Chapter 2
Principles of Analogue and True Microgravity Bioreactors to Tissue Engineering

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2.1 Introduction

2.1.1 The Early Days of Cell Culture in Microgravity

NASA, in the 1980s, embarked upon a program to enable pilot scale cell culture under microgravity conditions utilizing the Space Shuttle as a laboratory. The initial program goal was to enable culture of mammalian cells as a space-based bioprocess. It was ultimately discovered that both ground-based analogue and true microgravity offered powerful advantages for the in vitro reproduction of natural tissue cytoarchitecture. Initial work on ground-based analogues centered on development of continuous perfusion bioreactors and prototype microgravity compatible culture vessels with microcarrier beads as the cell attachment substrate. Fluid dynamic studies addressed optimizing internal vessel designs to simultaneously achieve, on Earth, low hydrodynamic stress, 3-dimensional spatial freedom, and co-location of particles with differing sedimentation rates while sustaining optimal mass transport and compatibility with microgravity operations [1, 2, 18]. It is these three simultaneous conditions that achieve the simulated microgravity conditions when operated on Earth in unit gravity.

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Initial designs led to a horizontal orientation of a cylindrical zero headspace culture vessel with an internal vane to achieve suspension of culture materials. This early design, while compatible with microgravity operations and enabling suspension of the culture, introduced damaging hydrodynamic forces similar to conventional vessels also utilizing internal lift mechanisms. Laser doppler measurements by Stan Kleis' team at the University of Houston verified the expected source of the damaging (to the cultured cells) hydrodynamic forces to be from high velocity gradients, and associated shear stress, through the boundary layer associated with fluid moving inside the vessel relative to the internal vessel walls.

Ray Schwarz, Tihn Trihn, and David Wolf, of the NASA design team, recognized that wall rotation alone, without any internal lift mechanism, such as a vane, was sufficient to achieve suspension. The internal vane was removed and the rotating fluid itself served as the lift mechanism. This wall rotation effectively negated the boundary layer velocity gradient near the vessel walls resulting in greatly reduced fluid shear stress in this region. These Rotating Wall Vessels (RWV's) were designed for continuous perfusion with controlled gas partial pressures and dissolved chemical species concentrations and also a simplified batch fed version was designed for use in an incubator.

The operational rotation rate typically was 10–30 rotations per minute (rpm) to avoid excessive sedimentation, minimize centrifugation, and achieved a homogenous distribution of culture materials including attachment substrates and cells. This new configuration, in 1986, demonstrated a dramatic improvement in cell viability for the cultured baby hamster kidney cell line (BHK-21) and primary human embryonic kidney cells (HEK). The very low hydrodynamic shear stress near the walls of the rotating zero headspace vessels produced an excellent microgravity analogue enabling projection of expected results in actual microgravity as well as enabling many of the benefits of space based microgravity culture on Earth.

Experience with microcarrier-based suspension mammalian cultures, using the rotational "low shear modelled microgravity" or as it is sometimes called "simulated microgravity" technique, demonstrated the formation of large viable three-dimensional (3-D) tissue-like aggregates bridging multiple beads. We recognized that the quiescent conditions within the RWV produced a uniformly distributed suspension of 3-D tissue-like aggregates similar to the anticipated behavior in microgravity. Thus, 3-D tissue modeling in analogue microgravity established an early form of 3-dimensional "Tissue Engineering."

2.1.2 Benefits of Ground-Based Analogue and True Spaceflight Bioreactor Culture

Earth-based analogue and actual Space-based microgravity Bioreactors, both based on the RWV technology, offer unique physical conditions that enable high fidelity in vitro culture of 3-D organotypic tissue. RWV technology is applicable over a wide range of normal and transformed mammalian cell types

(including cell lines and primary cells) to establish organotypic/organoid models that reproduce key aspects of the 3-D cytoarchitecture and function found in the parental tissue. Accurate models of tissues, which behave closely to the corresponding in vivo tissue, enable basic biological research to address understanding human disease mechanisms and treatments. As discussed in Chaps. 4–9, the application of these in vivo-like 3-D tissue aggregates to study the transition between normal cellular homeostasis and disease has unveiled novel mechanisms not possible using conventional flat 2-D monolayers grown on plastic.

