacements inside the cell, is put here in very general terms on purpose, because the detailed sequence of effects that causes the signal is not really important. What is important though, is that a minimal energy level is required to evoke any reaction in the cell. This translates into a minimal displacement of mass inside the cell and therefore to the notion that the weight vector has to have a constant direction for some minimal duration. Plant biologists call this minimal duration the perception time, and attempts have been made to measure this time for the specialised weight-sensing cells located in the tip of plant roots, at normal ground conditions. Values found in these studies are in a range between 0.5 sec and 13 sec, dependent on the species used and the limitations of methods applied. The important conclusion from these considerations is: the notion that cells require a minimal time of unidirectionality of the weight vector to sense weight at all, that for specialised plant cells this time seems to be in the order of seconds. The extracellular matrix (ECM) of mammalian, cells as mentioned in a previous chapter, provides a complex environment. In this environment seems for some unknown mechanisms that the cells behave as they do not feel weight. The physical basis for these effects of microgravity on cells is thought to be due to the low-shear fluid environment that the cells and tissues encounter in real or simulated microgravity. By eliminating sedimentation, buoyancy and density driven convection, the cells can be grown in a relatively quiescent fluid environment, where interactions between cells can occur with minimal disruption. This would allow cell aggregates and tissues to form as in vivo but also suggests that methods are required, by which the mechanical environment may be modified to control the development of engineered cell structures. Extensive research on the effect of mechanical stimuli on cell metabolism suggests that tissues may respond to mechanical stimulation via loading-induced flow of the interstitial fluids. In a bioreactor, the cells are subject to a flow of culture medium. Flow properties such as flow field, flow regime (e.g. turbulent or laminar), flow pattern (e.g. circular), entity and distribution of the shear stress acting on the cells greatly influence fundamental aspects of cell function, such as regulation and gene expression. This has been demonstrated for endothelial cells and significant research efforts are underway to elucidate these mechanisms in various other biological systems. The endothelial cell (EC) layer, which lines blood vessel provide the principal barrier to the transport of water and solutes between blood and underlining tissue. ECs are continuously exposed to the mechanical shearing force (shear stress) and normal force (pressure) imposed by flowing blood on their surface, and they adapted to this mechanical environment. In low gravity, the mechanical environment of ECs is perturbed drastically, and the transport properties of EC layers are altered in response. It is proposed that alterations in mechanical forces induced by microgravity, and their resultant influence on transendothelial transport of water and solute, are largely responsible for the characteristic cephalad fluid shift observed in humans experiencing low gravity. Understanding the mechanisms behind this fluid shift is crucial to developing countermeasures for crews cacing long-duration exposure to low-gravity. Another examples of flow and shear stress affecting mechanical and functional properties cells can be found in cartilage tissue.

5 Fluid Dynamics in Biology

Most of the biological, environmental, and industrial processes required to support life take place in a fluid phase. Fluid motion accounts for most transport and mixing in natural and industrial processes as well as in living organism. The ultimate goal of research in this area is to predict and even control this fluid behaviour. A detailed understanding of fluid dynamics over a range of length and time scale is essential for progress in many emerging research areas of physical and biological sciences.

The low -gravity environment of space offers a unique opportunity for the study of fluid physics and transport phenomena, as the nearly weightless conditions allow researchers to observe and control fluid phenomena in ways that are not possible on Earth.

Research on the behaviour of fluids and transport phenomena in weightlessness is also essential to the design and development of self-sustaining closed-loop system required for human life support. Self-assembly at cellular levels also provides a pathway to nanotechnology.

In this light, and considering that biological effects attributed to gravity must ultimately be established as a cascade of events beginning with the altered inertial environment, the aim to investigate fluid dynamics is for understand its rule in the balance of the forces in the inertial reference environment of microgravity and simulated microgravity. A first goal would be to design a Bioreactor that is capable to regulate several parameter of the flowfield inside the vessel. This capability would be of fundamental importance not only for better regulation of the force in the inertial environment in the vessel, but will also lead to knowledge on how cells are affected by different fluid flow. The possibility to investigate flow and shear effects on cells, provocated by controlled variation of flowfield and pressure, will help our understanding of differentiation, proliferation and organisation of cells.

Local fluid dynamics is also responsible of the mass transfer of nutrients and catabolites as well as oxygenation through the tissue. Most of the attempts to culture tissue-engineered constructs in vitro have utilized either stationary cultures or systems generating relatively small mechanical forces. For example, cartilage constructs have been cultured in spinner flasks under mixed or unmixed conditions, in simulated and in real microgravity. In these mixing studies, however, it is difficult to definitively quantify the effects of mixing-induced mechanical forces from those of convection-enhanced transport of nutrients to and of catabolites away from the cells. At the state of the art, the presence of a more controlled mechanical environment may be the condition required in order to study the biochemical and mechanical response of these biological systems. Such a controlled environment could lead to an advanced fluid dynamic design of the culture chamber that could both enhance the local mass transfer phenomena and match the needs of specific macroscopic mechanical effects in tissue development.

There has been few publication on how mechanical energy is transmitted to the cells and in turn how cells respond to these specific forces. One of major problem in literature data is that often investigator used a different measure of the effect of fluid flow on the cells, for instance death of the effect fluid on cells, for instance death rate (of non-growing cells), maximum cell density or multiplication ratio (from seeding density). Most do not give specific details of the bioreactor configuration either. Indeed, it is still an open question whether the changes in rate

of increase of cell population are caused by reduction of growth rate, i.e., by lengthening of the cell generation time, or instead by an independent death mechanism acting on a culture growing at a normal rate. The difference is significant since in the latter case nutrient and oxygen are consumed by cells that are later killed, giving lower net yields from these nutrients. Perhaps even more importantly, though, cells that are killed will lyse and contaminate the medium with their intracellular materials, complicating downstream separation and possibly causing enzymatic degradation of a desired product. A fundamental understanding of the damage mechanism is therefore essential for optimal design and scale-up of bioreactors.

Several studies demonstrate the sensitivity of cells to hydrodynamic stresses. Factor affecting the effects of fluid flow on animal cells are flow field (flow regime and flow patterns), level of 'shear', time of exposure, medium composition (eventual additives). One of the pourposes to study the shear sensitivity of cultured animal cells is to have information that will allow one to define an appropriate environment in which the cells can be cultured at large scale. This involves the identification of the relevant parameters of the flow field governing hydrodynamic related to cellular behaviour, e.g. injury, and their relationship with the equipment geometry (bioreactor design) and operation. In an ideal situation cell injury should be predicted by knowing the actual stress that the cell experiences and, from intrinsic cell mechanical properties, the resulting cell deformation. Should the cell deformation exceed a critical value, disruption of the cell structure would be expected.

Flows to study the cell response to hydrodynamic stresses have been characterised by different parameters. Shear stress and rate of share were used to characterise the flow in well-defined flow devices (parallel plate chambers, concentric cylinders, and plate-and-cone viscometers). Average wall shear stress and specific power dissipation, and Kolmogorov eddy length scale have been used for agitated small-scale bioreactors. Operation parameters such as gas flow rate, bubble frequency, and bubble size have also been used to characterise sparged bioreactors. Even though these parameters have proved to be useful to correlate cell damage under the conditions of the particular experiment, their application to reactor design questionable. For example, we do not know how to use parameter correlate cell damage in one particular system to predict cell damage in a different system. The problem reduces to asking what are the relevant parameters which govern hydrodynamic cell injury for any given flow. To date they have not been identified.

Parameter used to characterise hydrodynamic related cell injury should be of a general nature. These parameters cannot come from the geometry of the system producing the flow but from "intrinsic" characteristics of the flow itself. Any consideration of the geometry of the system producing the flow will limit will limit their generality. Once intrinsic characteristics of the flow have been identified, their relationship with the geometry and operation of a given system can then be considered for optimisation purposes. A second requirement is that we should consider the local and not 'average' parameters. Cell injury in a hydrodynamic environment (assuming no nutrient and no mass transfer limitations) must result from the application external stresses. Consequently, it must be a function of the local intensity of these stresses. Average values prevent the identification of those regions in the flow where cells can be killed. A flow may injure a cell at one point and have no effect at a different location. Thus, the values of the selected parameters must be local if general application must be accomplished.

Here the attention is focused on cell injury as measure of the effect of fluid flow on the cells. The reason of this choice is because of the amount of data on this effect coming from the great importance of it in cell culture. Flow parameter of a general nature that can be used to study hydrodynamic related cell injury are suggested. The first step is to propose how an individual cell or a cell attached to a surface of a microcarrier can be damaged as a result of fluid flow. Cell damage for suspended cells is defined as the disruption of the cell structure (possibly the rupture of the cell membrane) that results from cell deformation. For anchorage-dependent cells, cell damage is defined as the detachment of cells from the surface support. This definition is motivated by the fact that anchorage-dependent cells cannot divide when suspended. In microcarrier culture was observed that once cells detach from their surface support, they lyes in less then 2 hours.

An idea on how a suspended cell can be deformed, and disrupted, by flow field and which parameters govern cell deformation can be obtained from the classic experiments on deformation of liquid drops by shearing flows reported by Taylor. In interpreting the results of Taylor, the crucial assumption is whether a cell behaves as a liquid droplet. (Miguel A. et all, 1994) In this assumption suspended cells should be modelled as incompressible fluids having cortical tension (cell membrane tension).

The experience of Taylor demonstrate that the state of stress of suspending liquid alone should not be used to predict drop deformation, but the possibility of internal stress relaxation by the rotation (of the suspending particle) should also be considered. This internal stress relaxation by rotation is directly related to the vorticity of the suspended fluid. In this view two relevant flow parameters to study hydrodynamic related cell injury are the local state of stress and the vorticity of the flow (it is selected stress rather than rate of strain since it is obvious that viscosity of the suspending fluid should be considered).

The fact that some experimental evidence exists that cultured cells may behave as viscous liquid droplets does not imply that these cells have the same dynamics as true liquid droplets in an imposed flow. Because the cell interior is not a continuum, the measured viscosity is an apparent viscosity and the cortical tension is not a true interfacial tension. Nevertheless, considering a general deformable particle having a cortical tension and viscous interior, it is easy to see that a pure extensional flow will be more effective than a rotational one in deforming the particle. Flow characteristics associated with this "effectiveness" to produce deformation should be considered in our analysis of hydrodynamic related cell injury.