Typically, delicate anchorage dependent mammalian cells are seeded onto suspended microcarrier beads or other substrates in a media within a bioreactor vessel volume. Rotating bioreactors provide the physical, biochemical, and oxygen requirements to support 3-D volumetric culture [3–6]. Common to these are (1) a means to support a delicate culture against the force of gravity in 3 dimensions, and (2) minimize the introduction of undesired hydrodynamic forces, and (3) allow co-spatial location over extended periods of time of culture components with differing sedimentation rates. Conventional monolayers grown on flat flasks and plates inherently drive tissue growth to a surface not supportive of 3-D tissue growth. In contrast, spinner flask systems incorporate internal stirring mechanisms that introduce disruptive hydrodynamic forces. Hollow fiber systems introduce limitations on the free mobility, homogeneity, and sampling of the culture. Fluidized beds act to separate culture assembles by sedimentation properties and introduce fluid velocity gradients, shear stress, and instability. Gels and encapsulation techniques limit diffusion and mobility of culture constituents.

RWV bioreactors are fundamentally compatible with microgravity in that they are completely fluid filled, without gas headspace. When operated under gravity conditions they are rotated at an appropriate rate to achieve suspension of the culture constituents by viscous fluid coupling without internal mechanical lift mechanisms or associated fluid velocity gradients.

True microgravity bioreactor culture allows for optimal realization of the 3 simultaneous physical conditions in a free aqueous environment: (1) three-dimensional freedom for cell and substrate interaction, (2) low fluid mechanical stress, and (3) co-localization of particles with differing sedimentation rates. However, due to the absence of gravity driven culture component drift through the media (in actual microgravity) one must be careful to actively introduce a method to facilitate adequate and homogeneous mass transfer by induced fluid motion. This avoids a diffusion limited culture condition. The quiescent environment enabled by the 3 simultaneous conditions permit organized self-directed tissue assembly with minimal disruption or influence from the culture system per se. Counter to these extreme quiescent conditions is the necessity to introduce sufficient perfusion and mixing to meet the culture metabolic demand, support a “healthy” cell and tissue micro-environment, provide adequate mass transport and sustain a reasonably homogeneous overall vessel macro-environment minimizing deleterious dead spaces or shunts from the vessel fluid inlets to outlets. Essentially, the absence of dead spaces and shunts may be verified by how close to optimal is the residence distribution curve as measured at the vessel inlet and outlet under the specific

operating conditions and with the culture materials selected. In order to achieve these conditions, the Space Bioreactor baseline design incorporates independently rotatable inner and outer cylindrical walls enclosing a 125 ml culture volume in a concentric "couette" configuration. The inner cylinder is covered with a porous membrane providing the vessel media outlet. Differential rotation of the cylindrical walls sets up a forced convective flow pattern, particularly important in microgravity conditions, to effect mass transport of dissolved species including respiratory gases, nutrients, and waste products. Fixed to one end of the inner cylinder, adjacent to the media inlet, is a disk shaped "viscous pump," introduced by Dr. Kleis' laboratory, which introduces momentum asymmetry to incoming media to assure a single cell toroidal circulation pattern and reduce shunting of media directly from the inlet to the outlet. In the absence of this viscous pump the couette configuration results in a two cell flow pattern [10] with a rather astonishingly low crossover of dissolved species between cells and in a sense results in two separate volumes operating nearly independently. Numerical calculations over a range of operating conditions show that the fluid shear stress, when operating under actual microgravity conditions is quite low, typically on the order of 0.002 dynes/cm^2. This "residual" fluid shear stress, present even when operating under actual microgravity conditions, largely results from intentionally introduced forced perfusion flow and may be reduced to nearly arbitrarily low levels for significant periods of time by reducing the perfusion flow rate and "suffering" the resulting period of reduced mass transport. In unit gravity ($1 \times g$ on Earth), much of the fluid shear results from gravity driven continuous settling of tissue aggregates at very nearly terminal velocity through the fluid media and is much higher, on the order of 1 dynes/cm^2 for typical tissue aggregates [7, 8]. Ground-based RWV bioreactors utilize this gravity-induced sedimentation to effect mass transport often alleviating the need for additional forced convection by differential wall rotation or perfusion. This enables simplified batch fed reactors to operate, without differential wall rotation (as compared to a central annular cylinder – typically a gas exchange membrane) in an incubator environment. In contrast, the continuously perfused Space Bioreactor version does not require daily media change and can be perfused by a reservoir that is replenished at intervals, often weekly, and therefore is very advantageous during spaceflight operations. However, in this actual microgravity condition, means to actively induce mass transport by forced fluid flow are necessary to obtain a homogenous and well controlled microenvironment.

Accordingly, modeled (or simulated) microgravity in the ground-based RWV bioreactors is achieved in a calculable, deterministic, and defined fashion which enables methods for testable hypothesis driven experiments designed to causally link the output biological results to the input fluid dynamic conditions which are coupled to gravity in a defined way [1, 2, 5, 9–13].

True microgravity and analogue (Earth based RWV-generated) organotypic tissue constructs demonstrate the principles of gravitationally controlled culture techniques, and include 3-D organotypic models of human liver, intestine, lung, neuronal, cardiac muscle, prostate, cartilage, and lymphoid tissue, among numerous other organ sources [5, 14–16]. These 3-D tissue constructs have been applied in

interdisciplinary studies over the past several decades to advance our understanding of how cells behave normally or transition to disease (see Chaps. 4–9). The ability to culture primary and transformed human cells in 3-D reproduces key aspects of the morphology and function of the corresponding in vivo tissue and is a significant advancement to tissue engineering [3, 5, 14–17]. Importantly, mechanical as well as biochemical conditions imposed upon the tissue microenvironment are key factors in determining functionality and cytoarchitecture. These microenvironmental conditions can be different for each particular cell/tissue system.

NASA's development of RWV bioreactors provides a simultaneous combination of three-dimensionality, gentle and physiological fluid dynamics, and spatial-co location of mixed cell populations (and substrates) in free suspension sustaining adequate aeration and mass transport, [18]. Interestingly, the RWV provides conditions that are consistent with high mass transport analogues. Still, comparisons of data obtained in these bioreactors across unmatched platforms (i.e., on Earth versus in space) may introduce uncontrolled effects that weaken attribution of the RWV results to gravity. The NASA Space Bioreactor is designed to minimize confounding variables by providing a high degree of biochemical and fluid dynamic control, to be compatible with both Earth based control and spaceflight operations on the International Space Station (ISS) National Laboratory, as well as provide high performance analogue microgravity culture conditions on Earth, i.e. one is able to utilize the same bioreactor instrumentation both on Earth and in Space while also achieving simulated microgravity for the Earth based controls. The Space Bioreactor, and its Earth-based simulated microgravity RWV versions, have demonstrated high performance with respect to the ability to propagate and support functional, cytoarchitecturally correct, 3-D mammalian tissue constructs derived from a variety of tissue types [1, 3–6, 15, 17, 19] that have demonstrated their ability to serve as predictive human surrogate platforms (see Chaps. 4–9).

2.2 Operation and Examples of Ground-Based RWV Cultures and Comparison to Actual Spaceflight Cultures

To facilitate cell growth, microcarrier or other biocompatible anchorage substrates/scaffolds are added prior to cell culture. The vessel is then seeded with one or more cell types at the appropriate density, which then rapidly attach, spread, and multiply on the microcarrier or substrate. (Note: suspension cell culture in the RWV can also be performed without scaffolding, depending on the experimental objectives). This laboratory initially utilized baby hamster kidney (BHK21) cells and Cytodex-3 microcarrier beads as the anchorage dependent substrate to demonstrate the concept and feasibility of modeled (or equivalently stated, simulated) microgravity tissue culture in the RWV bioreactor. Initial observations revealed a high order of cellular assembly into a viable tissue-like cytoarchitecture [18] characterized by ordered and regular intercellular membrane close associations. Rapid growth, high metabolic activity, and uniform macroscopic tissue-like formations

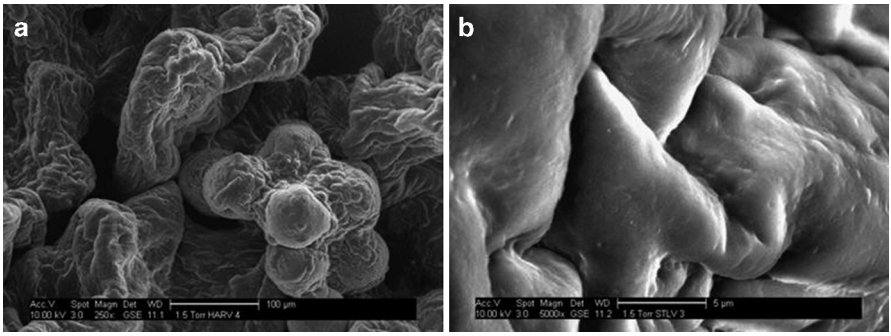


Fig. 2.1 Baby Hamster Kidney cell line, BHK21, cultured in the RWV in modeled microgravity on Cytodex-3 microcarrier beads. Rapidly proliferating cells on confluent 3-D cell-bead aggregates show tissue-like bridging across beads ((**a**) 250×; (**b**) 5000×) demonstrating the ability to form high order 3-D tissue-like assemblies; photo courtesy of NASA