Garcia-Briones and Chalmers (Garcia-Briones et all, 1994) elaborate the general applicability of this proposed parameters to characterise any flow in terms of its potential to produce cell damage. This is in fact a problem similar to the determination of the rheological behaviour of elastic liquid accumulates stresses if the direction of maximum strain is applied to the same line of particles. However, stress accumulation is less severe if the particles rotates such as same particles line is not always aligned to the direction of the maximum rate of strain. Viscometric and extensional flows have also been observed to have a different effect in the apparent viscosity of polymer solutions. Most polymer solutions undergo shear thinning with increasing shear rate and extension thickening as the rate extension increases. Since this effects results from the dynamic behaviour of polymer molecules with the flow field, it is of interest to have a general criteria for flow classification which will allow one to anticipate the effect of the flow accumulated by elastic liquids and to predict the viscosity of polymeric solutions. Astarita (Astarita, I., 1979) a flow classification which is: local, objective (invariant

under reference frame), general (not restricted to a particular class of flows), and kinematics (only depends on fluid kinematics). He pointed out that what distinguishes a viscometric flow from an extensional flow is not the magnitude of the principal rates of strain with respect to the liquid itself (given by the vorticity)

The forces to which a suspended cell or a cell on the surface of a microcarrier bead may be subjected have been analysed in detail. Analysis of the fluid mechanics occurring suggests that there are actually three potential damage mechanisms: collision of a cell with other cells (or cell-covered microcarrier with other beads in case of microcarrier culture), collision with parts of the bioreactor (primarily the impeller in case there is one), and interaction with turbulent eddies the size of the cells (or microcarrier beads). Review of the available quantitative information on agitation effects in cell culture does not establish which mechanism is predominant. The negative effects of excessive agitation on tissue cells culture have often been ascribed to "shear".

In a turbulent liquid exist Eddies of a range of sizes. This range of sizes is rather sharply bounded at one extreme by a smallest eddy size below which the kinetic energy of the eddies is rapidly viscously dissipated. The size and the velocity of these smallest eddies in isotropic turbulence are dependent by the kinematic viscosity of the medium and by the rate of turbulent energy dissipation per mass liquid. At steady state, the energy dissipated by turbulence equals that supplied by the agitator to the fluid, so the volume-averaged rate of turbulent energy can be calculated known the volume of the bioreactor.

A turbulent eddy much larger than the microcarrier beads can surround and rapidly accelerate a single bead to eddy's velocity or move groups of beads without creating a large relative velocity between them. With typical conditions the smallest eddy size is approximately 200 μm, or about the same as the bead size. In that case a bead would be too large for a smallest eddy to readily accelerate it, and the eddy would dissipate its kinetic energy against the cells on the bead's surface. Several eddies could also interact simultaneously with a single bead. In addition, under typical culture conditions of approximately 5g/L beads, the spacing between the beads is about equal to bead diameter. Eddies approximately the size of this spacing would also be capable of increasing the number of bead-bead collisions by accelerating a bead into its neighbours.

It can be shown that bead-bead collision are capable of delivering a physiological significant amount of energy to a cell. The effect of bead-bead collisions may be characterised by the turbulent collision severity (TCS), defined as the product of collision kinetic energy and frequency. Collision of the beads against internal part of the bioreactor have similar effects as bead-bead collision. In case of internal moving part the kinetic energy of the collision is much higher, because of the higher collision velocity.

Through an increase in viscosity, the turbulence can be damped and hydrodynamic effects can be reduced. Thickening agent which are completely soluble in cell culture medium and could significantly increase the viscosity in a flow with little or no toxic effect on cells, exists. However, for mammalian cells of interest, mechanical shear stress levels in the range 3 to 10 dyne/cm^2 cause damage to cells and reduce cell viability (Cherry RS, 1986). At shear levels as low as 0.92 dyne/cm^2 cell proliferation, morphology, and function are adversely affected (Goodwin et all. 1993). Thus, to encourage three dimensional growth and differentiation, and

further study the effects of shear on these cells, shear levels of 10^{-2} dyne/cm^2 are desired. To met this requirement, the flow field must be laminar.

6 Simulated Microgravity Bioreactors

In static flat culture flasks or dishes the 2-dimensional environment cells tend to sediment in bidimensional layer and tend to alter gene expression and prevent differentiation. As was already mentioned through the report various techniques can be used to manipulate, counteract or partially alter the net effect that gravity has on an object (e.g. agitation, freefall, centrifugation, diamagnetic levitation, time averaging). All this techniques represent important tools in biotechnology to allow the researcher to isolate specific individual components of the collective effects induced by gravity. In most dynamic systems, cells or tissues are suspend by physical forces that result in injury to the cells from contact with an impeller or by the shear forces caused by bubbles being sparged up through the culture for oxygenation and suspension. These conditions cause injury to cells or tissues.

Hereafter, a short overview of different ways to implement the technique of time averaging of the weight vector, here considered the most interesting one, are shown.

6.1 Clinostat and Rotating Wall Vessel

The fundamental difference between clinorotation and true freefall can be summed up as follows: both environments can effectively (or nearly) eliminate cumulative sedimentation for the given object of homogenous mass suspended in a sufficiently experiences chronic stimulation that is time averaged to zero, while an actual freefall environment essentially eliminates the stimulation (and can therefore be said to result in an instantaneous, rather than time averaged, net zero force). Particle motion within a clinostat reaches terminal velocity in which the accelerated motion induced by gravity is balanced by the viscous drag of the fluid medium. This is not the same as weightless freefall, in which an object travels unimpeded under constant acceleration (either centripetal or linear). In addition, coexisting objects of varying density will not be equally balanced within rotating system as they are in a state of true freefall. If the objects of concern are of small enough dimension and of a single density, they can for all practical purposes, theoretically be identically suspended in a sufficiently viscous liquid medium in either environment.

Clinostat and Rotating Wall Vessel (RWV) bioreactor are functionally similar devices insomuch as both operate on the premise that constant rotation of the system normal to the gravitational field of Earth will randomise the otherwise unidirectional pull of gravity, and both devices offer logical ground-based analogies for augmenting space flight studies. The clinostatic principle involved is that a fluid rotating (at the appropriate rate) about a horizontal or nearly horizontal axis (with respect to gravity) allows cells or cell attachment substrates having a density different from the fluid to travel in a nearly circular path and to deviate insignificantly from the fluid path. From the rotating reference frame the gravity vector is observed to rotate so that its time average is nearly zero. This allows for suspension of the

particles in a carrier medium with low fluid shear and with low interference. The vessel wall is rotated in order to reduce the adverse fluid velocity gradient through the boundary layer at this wall (which would occur at the interface between the moving fluid and fixed wall). The rotating wall is sufficient to cause fluid rotation due to viscosity. The operating limits are defined by the sedimentation rate of the particles in the fluid medium and the acceptable centrifugal force due to rotation The vessel rotating horizontally around its axis accomplish to a solid-body rotation of the fluid. In a rotating bioreactor, the cells can be fooled into thinking they are in a body. The RWV bioreactor consists of a cylindrical growth chamber with gas exchange membrane. The culture chamber with diffusion gas exchange is completely filled with culture medium (zero headspace). As the vessel rotates, the liquid inside accelerates until the entire fluid mass is rotating at the same angular rate as the wall. Thus, this environment eliminates most of the disruptive shear forces associated with a conventional bioreactor: Microcarrier beads and cells obey simple kinematics and are uniformly suspended in the culture medium. The suspended cells rotate as a solid body with minimal disruptive shear forces (about 0.2 dyn/cm^2), and the cells maintain their relative position for long periods, allowing them to touch one another or to construct bridges between the microcarrier beads. In addition, chamber rotation subjects the cells to a constantly changing angular gravity vector. Constant randomisation of the normal gravity vector subjects to a microgravity environment and akin to the free fall experienced for much shorter periods by aircraft in parabolic flight. In this environment, cells aggregate and undergo three-dimensional growth to form tissue-like spheroids. As aggregates grow during culture, the speed of vessel rotation is increased to counter gravitational sedimentation. This environment fosters tissue growth in three dimensions. This bioreactor enables the growth of clumps of cells that are larger in diameter.

The fluid dynamic operating principles of the RWV culture system thus encompass solid body rotation about a horizontal axis with some degree of three-dimensional spatial freedom, oxygenation without turbulence, high mass transfer rate, low fluid share forces, and the co-localisation of particles that have different sedimentation rates. The design requirement for the vessel to be used in space are similar to those of ground-based bioreactors, with a few exceptions. For the mammalian cells of interest, mechanical shear stress levels in the range of 3 to 10 dyne/cm^2 cause damage to cells and reduce cell viability. At shear level as low as 0.92 dyne/cm^2 cell proliferation, morphology, and function are adversely affected. Thus, to encourage three-dimensional growth and differentiation, and to further study the effect of shear on cells, shear levels of 10^{-2} dyne/cm^2 are desired. To meet this requirement, the flow field must be laminar. In the microgravity environment, the shapes of gas/liquid interfaces are dictated by surface tension. Gas bubbles form rather then the horizontal surface commonly used for gas exchange in bioreactors operated in Earth's gravity. This along with the concern of large stresses during dynamic coalescence of bubbles, lead to zero-headspace (no gas phase) requirement for the bioreactor. RWV in microgravity has also been shown to have limitation: It is difficult to remove air bubbles from the vessel on orbit without degrading the low-share culture environment or damaging the delicate three-dimensional tissue assemblies. Is limited in its ability to control the location of cells and tissue aggregates within the vessel. The RWV bioreactor, developed by NASA, was originally designed to protect cell culture from the high shear forces generated during the launch and the landing of the space shuttle.

6.2 The Rotary Cell Culture System™

The primary advantage of the RCCS™ over either dynamic or static tissue culture systems is that its gentle environment allows cells to aggregate, grow 3-dimensionally, and differentiate. This advantage results in cells or tissues that very closely resemble the *in vivo* tissue equivalent. In most dynamic systems, cells or tissues are suspend by physical forces that result in injury to the cells from contact with an impeller or by the shear forces caused by bubbles being sparged up through the culture for oxygenation and suspension. These conditions cause injury to cells or tissues.