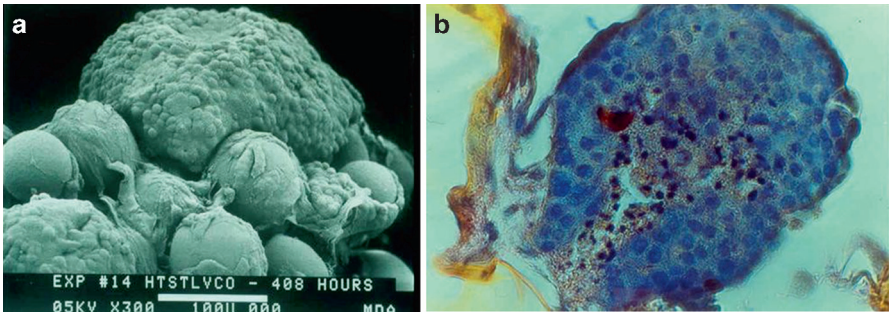


Fig. 2.2 Modeled microgravity co-culture of HT-29 colon carcinoma epithelial cell line with normal human colon fibroblasts. (**a**) SEM (200×), demonstrates assembly of macroscopic "polypoid" structures. The fibroblasts form a confluent feeder layer on Cytodex 3 microcarrier beads upon which the colon epithelial cells proliferate and differentiate. (**b**) Mucicarmine stain of a 10 μm section (200×) demonstrates fibroblast infiltration (*yellow color*) and sinusoid formation extending from the base feeder layer into the epithelial polypoid tumor [20]

were observed (Fig. 2.1a, b). Successful demonstration with BHK21 cells led to subsequent investigation of difficult to grow primary human embryonic kidney cells where the RWV growth conditions enabled rapid growth and high viability of 3-D tissue constructs (unpublished data).

This early work was extended to small and large intestine co-culture models, normal and neoplastic, respectively [20, 21]. Specifically, similar high order 3-D structure was achieved for colon carcinoma epithelial cell lines, including those derived from HT-29 cells both in monotypic culture (comprised of a single cell type, here HT-29 cells alone) and in co-culture (Fig. 2.2a, b) with normal human colon fibroblasts as a mesenchymal feeder layer [20, 21]. This study demonstrates the

typical pattern of high order self-organizing 3-D cytoarchitecture obtained for different cell types cultured in the RWV, and has since been found by many investigators to also occur in numerous other neoplastic, transformed cell lines, primary cells, and normal cells derived from cardiac, liver, prostate, skin, cornea, bone, cartilage, nerve, intestinal, lung, placental, vaginal, bladder, and lymphoid tissues [14, 17, 22–44]. While this chapter provides a general overview of the basic principles and applications of the RWV bioreactor in early 3-D cell culture studies, Chaps. 4–9 describe in detail (1) the specific 3-D cell culture models advanced using this technology (including immunocompetent co-culture models), (2) their molecular genetic and phenotypic validation demonstrating in vivo-like characteristics of the parental tissue, and (3) their application to serve as predictive platforms to study the transition between normal tissue organogenesis and disease phenotypes. This includes high fidelity models of infectious disease and cancer, the latter of which includes the establishment of models of both malignancy and phenotypic reversion to investigate mechanisms of tumorigenesis and cancer dormancy, respectively. In addition, RWV-derived 3-D cell cultures have shown promise for future use in translational studies in regenerative medicine and pharmaceutical development.

Initial true microgravity spaceflight testing of mammalian cell cultures conducted on the Space Shuttle and the MIR Space Station are largely consistent with those projected by use of RWV culture on Earth, particularly with respect to achieving a large and well-organized 3-D architecture and molecular and phenotypic indicators of proliferation, differentiation, and function [45–48]. For example, cultured lymphocytes showed a similar set of responses to both modeled and actual microgravity, characterized by reduced locomotion, blunted chemotaxis, and reduced mitogen response [47, 49]. Interestingly, these characteristics are similar to the responses of lymphocytes drawn from astronauts during actual spaceflight that are hypothesized as a potential risk of spaceflight induced immune dysfunction [50]. Conversely, some findings from actual microgravity cultures are not expressed under modeled microgravity conditions in the RWV, such as glycosaminoglycan distribution of highly mechanoreceptive cartilage tissue [6, 19], reflecting the differences between RWV and actual spaceflight growth conditions and presumably attributed to the more aggressive (but still very mild) hydrodynamic conditions introduced by RWV modeled microgravity as compared to actual microgravity. This “imperfect” modeling is rather dramatic to the naked eye when one observes the far more quiescent conditions of actual microgravity culture of cells as compared to the RWV (operated on Earth), as larger tissue aggregates form and begin to increasingly respond to the gravity vector (as their size and sedimentation rate increases). This loss of microgravity simulation becomes progressively more severe as tissue aggregates increase in size in the RWV and their Stokes terminal velocity of sedimentation attains an increased magnitude [1, 5]. This increased magnitude further degrades the 3-D tissue aggregates by damaging effects, such as vessel wall impacts and non-uniform shear stresses resulting from directional entrainment of the growing tissue aggregates and direct surface shear effects as they are pulled through the rotating culture media by gravity at very near terminal velocity [12].

2.3 Ground Based and Spaceflight Bioreactor Design

In addition to deterministic gravity coupling, a defined, spatially homogeneous and time invariant mass transport condition in a cell culture vessel was determined to be beneficial in order to conduct meaningful experiments with results causally attributable to the unique "microgravity" fluid mechanics. These conditions drove the fundamental bioreactor design requirements to be a continuous flow perfused zero headspace cylindrical rotating wall reactor vessel, 250 ml in volume, composed of independently rotating concentric couette configured inner and outer walls designated as the "Rotating Wall Perfused Vessel" (RWPV). The perfused culture system provided for respiratory gas exchange, external to the culture vessel, by means of a high surface area gas exchanger in an external media flow loop and offered reduced temporal and spatial variability and improved control for dissolved gas species, as well as the opportunity for introducing the benefits of direct organoid perfusion [29]. A simplified batch fed version, for incubator use, enabled low overhead operation for developmental testing and use by conventional laboratories without extensive engineering staff. This batch system was designated the "Slow Turning Lateral Vessel" (STLV). This simplified instrument, and it's related "High Aspect Rotating Vessel" (HARV) which enables greater diffusional respiratory gas exchange, provides less defined and less controllable fluid mechanical properties but functions well for initial Earth-based studies while still providing basic modeled microgravity conditions. These incubator batch fed systems provide respiratory gas exchange with incubator atmosphere by means of a membrane within the vessels themselves. One approach for development and optimization of a spaceflight mammalian cell culture experiment is to begin with the ground-based STLV and HARV systems and then progress to cultures in the RWPV to optimize for flight (Fig. 2.3). Detailed methods for conducting such work may be referenced in Freed

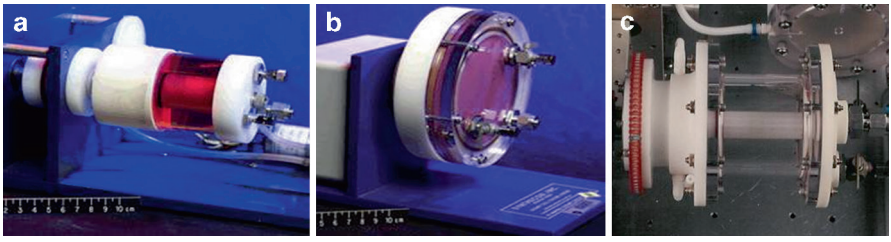


Fig. 2.3 The three primary RWV bioreactors which produce modeled microgravity conditions by zero head space rotation. The Slow Turning Lateral Vessel (STLV) (**a**) with a center core cylindrical gas exchange membrane, and the High Aspect Rotating Vessel (HARV) (**b**) with a high surface-to-volume ratio gas exchange membrane, are batch fed and used in a conventional incubator. The Rotating Wall Perfused Vessel (RWPV) (**c**) supports an external media perfusion loop with associated external oxygenator (not shown). Actual microgravity bioreactors incorporate the RWPV that allows independently adjustable rotation of the inner wall (the spin filter media exit) and outer walls in order to set up controlled fluid dynamic conditions including mass transport and fluid shear