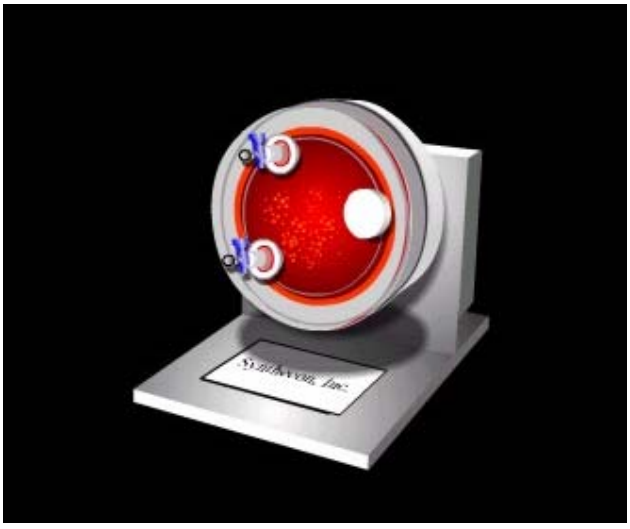

Figure 3: The Rotary Cell Culture System™ Synthecon (photo Synthecon)

In static flat culture flasks or dishes the 2-dimensional environment and plastic substrate tend to alter gene expression and prevent differentiation. Conversely, cells or tissues grown in the Rotary Cell Culture System™ are suspended by randomising the gravity vector so they are, in effect, in continuous free-fall through the culture medium. A good analogy would to imagine a cell falling through a fluid-filled tube of infinite height. The tissue would fall, tumble, mix in the media and without any single gravity vector dictating growth direction; the tissue would grow in all directions. That is mechanism of the RCCS™. Destructive stress forces are insignificant because this system has no propellers, airlifts, bubbles, or agitators. Because the cells are maintained in a gentle fluid orbit, the cells are allowed to co-localise, communicate with each other and form three-dimensional structures. Furthermore, the RCCS™ is the only system currently available that allows researchers to introduce co-cultures that provide spontaneous tissue differentiation, higher cell multiplication, decreased cell death, and increased secretion of cell products. More than 50 cell and tissue types have been successfully cultured in the RCCS™. Cells not only grow, but also develop into functional specialised tissue organoids. Scientists from academia, industry and government have published over 70 peer-reviewed articles utilising RCCS™ technology the in scientific literature. These publications clearly show that the RCCS™ is the best technology available for growing differentiated mammalian tissue in vitro. (www.synthecon.com)

6.3 Rotating Wall Perfused Vessel

The RWPV was designed specifically to allow the culture of shear-sensitive mammalian cells in a microgravity environment. The culture times vary from several days on the Space Shuttle to several months to the Space Station. Thus, the bioreactor system provides capabilities for replenishing fresh medium, as well as monitoring and control oxygen, pH, and temperature through the use of a recirculating (perfusion) flow loop. As with all bioreactor designs the bioreactor system must provide a proper biological environment for the cells. A best environment is furnished by a perfused system, the vessel is provided of an adequate distribution of inlet fluid throughout the vessel. This requirement in the RWPV is even more stringent. To perform meaningful cell science experiments, the goal is to provide a nearly spatially uniform environment, with adequate mass transport. That is, the fluid in the vessel should be well mixed, with a mixing time that is short compared with the time scale of the cell responses. All cells should experience nearly the same environment so that responses to particular environment can be isolated. The cells on microcarrier and cell assemblages must be suspended in the fluid medium. Interaction with solid walls result in large shear and normal stresses and should be minimised. Settling or centrifuging of cells onto walls results in poor mass transport and/or high stress levels. Unfortunately, the operating conditions for suspension in unit gravity and in microgravity are quite different and result in differences in hydrodynamic environment between cell science experiments in space and ground control experiments on Earth.

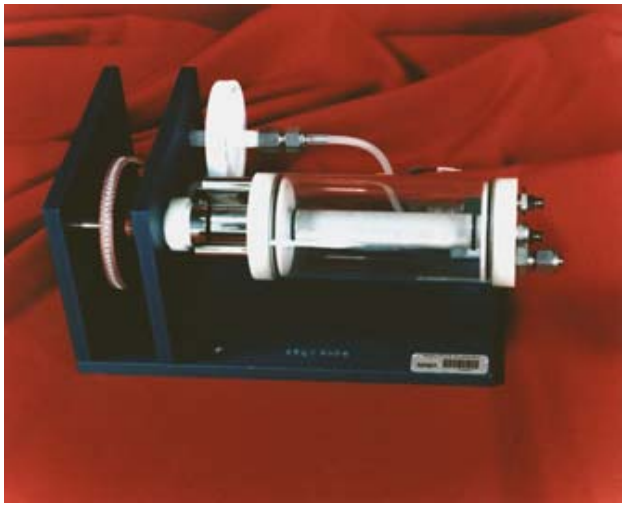

Figure 4: Syntecon Rotating Wall Perfused Vessel geometry Oxygenated by a center core membrane oxygenator, these reusable vessels are used for microcarrier cell culture and explant tissue cultures.(Photo Synthecon)

The RWPV bioreactor, based on a viscous pump design, has successfully cultured mammalian cells both on Earth and in microgravity. The vessel has a concentric cylinder arrangement, with length of 7 cm, an outer diameter of 5 cm, and inner cylinder of 1.5 cm (the lengths are given just for fix a proportion of the model bat they can change for different configurations). The inner cylinder is porous, with a filter material covering the exit holes for

extracting fluid into the external perfusion flow loop. There is a disk attached to one end of the inner cylinder. Both the inner and the outer cylinders can be independently rotated. The inlet for the fluid from the external perfusion flow loop is located between the disk and vessel end cap, providing a means of keeping cells on microcarriers from entering the narrow (high shear) region behind the disk. When operated on Earth, the solid body rotation mode is used where the inner cylinder, disk, and outer cylinder are all rotated at the same rate, between 15 and 35 rpm. As the cells grow and form aggregates, the rotation rate is increased in order to maintain off-bottom suspension as the cells fall through the fluid under influence of gravity. For most cell types, these rotation rates are high enough to keep cells moving in circular orbits about the spin filter. For larger, more-dense cell cultures, a hover mode is used in which the rotation rate is just high enough to keep cell structures suspended in a stationary location.

In microgravity, rotation is not required to suspend the cells; however, some fluid motion is required for adequate mass transport. A differential rotation mode is used where the inner cylinder and disk rotate together at a higher rate than the outer wall. The disk rotation drives a secondary flow pattern in the radial-axial plane that enhances mixing and mass transport. However, care must be taken to insure that rotation transport. However, care must be taken to insure that rotation rates are chosen that will provide adequate mass transport and proper cell suspension without clustering cells in one end or centrifuging them to the wall.

Referring to Figure 5 , the general organisation of the device is illustrated and some technical detail are reported from the U.S. patent n. 5,026,650 ; Horizontally rotated cell culture system with a coaxial tubular oxygenator from Schwarz Ray P. A frame means 10 has vertical and spaced apart plates 11, 12 which support a motor pulley 14 and a housing pulley 13 where the pulleys 13, 14 are connected by a belt drive 15. The motor pulley 14 is coupled to a motor 16 which can be controlled in a well known manner to provide a desired drive speed.

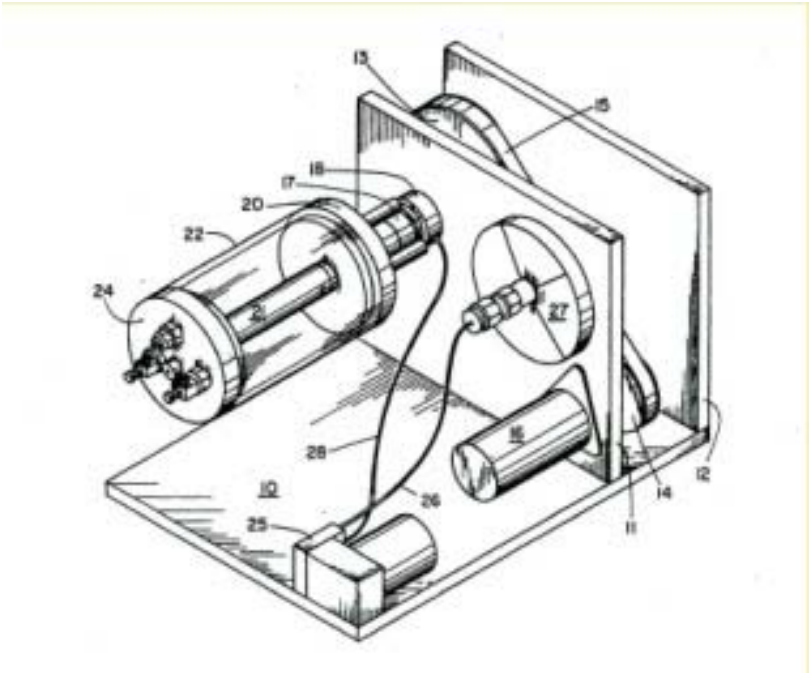

Figure 5: Synthecon's Rotating Wall Perfused Vessel schematic diagram

The housing pulley 13 is connected to a drive shaft 17 which extends through a rotative coupling 18 to an inlet end cap 20. The inlet end cap 20 is attached to a central assembly 21 and to a tubular outer culture cylinder 22. At the other end of the central assembly 21 and the culture cylinder 22 is an outlet end cap 24. An air pump 25 on the frame means 10 is connected by input tubing 26 to a filter 27. An output tubing 28 from the pump 25 couples to the rotative coupling 18 where the air input is coupled from a stationary annular collar to an internal passageway in the rotating drive shaft 17.

Referring now to Figure 6 , the cell culture system of the present invention is illustrated in partial cross section where the rotative coupling 18 receives the output tubing 28 and the drive shaft 17 has a central air inlet passageway 30 for the passage of air.

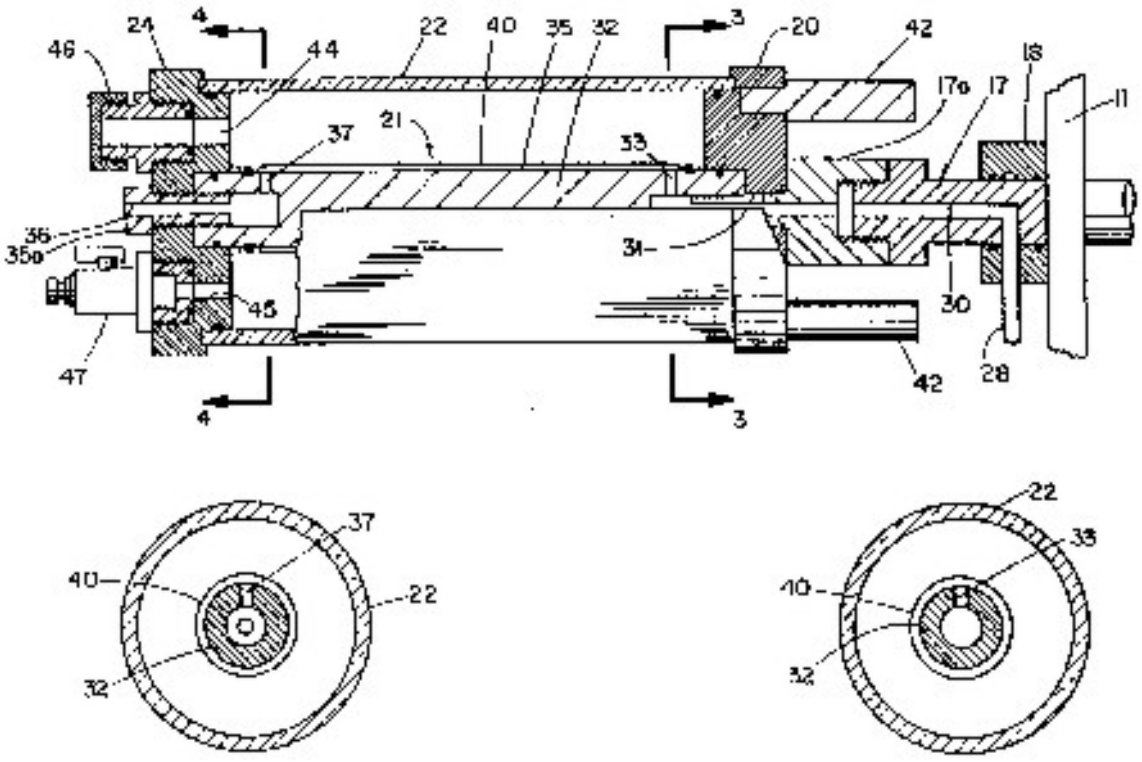

Figure 6: Schematic diagram of the vessel of the Synthecon Rotating Wall Perfused Vessel