Fig. 2.4 The Biospecimen Temperature Controller (BSTC) provides a CO_2 controlled environment to hold up to 32 Tissue Culture Modules (TCMs), of 25 ml volume each, for static mammalian tissue culture in space. Media may be manually exchanged and cell samples extracted for fixation with syringes from a support kit (not shown). High order tissue structure is demonstrated by the neuroendocrine pheochromocytoma derived PC12 cell line. This structure is estimated at 3 mm in length and was obtained by placing a drop of culture directly on a zero focus distance video camera lens to obtain macro-photography. These frond-like structures do not appear in Earth-based simulated microgravity or conventional culture. Photos courtesy of NASA

et al. [19], describing both ground-based controls and inflight cultures of bovine cartilage tissue on polyglycolic acid matrix during the MIR 3 mission. An excellent fluid dynamic numerical analysis of this experiment is provided by Lappa [12] in which the physical conditions are related to the tissue morphological development. In this case, several parameters, such as glycosaminoglycan content, were found superior in the Earth-based simulated microgravity controls, as compared to actual microgravity for this mechanosensitive tissue. While both culture in the RWPV and actual microgravity provided models that were superior to conventional culture techniques, this experiment highlighted the necessity to modulate the extreme quiescence of actual microgravity to the desired mass transfer and mechanical stress operating points to optimize for the particular tissue system. This greatly extended operating range enabled for actual microgravity tissue culture is an important use of the Space-based laboratory to conduct studies not possible on Earth.

A spaceflight certified companion static tissue culture device, the Biotechnology Specimen Temperature Controller (BSTC, Fig. 2.4) system, was also developed and operationally deployed. This instrument sustains a 5 % CO_2 gas mix in a 37° chamber around a maximum of 32 gas permeable Tissue Culture Modules (TCM's, Fig. 2.4) each of 25 ml volume. Although the fluid dynamics are not specifically controlled or adjustable, nor is modeled microgravity achieved for Earth based controls, these modules have the advantage over the Space Bioreactor of multiple replicates. The media may be exchanged manually via syringes and the cells may be similarly sampled for preservation. The neuroendocrine PC12 cell line derived from human pheochromocytoma cells (Fig. 2.4) was successfully cultured on the MIR Space Station during the NASA 6 mission demonstrating the capacity for delicate high order structure of cell cultures even under less than fully controlled conditions.

2.4 Fluid Dynamics of RWV in Unit Gravity (1×*g* Ground Based) and Microgravity (Spaceflight)

Fluid dynamics is an important aspect of the mechanical interaction between the biological cell/tissue culture and its environment. When designing a vessel for the culture of anchorage dependent cells in microgravity, special care must be paid to avoid conditions leading to inadequate mass transport. The convective transport conditions nearly always present in Earth's gravity (1×*g*) due to slight density differences between culture media and cells/microcarriers or cell aggregates are reduced by several orders of magnitude in microgravity. As such, it is important to partially replace this enhanced mass transport by forced or even natural convection present in 1 *g* by a controlled three-dimensional flow field in microgravity to provide adequate transport of scalar quantities such as dissolved oxygen, CO_2, nutrients, and cellular waste products.

The dominant feature in the presence of gravity is the distortion of the paths of suspended particles (including cells/aggregates) from the background circular fluid streamlines within an RWV, as these particles track the gravity vector (Fig. 2.5a). Analysis and experimental verification of this first order effect is provided by NASA Technical Papers [1, 2] that explored various rotational rates and particle terminal velocities under unit gravity and during low gravity aircraft parabolic flight. Cell/tissue aggregates and their associated substrates/scaffolds accelerate to terminal velocity very quickly, on the order of milliseconds. The RWV rotates through a negligible angle during the time required for these constituents to reach 98 % of terminal velocity. Essentially, the 3-D tissue aggregates are always settling through the media at terminal velocity (in a circular motion unless there is contact with structure) at the same speed as if they were settling linearly downwards in a non-

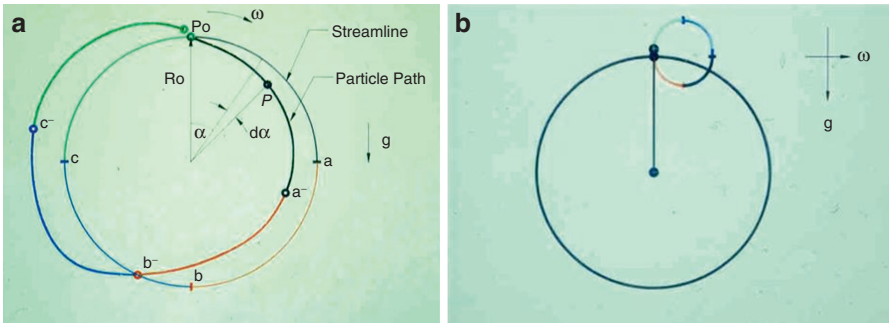


Fig. 2.5 Observation of a particle within the RWV bioreactor. (**a**) As viewed from the external (non-rotating) reference frame, one observes a rotating culture vessel with the path of particles suspended inside deviating from the background circular streamline. In single revolution, the particles do not quite return to their original location due to centrifugal and Coriolis pseudo forces. They slowly drift outwards and lag in phase, cumulatively over time. (**b**) The same physical situation as viewed from the rotating reference frame (i.e., spinning at the same rate as the RWV). In this case, one observes particles tracking the rotating gravity vector at very nearly terminal velocity [11, 13]

rotating system. Several investigators have verified this gravity-induced motion by various analytical, numerical, and empirical imaging techniques [11, 13]. This "path distortion" may be visualized more clearly as observed from the rotating reference frame of the RWV vessel (Fig. 2.5b). In this reference frame, a rotating gravity vector, *g*, is observed from the point of view of an apparently non-rotating vessel, i.e. as one would observe if one was inside the vessel rotating with it. The 3-D tissue constructs are observed to follow a circular path with a tangential speed very close to terminal velocity. The circle diameter increases with increasing terminal velocity and with decreasing rotation rate. Note that all the particles in the RWV move in phase with each other. In this regard, a smaller slower settling particle and a larger aggregate which settles more rapidly remain co-located in the same spatial region, co-orbiting for extended periods of time. This allows cells, substrates, and growing tissue aggregates to interact without encountering disrupting regions of high fluid shear stress. However, particles near the vessel wall and those that sediment rapidly will be exposed to wall impacts at speeds as high as their terminal sedimentation rates. Residual fluid shear stress in unit gravity is the result of particles settling through the culture media at terminal velocity. Under the true microgravity conditions of spaceflight, sedimentation is negligible and a much lower residual fluid shear stress is governed by the intentionally introduced background forced convective flow field as introduced to achieve necessary mass transport. Centrifugal and Coriolis pseudo-forces, apparent in the circularly accelerated reference frame, are much lower than forces resulting from gravity, but these do have a cumulative effect over time causing particles denser than the media to migrate outwards, or those less dense than media to migrate inwards [11]. In practice, as 3-D tissue aggregates progressively increase in size and density, the microgravity simulation is progressively lost, and the advantages of RWV cell culture are progressively diminished. However, this gravity-induced motion through the culture media does serve to increase mass transport and to reduce the size of the local region around a metabolizing tissue aggregate through which diffusion must occur. For example, mechanically sensitive tissue such as cartilage will respond to these forces [12, 19].

In Fig. 2.6, Lappa [12] shows flow and relative fluid shear calculations superimposed with cartilage tissue, approximately 1 cm long, that were originally grown by Martin et al. [51] under RWV conditions. Hypertrophic growth response of the tissue is in agreement with local regions of higher shear stress at the tissue surface. In Fig. 2.7, Nauman et al., demonstrated the local disturbance to circular flow and resulting fluid shear stress introduced by beads of differing sizes in bacterial cells cultured in the RWV [52]. The authors made use of this property to intentionally introduce known regions of fluid shear to measure bacterial molecular genetic and pathogenesis-related stress responses to that stimulus.

The gravitationally induced particle motions, as described above, contribute to mass transport of the scalar dissolved concentrations under unit gravity conditions. The motion may be considered to be convection. Modeling of the scalar transport may be accomplished for microgravity conditions. Mass transport in microgravity, as in 1 *g*, can be thought of as a diffusion process due to concentration gradients and a convection process due to relative motions between the fluid and particles.

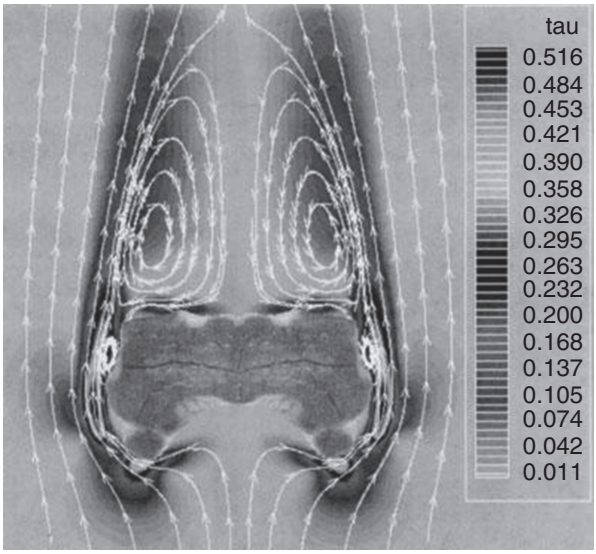


Fig. 2.6 Lappa [12] mathematically evaluated the expected tissue shape, fluid shear stress distribution, and flow velocity field and compared these calculations to the histologic cross section of cartilage grown for 60 days in RWV conditions (in unit gravity) by Martin et al. [51]