The drive shaft 17 is attached to a coupling shaft 17a which extends through a central opening 31 in the inlet end cap 20. The coupling shaft 17a is threadedly attached to a cylindrically shaped, central support member 32. The central passageway 30 extends inwardly through the shafts 17, 17a to a transverse opening 33 which couples the air inlet passageway 30 to the exterior surface 35 of the central support member 32. The central support member 32 is sealingly received in a counterbore in the inlet end cap 20 and at its opposite end, the support

member 32 is sealingly received in a counterbore of the outlet end cap 24. A tubular outlet member 35a is threadedly attached through a bore in the outlet end cap 24 to a blind bore in the support member 32 and an air exit passageway 36 in the outlet coupling is connected by a transverse opening 37 to the exterior surface 35 of the central support member 32. A tubular oxygen permeable membrane 40 is disposed over the central support member 32 and has its ends extending over the openings 33 and 37 in the central support member 32 so that the membrane 40 can be sealingly attached to the central support member 32 by 0-rings or the like. Thus an air passageway is provided for an input of air through the passageway 30 and the transverse opening 33, through the annular space between the inner wall of the membrane 40 and the outer wall of the central support member 32 to the exit transverse opening 37 and to the exit passageway 36. The membrane 40 may be made of silicone rubber which operates under air pressure to permit oxygen to permeate through the wall of the membrane into the annulus of fluid medium surrounding the membrane and carbon dioxide to diffuse in the opposite direction. Coaxially disposed about the central support shaft 32 is a tubular outer cylinder 22 which can be glass. The cylinder 22 is sealing received on the end caps 20, 24 and defines an annular culture chamber between the inner wall of the cylinder 22 and the outer surface of the membrane 40. On the inlet end cap 20 are circumferentially spaced apart cylindrical members 42. When the coupling shaft 17a is detached from the shaft 17, the members 42 provide a base for standing the cylinder 22 upright or in a vertical position for sampling, changing or adding fluids to the system. In the outlet end cap 24, there are two or more access ports 44, 45 each having closure means 46. A hypodermic needle with fluid medium can be inserted through one access port to inject fluid when withdrawing fluid from the other port. In this regard samples or media can be withdrawn without forming an air space, thereby preserving the zero head space. The present device thus involves the central cylindrical core which is a source of oxygenation through the cylindrical membrane and the membrane and outer wall of the vessel are rotated about a horizontal axis. This involves a type of clinostat principal, i.e. a principal that fluid rotated about a horizontal or nearly horizontal axis can effectively suspend particles in the fluid independent of the effects of gravity. The rotational speed of the cylinder 22 effectively eliminates the velocity gradient at the boundary layer between the fluid and the cylinder wall. Thus, shear effects caused with a rotating fluid and stationary wall are significantly reduced or eliminated.

In an experimental use a high density culture of baby hamster kidney cells were produced by utilising beads constructed from cytodex 3 material and utilising a microcarrier medium prepared with low bicarbonate and 20 mM HEPES buffer, 10% fetal calf serum and antibiotics (pH 7.35+10 ml/1 NaOH (ln) where the seeding density of the cells were 6 to 8 cells per bead and the bead density was 15 mg/ml. In the process the vessel was soaked in ethyl alcohol overnight, rinsed with Millipore quality water, wrapped and autoclaved. After cooling, the vessel was rinsed with complete medium prior to loading the beads. The beads were loaded into the vessel via the syringe barrel attached to one of the ports and the vessel was filled with medium and placed in an incubator and allowed to run for about 30 minutes to equilibrate while preparing the cells. The stored frozen cells were thawed, suspended in the medium, counted, diluted and loaded into the vessel via a syringe attached to one of the ports. The vessel was subsequently filled with culture grown medium and rotation rate was set at 10 rpm. The air pump was actuated to move incubator air across the exchange membrane. Samples were withdrawn for cell counts at 1, 3 and 5 hours and approximately 24 hours thereafter. The results of the tests were that the cells attach and grow on the beads at the high

density of 15 mg/ml. The total cell counts at maximum cell number showed about 1.1.times.10.sup.9 cells at 168 hours. The attachment of cells to the beads occurred normally within the first hour. Fluid media change was made every 48 hours. Cell growth cultures have been successfully produced by use of the present configuration in development of human colon carcinoma cells, human embryonic kidney cells, tobacco callus cells, normal human embryonic kerotinocytes cells, normal human colon fibroblast cells, human promyleocytic leukemia cells, bovine embryonic kidney cells, normal embryonic lung cells, mouse melanoma cells, mouse hybridoma cells. As can be appreciated, the ability to grow mammalian cell cultures in a controlled environment is significant. The present configuration has excellent operating characteristics between 5 and 40 RPM for culture of anchorage dependent cells on microcarrier beads. It is possible to vary the angular rotation rate in order to induce secondary flow patterns within the vessel, at the expense of transient fluid velocity gradients at the wall boundary layers, useful for distributing nutrients or waste products.

The entire cylindrical vessel is rotated to suspend the cells by rotation of the vessel so that suspension of the cells is such that the beads are not agitated into contact with one another. At the same time the velocity gradient at the boundary layer of the fluid and the vessel wall is minimised. As a result of the horizontal orientation and clinostatic suspension of the particles, less mixing force is required to move a particle from the bottom to the top of the vessel. The zero head space results in no air bubbles which cause disruption of fluid streamlines thus subjecting the culture to adverse shear effects. The central gas exchange membrane permits a uniform dispersal of component gases from a central core to the fluid medium and reverse to allow culture respiration.

6.3.1 Fluid Dynamic within a Rotating Vessel

There has been very large number of experimental and theoretical studies of flow between concentric rotating cylinder (circular Cuette flow) in the century since the earliest studies were conducted by Mallock (1888, 1896) and Cuette (1890). The Navier-Stokes equations for flows far from equilibrium of course have in general multiple stable solutions. A graphic demonstration of this non-uniqueness was provided by Coles' (1965) discovery that, depending on the path followed in junction of the transition curves can be studied by bifurcation theory (normal forms in codimension-2 theory). The transition at large Reynolds number should serve as guide for later theoretical and numerical work.

The mathematical model and numerical solution for the flow field is shortly overviewed. Assumption for this mathematical simulation were as follows:

1. The culturing medium, or substrate, was a Newtonian fluid with constant density ρ_s = 1.02 g/cm^3 , and a constant viscosity μ = 0.0097 g/cm s
2. Particles were spherical in shape with a diameter d = 175 μm and a density ρ_p = 1.04 g/cm^3 , did not interact with one another, and did not affect the flow of the culturing medium.
3. The flow of the culturing medium caused by the rotation of the concentric cylinders was a laminar, axial symmetric Couette flow governed by the Navier-Stokes equation.
4. The forces acting on a particle were drag from the fluid’s circulation, buoyancy from the gravitational force relative to the difference between the densities of a particle and

the fluid, and centrifugal force from the rotation of the vessel. The numerical values in assumption 1 and 2 correspond to anticipated early stages of cellular growth in the bioreactor using Cytodex-3 microcarriers in a standard medium with 10% fetal calf serum.

The unique advantage of this concentric rotating bioreactor was that the fluid shear force created by the differential rotation of the two cylinders would be more uniform and controllable, and thereby providing better-controlled environment for understanding the effects of fluid shear on three-dimensional cell culture. When there is a steady flow of constant density around a submerged spherical bead, the fluid will exert a force on the solid bead surface . This force maybe categorised in two parts: the background force and the kinetic force. To describe the background force, the flowfield between two concentric rotating cylinders is used. The velocity profile can be derived by the balance of the centrifugal force, pressure gradient force, and viscous force. The background shear stress can be estimated from the velocity gradient, and we projected this force onto the plane of characteristic area for convenience of comparison with the kinetic force. The kinetic force point in the same direction as the approach velocity and is defined as a product of a characteristic area, the kinetic energy, and the drag coefficient. This force can also be called the drag force. In our calculations, we use the terminal velocity for the steady-state fall of a spherical bead as the approach velocity. The total force experienced by a 175 μm particle was about 0.6 dyne/cm^2. When the approach flow velocity to a particle was kept constant, smaller particles experienced stronger viscous effect. Therefore, at the beginning of cell culturing the cell would experience a stronger drag force per unit area from the same approaching flow velocity. Hence, minimising the flow velocity in the early stages of the cell culturing becomes important. This represents an ideal use of microgravity, since during the early stages of the cell culturing there is already a sufficient supply of oxygen and nutrients so that with the absence of sedimentation the bioreactor does not need to be rotated at all, i.e. quiescent environment can be maintained while the initial aggregates of cells and microcarriers become established.

Because of the very limited rotation rates required to maintain low mechanical stress levels, the flow must remain laminar. The fluid is assumed to be incompressible and of uniform density and viscosity. The symmetry of the geometry and boundary conditions leads to the assumption of an axisymmetric flow field. The solution is independent of the azimutal direction and can be obtained in a radial-axial plane. The flow pattern can be visualised using the interactions of stream surfaces with a radial-axial plane. For the steady-state solution, these lines would coincide with fluid element's paths viewed in a plane rotating in the azimuthal direction with the fluid element. These lines are called streamlines, although they are not tangent to the local velocity vectors (only the radial and axial components). It should always be remembered that the primary fluid motion is in the azimuthal direction. It is useful define a measure of the mechanical shear forces that exist in the flow field. The three shear stress components in the fluid are:

$$S_{r\theta} = \mu\left[r\frac{\partial}{\partial r}\left(\frac{v}{r}\right)\right]$$

$$S_{\theta z} = \mu\left[\frac{\partial v}{\partial z}\right]$$

$$S_{rz} = \mu\left[\frac{\partial w}{\partial r} + \frac{\partial u}{\partial z}\right]$$

These act on three mutually perpendicular (co-ordinate plane) areas to produce the resulting traction forces. Thus, a meaningful metric of the shear force per unit area is;

$$S_m = \frac{1}{3}\left(S_{r\theta}^2 + S_{\theta z}^2 + S_{rz}^2\right)^{1/2}$$

This measure can be used to compare the stress levels in the RWPV operated under different operating conditions. It must be point out that this measure does not include shear forces resulting from relative motions due to density differences existing in acceleration fields.

6.4 The Rotary Culture Max

The new "Rotary Culture Max" version of the Rotary Cell Culture System [TM] uses the proven, patented NASA tissue growth technology.

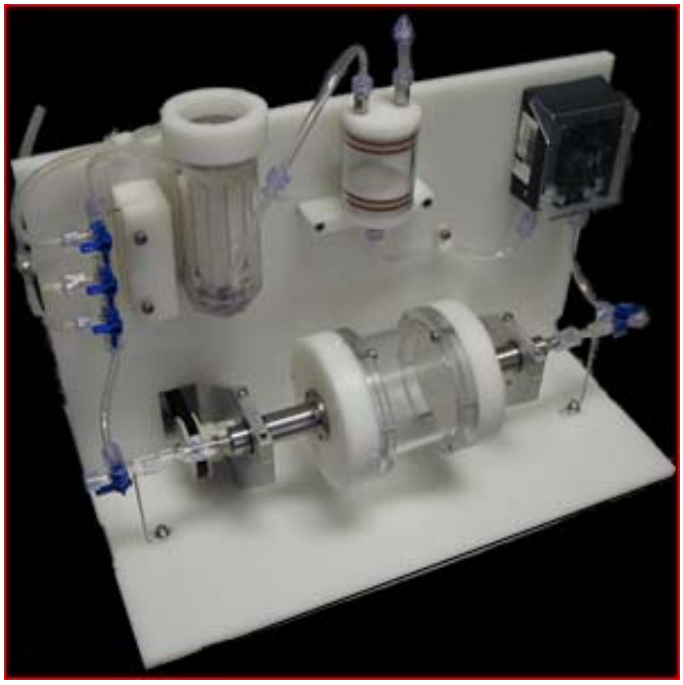

Figure 7: Synthecon's New Rotary Culture Max (photo Synthecon)

In this new design, the central tubular filter of the previous perfused system is replaced with a large flat filter that covers the inside surface of the leeward endcap, creating an unobstructed culture chamber. The volumes of this open chamber design "Rotary Culture Max" can be

scaled up to grow larger three-dimensional tissues and organoids and enhance production of commercial quantities of hormones, enzymes and biopharmaceutical proteins from human cells and tissues. The "Rotary Culture Max" uses an external gas transfer oxygenator that functions very much like the membrane of the lungs to deliver dissolved oxygen into and remove carbon dioxide from the fluid culture medium. The cells and tissues draw dissolved oxygen out of the recirculating culture fluid much as human tissue cells obtain dissolved oxygen from the blood. The flowing culture fluid also feeds the growing tissues. This growth medium is changed as necessary to replenish nutrients, remove metabolic wastes, control acidity or to harvest secreted products.

Operation is beautifully simple. The cylindrical growth chamber is filled with the growth fluid medium and the cells or tissue materials are added. The filled cylinder is rotated about its horizontal axis to suspend the tissue particles. Cells or tissue particles go into uniform fluid suspension orbits within the horizontally rotating culture vessel. Destructive turbulence and collision forces are minimised because the culture medium, cells and tissue particles all rotate with the vessel and do not collide with the vessel or any other damaging objects. The absence of damaging stress allows the tissue to grow into three-dimensional "organoids" that have the differentiated structure and function of human tissue in the body.

6.5 Hydrodynamic Focusing Bioreactor

The HDFB developed by NASA provide a unique hydrofocusing capability that simultaneously enables a low-shear culture environment and unique hydrofocusing-based "herding" of suspended cells, cell aggregates, and air bubbles.

Two main limitation where observed in the NASA Rotating Wall Vessel, the first is the difficulty to remove air bubbles from the vessel without degrading the low-shear culture environment or damaging the delicate three-dimensional tissue assemblies. Second is in its ability to control the location of cells and tissue aggregates within the vessel. The HDFB, based on the principle of hydrodynamic focusing, overcomes this limitations. The HDFB provides the ability to control and remove bubbles from the bioreactor without degrading the low-share culture environment or the delicate three-dimensional tissue assembling. It also provides unparalleled control over location of cells and tissue within the bioreactor vessel during operating and during sampling. The HDFB is a rotating dome-shaped cell culture vessel with a centrally located sampling port and an internal rotating viscous spinner attached to a rotating base. The vessel and viscous spinner can rotate at different speeds and in either the same or different directions. Adjusting the differential rotation rate between the vessel and spinner results in a controllable hydrodynamic focusing force. The resultant hydrodynamic force suspend the cell in low-shear fluid environment that supports the formation of delicate three-dimensional tissue assemblies. For three-dimensional cell culture, cells are inoculated into the vessel either with or without beads or scaffolds for cellular attachment. The vessel is filled completely with culture medium and rotation rates are set to maintain suspension. Oxygenation is provided to the cell culture through an oxygenator membrane that is integral to the vessel.

With reference to Figure 8, the configuration of the bioreactor is described in order to fix some idea on technical details. The bioreactor comprises a dome-shaped culture chamber 1 having an interior volume 2, a bottom circular ridge 3, walls having at least a partially curved surface 4 and an apex 5. The circular ridge of the culture chamber is fluidtightly integrated with a gas-permeable membrane 6. The membrane-side of the culture chamber is fluidtightly integrated with a rotating base 7 in an orientation where the axis of symmetry of the dome-shaped culture chamber 8 is substantially horizontal. The culture chamber can be made of any material that is compatible with the culturing system, including glass, plastic, or stainless steel. Preferably, the vessel comprises a material that can be sterilised by ethylene oxide,

gamma irradiation, or autoclaving, or, alternatively, is disposable. The interior volume 2 of the culture vessel can vary greatly, depending upon the types of cells, amount of cells to be produced and available laboratory space. In a preferred embodiment, the volume of the substantially dome-shaped vessel is between about 10 mL and about 10 L. Small and medium

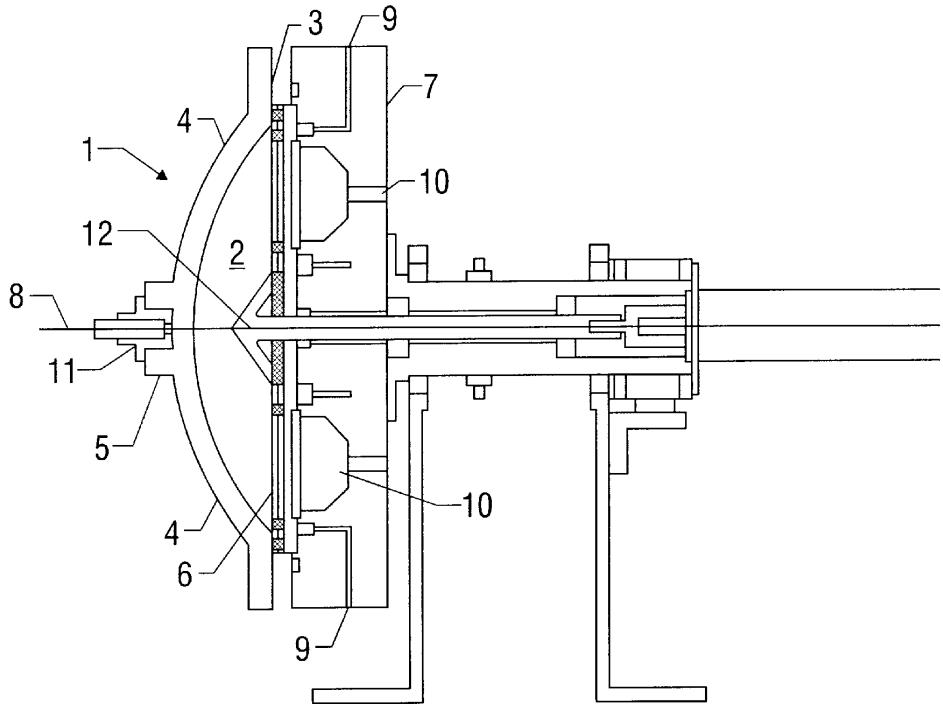

Figure 8:Schematic diagram of a bioreactor having a dome-shape culture vessel (U.S. Patent Office n. 6,001,642)

scale laboratory cultures can preferably be performed in vessels of 100 mL, 250 mL, and 500 mL volumes. Larger preparative scale cultures can preferably be performed in vessels of 1 L, 5 L, and 10 L volumes. The gas permeable material 3 can generally be any material compatible with the culturing system, i.e., to allow flow of appropriate gases and to restrict flow of cells and liquid nutrients across the membrane. For example, the gas permeable

membrane can comprise polytetrafluoroethylene, polyethylene, or porous hydrophobic TEFLON.RTM. membrane. The gas permeable membrane may additionally allow flow of liquids, nutrients, and metabolites across the membrane. The bioreactor can further comprise an access port or a plurality of access ports to the culture vessel 9, 10, 11. The access ports allow transfer of materials and gases into and out of the bioreactor. Access ports can lead to the surface of the gas-permeable membrane 10 or can lead directly into the culture vessel 9, 11. The access ports leading directly into the culture vessel can go through the gas permeable membrane 9 or can go through the wall or apex of the culture vessel 11. The access ports can be any type of port used with culture or reaction vessels, including valves and membranes that can be penetrated by tubing, syringe, pipette or other sampling device. The bioreactor can further comprise a agitator within the culture vessel 12. This agitator can be any type of device that provides additional agitation to the culture mixture, including a magnetic spinner or a mechanically driven propeller 12. The invention is further directed to a method for culturing cells. The method according to the invention comprises providing the inventive bioreactor, adding cells and appropriate nutrients to the interior volume of the dome-shaped culture chamber, and rotating the base at a rate that generates suitable mass transfer and low shear stress. Where the mass transfer of the culture solution is provided by both the rotation of the culture chamber and by spinning an agitator in the culture vessel, the combined effects of these means must be weighed in determining the respective rotation rates. The culturing of cells is preferably performed under conditions that optimise mass transfer and create low shear forces. Preferably, the culturing cells are subjected to average shear forces of less than about 1 dyne/cm.sup.2, more preferably less than about 0.75 dyne/cm.sup.2, and most preferably less than about 0.5 dyne/cm.sup.2. The bioreactor and culturing method can be use for culturing any type of cell. In a preferred embodiment, the cells are preferably mammalian cells. The cells can be adherent cells or non-adherent cells, single cells or multicellular aggregates. Adherent cells are attached to a substrate, such as microcarriers, fibrous supports or other cells. The culturing method further comprise adding nutrients that enhance cell production and growth and removing waste products from the culturing process. Preferably, the gases are transferred across the gas permeable membrane 3. Alternatively, gases, nutrients or cell samples can be extracted from a port extending through the gas permeable membrane 9 or other access ports in the culture vessel wall or at its apex 11. For example, gas bubbles can be removed or culture fluid samples taken from an access port at the culture vessel apex to avoid disrupting the operation of the bioreactor. The culture vessel can be temporarily tilted to position the apex of the culture vessel so that bubbles rise to the apex access port.

The rates of rotation of the vessel and spinner are limited to a range in which the radial, circumferential, and axial velocity were large enough to suspend particles and provide adequate mixing, but small enough to prevent turbulence. Relative rotation rates may be determined and optimised by simulating flow fields with software such as FLUENT or FIDAP (both are commercially available from Fluent, Inc., Lebanon, N.H.). The unique advantage of this rotational perfuised hemispherical bioreactor is that the fluid shear force created by the differential rate of rotation of the dome and spinner would be more uniform and controllable. Numerical studies have been conducted to evaluate the flow field distribution within the HDF bioreactor vessel and to determine the magnitude of the hydrodynamic forces applied to cellular structures within the reactor. The following assumption were applied to the numerical model:

1. The culture medium was a Newtonian fluid with a constant density of 1.02 g/cm^3 and a constant viscosity of 0.0097 g/cm s.
2. Cellular structure were spherical in shape with diameter of 175 microns and a density of 1.04 g/cm^3. They did not interact with one another, and did not affect the flow of the culture medium.
3. The flow of the culture medium was laminar, axially symmetric flow governed by Navier-Stokes equation.

The forces acting on a cellular aggregate are drag from the fluid's circulation, buoyancy from the gravitational force relative to the difference between the densities of cellular structures and the fluid, and centrifugal force from rotation of the vessel. Rotation of the vessel under the influence of gravity, combined with changing particle velocity due to interaction with the spinner, keeps the particles focused between the dome and the viscous spinner and causes the heavier particles to move farther from the tip of the viscous spinner.

6.6 Random Positioning Machine

What the Clinostat AND Rotating Wall Vessel achieves in two- dimensional space, the RPM achieves in three-dimensional space: the weight vector experienced by the organisms of an experiment continuously changes its direction in three-dimensional space with a frequency of more than 1 Hz (Fig. 1).

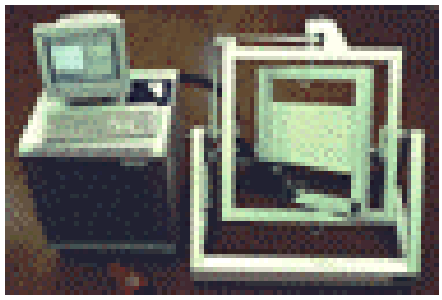

Figure 9: Prototype of the Random Positioning Machine at Fokker Space and Systems in Leiden, The Netherlands. (photo Fokker Space)

An experiment is mounted at the centre of a cardanic framework, the rotations of which are driven by two independent motors. The movement of the experiment is controlled by software providing four basic modes of operation:

1. centrifuge mode around one vertical axis only,

2. & 3. two clinostat modes around one horizontal axis or around the other horizontal axis , perpendicular to the first one,

4. random positioning mode, following a path of calculated positions based on numbers from a random generator.

In all cases, the so-called walk speed, defined as the speed of a given point on the experiment projected on a sphere of fixed diameter, can be set at a constant value. By adding selection

criteria of the random positions generated, the machine can be instructed to avoid a succession of positions in the same space segment, which might distort the time-averaged microweight condition. Similarly, the machine can be instructed to spend more accumulated time in a particular space segment, resulting in average weight vectors of some pre-established values. Such a capability allows the investigator to impose onto the experiment values in between normal weight and microweight for the purpose of establishing threshold levels of the organism under study.

7 Modelling of Flow Field in Computer Simulation

An other important method of research is 'computer simulation'. This chapter is a short introduction on computer simulation. The goal of simulation is to quantify the flow within the culture chambers and calculate important flow parameter related to cell behaviour. The simulation are conducted with commercial software implemented for various Fluid Dynamic research. Two example of finite element programs are POLYFLOW and FLUENT. These two programs use mathematical models derived from conservation equations for mass, momentum and energy using a control volume-based, finite-difference method to simulate fluid flows. POLYFLOW is optimised for simulation of non-Newtonian and viscoelastic flows, and FLUENT is a more general program for Newtonian flow and simple non- Newtonian flow. In each program, the Navier-Stokes equations provides velocities, pressures, solutions of values of the stream function, and components of the rate of deformation and vorticity tensors for specific control elements given specific input conditions. Both the programs allows three-dimensional simulation of the flow field. However often the problem can be simulated as a two-dimensional model because of axisymmetric geometry. The mesh density of the simulation should be adapted at the accuracy of the investigation. However a phase of tuning is required till is reached a value of the mesh significantly independent from variation in its density. The concentration of the nodes should be concentrated in critical region that are proper of each geometry (e.g. orifice regions).

In order to give an example an experience of Gregorides is reported. In his study he analysed the cell damage within in a flow-contraction apparatus (Gregorides N. 1999). The contraction flow used to induce cell damage is a fast transient extensional flow. It is characterised by finite spatial regions of high extension and short-residence time. The flow into a contraction is simple, but the flow field is inhomogeneous, with pure extensional flow only along the centerline. The flow is predominantly extensional very close to the orifice, but the simulation domain is defined with long entry and exit sections. Two flow field parameter were used to characterise the flow with respect to cell damage: energy dissipation and dimensionless ratio, W_{rel}/S, which is a ration of the time scale of liquid deformation to time scale of rotation and similar to the flow classification parameter developed by Garcia-Briones already mentioned through the report. The energy dissipation, which is commonly used by mixing researchers and practitioners, is a measure of the energy transferred to a control volume.

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Appendix I

CNR research on Endothelial Cells

CNR research on Endothelial Cells

One of Major problems of growing tissue in microgravity remains the absence of spontaneus formation of blood vassals

The CNR research, propose to study cultured endothelial cells on Earth in simulated microgravity (RWV) and in hypergravity conditions. Due to the complexity of this study, a multiple approach is intended to use: NMR spettroscopy will even be performed to focus energy metabolism and molecular biology to investigate gene expression.

Tissue engineering is focused on the creation of properly assembled and oriented tissue with the specific aim of generating functional 3D structures of suitable size to be used in transplantation or in vitro studies as drug toxicity.

In microgravity, either gravity induced low forces (buoyancy, sedimentation and hydrostatic pressure) act on cellular structures and absence of fluid movements (no differences in density) produces abnormal distribution of cellular nutrients and metabolites. Microgravity may faster a condition in which cells mainly turn energy utilisation to structural and functional aspects of growth. Microgravity seems to affect the organisation of the cytoskeleton.

The mechanical environment of the endothelial cell influences its structure and function: flow and the associated shear stress plays an important role in its regulation and influence its gene expression.

One of the major problem of growing tissue in microgravity (in spite of low shear stress) is that spontaneous formation of blood vassal has failed so far**.**

There are several limitation in the 3D tissue, such as the restricted diffusion of nutrients and oxigen that limits their size. Spheroids larger then 1 mm in diameter generally contain a hypoxic, necrotic center. A major focus of tissue engineering is how to best generate functional 3D constructs large enough to save as replacement organs or suitable for in vitro studies of for example, drug toxicity or oxigen diffusion in tissues.

All Tissue, will eventually require internal blood vassal like condutcs for the delivery of oxigen and nutrients as well as for the removal of waste products. Inclusion of endothelial cells in the mixture of cells cultured in the RWV bioreactors, with the stated purpose of generating vassal-like conduits, has so far failed to yeld spontaneous formation of blood vassal in situ.

In these co-culture, endothelial cells remain viable and organize into hemotypic clusters without forming tubular structures…

Appendix II

Proposal to the Italian Space Agency (ASI)

"Ricerche di base per le Tecnologie Spaziali e ricerche in microgravità"

Agenzia Spaziale Italiana
Viale di Villa Grazioli, 23 - 00198 Roma

8.1 Tel. 068567306 - Fax 068567304

PROPOSAL APPLICATION
IN RESPONSE TO ANNOUNCEMENT 2001

NEW PROPOSAL

1. AREA: Ricerche di base per le Tecnologie Spaziali e ricerche in microgravità Linea strategica: Tecnologie Futuribili Indirizzo di ricerca: /	**8.1.1.1.1 Riservato ASI** Numero:

2.Title of project: Development of a new bioreactor for mechanobiologic research in a microgravity environment.

3. Principal Investigator: *8.1.1.1.1.1.1.1 Position:* **Institution:** **Office /Organization Division:** **Address:** **Telephone:** **Fax:** **e-mail:**	*First name*: Gabriele *Last name*: Dubini *Degree: Ph.D.* *Associate Professor* Politecnico di Milano Dipartimento di Ingegneria Strutturale Piazza Leonardo da Vinci 32, 20133 Milano, Italy +39.02.2399.4283 +39.02.2399.4286 gabriele.dubini@polimi.it

9

4. If the project has passed/presented to an international announcement of opportunity (AO), specify name, date and origin of the AO.

5. Research group(s) (duplicare la tabella per ciascuna Unità Operativa)

U.O. N. 1 ***Politecnico di Milano***	*First name Last name*	*Institution*	*Position*	*Time (months)*	*Other ASI project*
Responsible	*Gabriele Dubini*	*Dip. Ing. Strutturale*	*Associate Professor*	*3*	*-*
Participants	*Roberto Contro*	*Dip. Ing. Strutturale*	*Full Professor*	*1*	*-*
	Riccardo Pietrabissa	*Dip. Bioingegneria*	*Full Professor*	*1*	*-*
	Pasquale Vena	*Dip. Ing. Strutturale*	*Assistant Professor*	*2*	*-*
	Manuela Teresa Raimondi	*Dip. Bioingegneria*	*Research Fellow*	*2*	*-*
	Federica Boschetti	*Dip. Ing. Strutturale*	*Research Fellow*	*2*	*-*
	Francesco Migliavacca	*Dip. Bioingegneria*	*Research Fellow*	*2*	*-*
	Borsista	*Dip. Bioingegneria*	*Fellowship*	*11*	

U.O. N. 2 ***Carlo Gavazzi Space S.p.A.***	*First name Last name*	*Institution*	*Position*	*Time (months)*	*Other ASI project*
Responsible	*9.1.1.1.1.1 Fabio Tominetti*	*CGS*	*System Engineer*	*4*	*-*
Participants		*CGS*	*System Engineer*	*3*	*-*
		CGS	*System Engineer*	*3*	*-*
	9.1.1.1.2 Silvano Campiotti	*CGS*	*System Engineer*	*3*	*1*
		CGS	*Fellowship*	*11*	
	9.1.1.1.3 Gianfranco Santoruvo				
	9.1.1.1.4 Mauro Villa				
	9.1.1.1.5 Andrea Guidi				

U.O. N. 3 ***CNR***	*First name Last name*	*Institution*	*Position*	*Time (months)*	*Other ASI project*
Responsible	*Silvia Bradamante*	*CNR*	*Senior scientist*	*2*	
Participants	*Jannette M. Maier*	*UNI*	*Research Fellow*	*2*	
	Livia Barenghi	*UNI*	*Collaborator*	*3*	
	Maria Bonfa'	*CNR*	*Technician IX*	*3*	
		UNI	*Fellowship*	*2*	
	9.1.1.1.5.1 Sofia Carlsson	*UNI*	*Fellowship*	*11*	
	Borsista				

6. Personnel dedicated to the project in 2002

	Number	months
Researchers:	10	15
Students (PhD, fellowship)	5	37
Collaborators	1	3
Technicians:	5	16

7. Estimate of the expenditure
(duplicare la tabella per ciascuna Unità Operativa)

9.1.1.2 UNITA' OPERATIVA N. 1 (Politecnico di Milano)		9.1.1.3 **ESTIMATE OF THE EXPENDITURE FOR 2002**	
	DESCRIPTION	***MLT***	***EURO***
Consultants/Fellowships	*Research fellowship*	30	15,494
Industrial activities			
Equipments *	*Metering micropumps* *Rotatory viscosimeter* *Microscopy optics*	30	15,494
Supplies	*Row materials*	3	1,549
Travels	*Italy* *Europe* *No Europe*	4 5 5	2,066 2,582 2,582
Other expenses	*Overheads*	3	1,549
10 TOTAL		80	41,317

10.1.1.1 UNITA' OPERATIVA N. 2 (Carlo Gavazzi Space S.p.A.)		10.1.1.2 **ESTIMATE OF THE EXPENDITURE FOR 2002**	
	DESCRIPTION	***MLT***	***EURO***
Consultants/Fellowships	*Research Fellowship*	30	15,494
Industrial activities	*Assessment of Scientific Requirements, Definition of Engineering Requirements, Realization of the prototype*	45	23,241
Equipments *	*Rotating Positioning Machine (Fokker Space), tools and jig*	40	20,658
Supplies	*Row Materials*	15	7,747
Travels	*Italy* *Europe* *No Europe*	1 2 4	516 1033 2066
Other expenses	*Overheads*	5	2,582
11 TOTAL		142	73,337

11.1.1.1 UNITA' OPERATIVA N. 3 (CNR)		11.1.1.2 **ESTIMATE OF THE EXPENDITURE FOR 2002**	
	DESCRIPTION	***MLT***	***EURO***

Consultants/Fellowships	*Research Fellowship*	20	10,329
Industrial activities			
Equipments *	*Bioreactor RWV (Synthecon)*	20	10,329
Supplies	*Biological cultures* *Chemical supplies*	35	18,076
Travels	*Italy* *Europe* *No Europe*	0 2 4	0 1,033 2,066
Other expenses		0	0
12 TOTAL		81	41,833

* specify each equipment with value > 10 Mlt

8. Equipment and services available for the project
(organizzato per Unità Operative)

UNITA' OPERATIVA N. 1 (Politecnico di Milano)
The activity of the Politecnico di Milano Research Unit is based at the Laboratory of Biological Structure Mechanics (LaBS). The LaBS, formed by 7 locals extending on a 500 squared meters area, accommodates a computer centre and an experimental laboratory. The computer centre has two HP workstations and 18 PCs in a local area network and several peripherals. The available software enables Computer Aided Design, solid modelling from diagnostic images, finite element and simulations of both solid and fluid continua (structural and fluid dynamics analyses). The experimental lab has 6 mechanical testing machines equipped also with a hip and a knee MTS simulators, devices for fatigue testing on hip and dental prostheses, a femoral test bench for trauma devices. There are also test benches for cardiac surgery medical devices, such as blood oxygenators, cardiac valves, vascular prostheses and a mock loop of the human circulation to experience in using artificial ventricles. More in detail, the equipment available for the project is:
- Leica Stereoscan S260 electron microscope
- MTS Minibionix 858 axial-torsional servohydraulic testing machine with equipments
- MTS Synergie 200H mechanical testing machine with equipments
- HP Visualize C3000 workstation
- Stereoscopic Zoom Microscope SMZ800 with digital camera and accessories
- Bohlin CVO 120 Rheometer

UNITA' OPERATIVA N. 2 (Carlo Gavazzi Space S.p.A.)
- Unigraphics 3D CAD
- Laboratory Instruments
- Equipment and tools for prototype development

UNITA' OPERATIVA N. 3 (CNR)
- Facilities:
Tissue culture; stabulary and radioisotope facilities; molecular biology and biochemistry labs with basic equipment. NMR Spectroscopy Laboratory
- Laboratory equipment:
Low-speed centrifuges; ultracentrifuge; -80°C freezer
PAGE and nucleic acids electrophoretic devices; transblot and pulse-field apparatus; automated DNA sequencing equipment; hybridization oven and cross-linker; Vacuum Savant centrifuges; Perkin Elmer thermal cycler; fluorescence microscopes
Rotating Wall Vessel (RWV)

12.1.1.1 9. REPORT AND ESTIMATE OF THE EXPENDITURE FOR THE PROJECT IN MLIT/EURO						
	1° *YEAR (….)*	**2° *YEAR (….)***	**3°*YEAR (….)***	**4° *YEAR* (….)***	**5° *YEAR* (….)***	**TOTAL**
Consultants/Fellowships *12.1.1.1.1.1*	*80/41,317*	*85/43,899*	*90/41,317*			*255/131,697*
12.1.1.1.1.2 Industrial activities *12.1.1.1.1.3*	*45/23,241*	*20/10,329*	*65/33,570*			*130/67,139*
Equipment *12.1.1.1.1.4*	*90/46,481*	*20/10,329*	*50/25,823*			*160/82,633*
Supplies *12.1.1.1.1.5*	*53/27,372*	*100/51,646*	*50/25,823*			*203/104,841*
Travels *12.1.1.1.1.6*	*27/13,944*	*25/12,911*	*30/15,494*			*82/42,349*
Other expenses	*8/4,132*	*8/4,132*	*10/5,165*			*26/13,428*
TOTAL	***303/156,486***	***258/133,246***	***295/152,355***			***856/442,087***

***** per l'area "Ricerche di base per le tecnologie spaziali e ricerche in microgravità" sono ammesse richieste della durata massima di 3 anni

10. Proposte "cofinanziate" e "partecipate"

In caso di ricerca "cofinanziata" (una proposta che riceve finanziamenti da ASI e da altri enti, istituti o agenzie diversi dai proponenti) e/o "partecipata" (una proposta che riceve finanziamenti da ASI, e altri enti, istituti o agenzie, diversi dai proponenti, mettono a disposizione strutture e/o attrezzatura e/o personale), si debbono riportare i finanziamenti e/o investimenti e/o entità della partecipazione, espressi in percentuale, dei singoli enti, istituti o agenzie (detta partecipazione può consistere anche nella messa a disposizione di ricercatori).

	Ente	*Finanziamento*
Cofinanziamento (%):		
Cofinanziamento (Mlit.):		
Partecipazione (%):		

11. Scientific rationale (max 2 page)

The general target of the project is the development of a new bioreactor for mechanobiologic research in a microgravity environment. Space flight provides a unique test bed for developing new technologies and products with ultimate benefits targeted towards terrestrial biotechnology markets. The weightless environment can be used to better understand, accelerate or retard biophysical / biochemical processes, to create new biologically based products or to improve existing ones, via process-oriented applied research. As such, each investigation focuses on how space flight can be used as a viable research tool that will eventually provide a value added factor to a commercial product, process or service.

A bioreactor is generally a tool or device for generating products using a biological system. They can be classical or advanced fermentors, cell culture perfusion systems, or enzyme bioreactors. Examples of biotechnology studies conducted in microgravity are the investigation on the effects of space flight on antibiotic fermentation processes, on bacterial growth kinetics, on cell cultures and tissue engineering (e.g. regulation and gene expression studies). Initial microgravity experiments demonstrated relative increases in antibiotic production of between 75%-200% over ground-based controls. However, the absolute production level obtained in the flight hardware used for these initial experiments was significantly less than that when produced using a flask in a typical laboratory setting. Moreover, space flight has been shown to induce a number of changes in bacterial growth kinetics, such as increased final cell concentration and reduced lag phase duration. Recently, tissues grown in microgravity or environments that simulate certain aspects of microgravity have been shown to retain or develop differentiated cell functions. Thus, microgravity may be an ideal environment to conduct critical biotechnology research, such as tissue-engineering research.

The physical basis for these effects is thought to be due to the low-shear fluid environment that the cells and tissues encounter in real or simulated microgravity. By eliminating sedimentation, buoyancy and density driven convection, the cells can be grown in a relatively quiescent fluid environment, where interactions between cells can occur with minimal disruption. This would allow cell aggregates and tissues to form as in vivo but also suggests that methods are required, by which the mechanical environment may be modified to control the development of engineered cell structures. Extensive research on the effect of mechanical stimuli on cell metabolism suggests that tissues may respond to mechanical stimulation via loading-induced flow of the interstitial fluids. In a bioreactor, the cells are subject to a flow of culture medium. Flow properties such as flow field, flow regime (e.g. turbulent or laminar), flow pattern (e.g. circular), entity and distribution of the shear stress acting on the cells greatly influence fundamental aspects of cell function, such as regulation and gene expression. This has been demonstrated for endothelial cells and significant research efforts are underway to elucidate these mechanisms in various other biological systems. Local fluid dynamics is also responsible of the mass transfer of nutrients and catabolites as well as oxygenation through the tissue. Most of the attempts to culture tissue-engineered costructs in vitro have utilized either stationary cultures or systems generating relatively small mechanical forces. For example, cartilage constructs have been cultured in spinner flasks under mixed or unmixed conditions, in simulated and in real microgravity. In these mixing studies, however, it is difficult to definitively quantify the effects of mixing-induced mechanical forces from those of convection-enhanced transport of nutrients to and of catabolites away from the cells. At the state of the art, the presence of a more controlled mechanical environment may be the condition required in order to study the biochemical and mechanical response of these biological systems. Such a controlled environment could lead to an advanced fluid dynamic design of the culture chamber that could both enhance the local mass transfer phenomena and match the needs of specific macroscopic mechanical effects in tissue development.

Currently two bioreactors for the generation of simulated microgravity have been developed: the rotating wall vessel (RWV) developed by NASA and the random positioning machine (RPM) developed by Fokker Space. Both are based on time averaging of the weight vector acting on the biological particles, which are suspended in the culture medium inside the bioreactor. The time averaging is obtained through a slow uniaxial rotation of the vessel chamber in the RWV, or by a guided triaxial rotation in the RPM. In the microgravity environment simulated by these devices, the particles are strongly influenced by a viscous drag and tend to rotate with the fluid medium. The particle's motion relative to the rotating fluid is determined by a balance of gravitational and centrifugal forces experienced with those exerted by the fluid (pressure and drag) and by the buoyancy. Because the time averaging technique is effective on the particles also in hypergravity, the RWV device was originally designed to protect cell cultures from the high shear forces generated during launch and landing of the Space Shuttle. Therefore, it can be used both for simulated and true microgravity experiments, thus providing the additional benefit of isolating gravity as an experimental variable. The European Biologic Laboratory approach to cell culture protection during launch and landing is to require the launch in orbit of frozen experiments, which are then unfrozen and set up by the ISS crew.

This project is targeted at the development of a new bioreactor for advanced biotechnology research, in which the limitations shown by the devices currently available could be overcome at least in part. The new device will allow the biological systems studied to be subject to controlled and quantified stresses and transport phenomena. The device will allow ground research and experiment setup in simulated microgravity, experiment protection during launch and landing, setup and use in microgravity with minimal interaction by the ISS crew.

12. Methods and technical description (max 1 page)

The general target of the project is the development of a new bioreactor for advanced biotechnology research in simulated and true microgravity. The biological samples to be studied inside the device range from bacteria to cells and engineered tissues.

Specific design requirements are:

- controlled and quantified fluid dynamics inside the chamber
- simulation of a microgravity environment
- experiment protection during launch and landing
- use in microgravity
- minimal interaction by the ISS crew during setup and use
- automation and remote controlling of specific functions
- any requirement applicable to space missions

The general target will be achieved through the following main activities:

1. design of a prototype bioreactor for simulated and true microgravity experiments in biotechnology according to the design requirements from previous research;
2. fluid dynamic design and optimization of the culture chamber;
3. assembly of an engineering model;
4. comparative evaluation of the prototype with different commercial bioreactors currently on the market, with reference to the mechanical environment induced on the biological systems;
5. evaluation of the influence of the bioreactor design on different experimental models;
6. tradeoff of the prototype specifications according to the experimental results and to ISS requirements;
7. engineering of a final prototype.

The bioreactor for microgravity studies will be designed at the Laboratory of Biological Structure Mechanics (LaBS) at Politecnico di Milano, using the weight vector time averaging approach to achieve a simulated microgravity environment. Computational fluid dynamics models of the chamber will be developed using commercially available codes, in order to predict the mechanical loads induced on the cultured biological systems, for different designs and for different properties of the culture medium. Based on the fluid dynamic design optimisation, an engineering model will be assembled by Carlo Gavazzi Space (CGS) and tested against the commercially available bioreactors. Comparative evaluation of the prototype with different commercial bioreactors will be performed at LaBS and CNR facilities. The engineering model will be tested by the 3rd U.O. (CNR and LITA Vialba facilities) against different experimental models (e.g. anchored and suspended cell cultures, different fluid dynamic environments). The experiments will be conducted on various biological systems and will focus on endothelial cells, on which the CNR research unit is active with numerous research projects since years. The process described above will be iterated, identifying the role and effects of the relevant design parameters, until all the design requirements are met.
Because bioreactors usable under space flight conditions impose constructional principles, which are different from those intended solely for ground applications, the bioreactor will be designed in order to meet the requirement to be flown on the Columbus Laboratory.
The final prototype will be manufactured by CGS. It will have the capability of monitoring and recording specific experiment parameters and conditions on-line, using state of the art subsystems, and will as well meet all the original design requirements plus those generated or modified by the research results.

13. Bibliography of the project (max 20 references)

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14. List of publications of the Principal Investigator (Max 5 in the last 5 years)

1. Dubini G, Redaelli A. Mesh updating in fluid-structure interactions in biomechanics: an iterative method based on an uncoupled approach. *Ann. Biomed. Eng.*, 1997; 25(1): 218-231.
2. Migliavacca F, Kilner PJ, Pennati G, Dubini G, Pietrabissa R, Fumero R, de Leval MR. Comparison between computational fluid dynamic and magnetic resonance analyses on a case of total cavopulmonary connection. *IEEE Trans. Biomed. Eng.*, 1999; 46(4): 393-399.
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4. Migliavacca F, Pennati G, Dubini G, Pietrabissa R, Fumero R, Urcelay G, Bove EL, Hsia TY, de Leval MR. Modeling of the Norwood circulation: effects of shunt size, vascular resistances and heart rate. *Am. J. Physiol.*, 2001; 280(5): H2076-H2086.
5. Pennati G, Fiore GB, Migliavacca F, Lagana' K, Fumero R, Dubini G. In vitro steady-flow analysis of systemic-to-pulmonary shunt haemodynamics. *J. Biomechanics*, 2001; 34(1): 23-30.

15. Publications of the responsible of each sub-project (max 5 in the last 5 years)

RIASSUNTO DELLA PROPOSTA (IN ITALIANO)

Linea Strategica	12.1.2 Indirizzo di ricerca	12.1.3 Anno 2001 12.1.4

Il progetto o parte di esso è stato selezionato nell'ambito di bandi internazionali? (specificare)

Coordinatore: Gabriele Dubini
Responsabili delle U.O.: Fabio Tominetti (U.O.N.2) **Silvia Bradamante (U.O.N.3)**
Titolo della proposta: Sviluppo di un bioreattore per ricerca meccanobiologica in microgravità.

12.1.4.1.1.1.1.1.1 Riassunto

Descrivere brevemente gli obiettivi strategici e i risultati che si ritiene di raggiungere. Il riassunto deve servire come una descrizione succinta del programma, comprensibile anche ai non esperti del settore (massimo mezza pagina)

Oggetto del presente progetto è la progettazione e la realizzazione di un bioreattore per ricerche di meccanobiologia in microgravità. Un bioreattore è un dispositivo che permette di tenere in vita un sistema biologico. Recenti ricerche hanno mostrato come la riduzione dell'entità della forza di gravità abbia effetti diversi sulla crescita di sistemi biologici, che vanno, per esempio, dall'aumento della velocità di proliferazione di batteri alla differenziazione di cellule in un tessuto. Il bioreattore in progetto verrà utilizzato nello studio della proliferazione di cellule e della formazione di tessuti, in condizioni simulate di microgravità a terra e in presenza di microgravità reale in orbita.

Il progetto prevede lo sviluppo delle seguenti attività:

1) progettazione di un bioreattore protitipale per esperimenti in microgravità simulata e reale nell'ambito della biotecnologia, basata su specifiche derivanti da ricerca pregressa.
2) Progettazione fuidodinamica e ottimizzazione della camera di coltura.
3) Assemblaggio di un modello di laboratorio
4) Valutazione comparativa del prototipo con vari bioreattori commerciali attualmente presenti sul mercato, con riferimento all'ambiente meccanico prodotto sui sistemi biologici in coltura.
5) Valutazione dell'influenza della progettazione del bioreattore su diversi modelli sperimentali.
6) Ottimizzazione tra specifiche di prototipo e risultati sperimentali.
7) Produzione del prototipo definitivo rispettando le specifiche della ISS.

Le attività di simulazione fluidodinamica e definizione delle specifiche di progetto e del prototipo di massima, saranno condotte dall'unita operativa che coordina il progetto, che appoggia la propria attività presso il Laboratorio di Meccanica delle Strutture Biologiche (LaBS) del Politecnico di Milano. Le attività di sviluppo e ingegnerizzazione dei prototipi da laboratorio e di quello finale, saranno condotte dall'unità operativa industriale Carlo Gavazzi Space. Le attività di sviluppo e implementazione di modelli sperimentali comparativi su bioreattori commerciali e sui prototipi saranno condotte dall'unità operativa che appoggia la propria attività presso il CNR. Per lo sviluppo totale del progetto è previsto un impegno di 3 anni.

_____ _____

Firma del coordinatore del progetto (specificare statuto o incarico)

Firma del responsabile amministrativo (specificare statuto o incarico)

Redatto il

Titolo del progetto: Sviluppo di un bioreattore per ricerca meccanobiologica in microgravità.

P.I.: *Nome*: *Titolo*: *Cognome*:

Descrizione del prodotto - allegato tecnico (MASSIMO una pagina)

Obiettivo del progetto è lo sviluppo di un nuovo bioreattore per ricerche avanzate nel campo delle biotecnologie in microgravità simulata e in microgravità reale. I campioni biologici da studiare variano da batteri a cellule a tessuti rendendo il bioreattore da realizzare un bioreattore multiutente.

Specifiche di progetto sono:
Controllo della fluido dinamica all'interno della camera di coltura.
Simulazione della microgravità per ricerche in laboratorio sulla terra.
Capacita di proteggere l'esperimento da mandare in orbita, durante le fasi di lancio e di atterraggio.
Possibilità di utilizzo, dello stesso dispositivo, in microgravità.
Interazione minima da parte dell'equipaggio della stazione spaziale, durante un esperimento.
Automazione e controllo remoto di specifiche funzioni.
Ogni specifica necessaria a missioni spaziali.

Gli obiettivi tecnici che ci si prefigge di raggiungere nel primo anno del progetto sono:
la progettazione di un bioreattore protitipale per esperimenti in microgravità simulata e reale nell'ambito della biotecnologia, basata su specifiche derivanti da ricerca pregressa, la progettazione fuidodinamica e ottimizzazione della camera di coltura.
E l'assemblaggio di un modello da laboratorio

L'approccio previsto per ottenere la microgravità simulata è quello di mediare nel tempo il vettore peso attraverso la rotazione della vasca. Analisi fluido dinamiche verranno effettuate principalmente mediante l'utilizzo di codici di fluido dinamica computazionale (CFD) disponibili in commercio. L'assemblaggio del modello da Laboratorio sarà anche momento di analisi e ottimizzazioni per diverse soluzioni derivanti dalle ricerche fluido dinamiche.