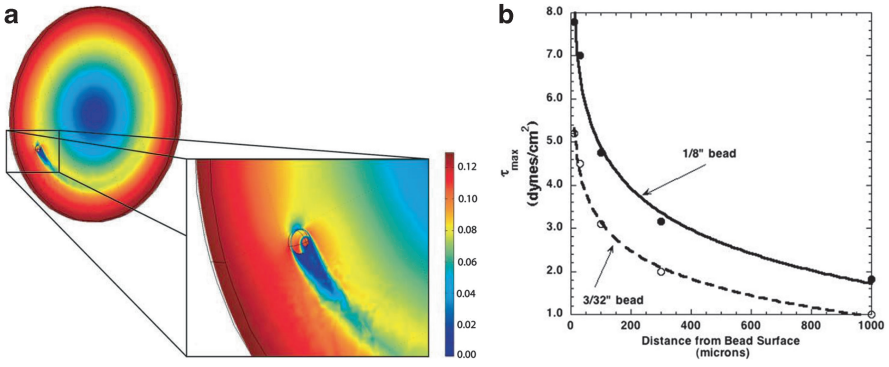


Fig. 2.7 Fluid velocity distribution and maximum shear stress in the RWV bioreactor. (**a**) Nauman et al. [52] model the distribution of fluid velocity in the RWV when a spherical bead is added to disrupt the velocity field, thus inducing elevated shear stress. (**b**) The induced fluid shear stress surrounding beads of two sizes (1/8 and 3/32 in. diameter) when added to the RWV. Copyright © American Society for Microbiology, [*Applied and Environmental Microbiology*, 73, 699–705, 2011]

However, in microgravity, convection must be intentionally introduced. Dependence on diffusion for mass transport quickly leads to limiting the culture to smaller quantities of cells and influences the cell microenvironment and health of the cultures. In microgravity, the relative motion to achieve sufficient mass transport is due only to the nature of the flow field, since the dominant effect of gravity is absent. Introduction of convective fluid flow also serves to reduce the size of the local viscous layer at the tissue surface through which diffusion must ultimately occur to effect transport at the cell surface.

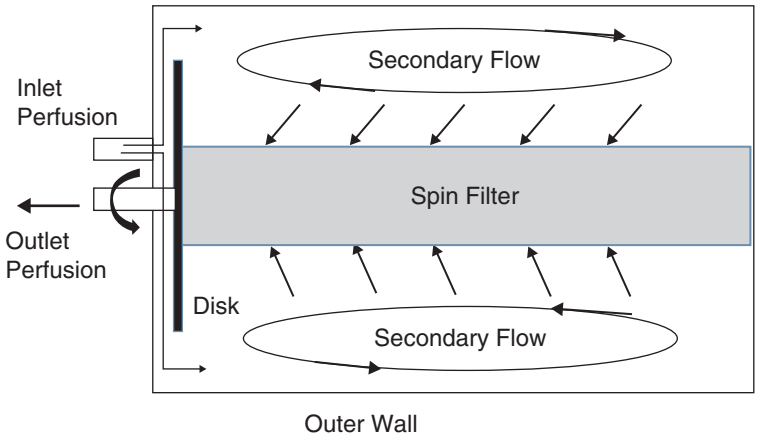


Fig. 2.8 Rotating Wall Perfused Vessel as used in true microgravity and on Earth in unit gravity. The disk on the left side of the spin filter acts as a "viscous pump" and promotes a single cell secondary flow. In true microgravity, the spin filter is typically operated from 5 to 10 rpm and the outer vessel wall at 1 rpm. Some outer wall rotation is required to stabilize the secondary flow cell. On Earth, to sustain tissue suspension, these rates are biased upwards by 10–20 rpm

In space, in the absence of a dominant gravitational acceleration and resulting particle tracking of the gravity vector, simple RWV bioreactors such as the High Aspect Ratio Vessel (HARV) would move the cells on microcarriers to the outside wall of the vessel. To avoid this situation, a three-dimensional flow field is required. A controllable three-dimensional flow field is obtained by differential rotation of concentric cylinders similar to the Taylor/Couette geometry. However, the symmetry of this geometry leads to two or more counter rotating fluid zones, with very little scalar transport between these "fluid cells." To avoid this situation, the RWPS was designed with a solid disk, termed a "viscous pump" attached to one end of the inner cylinder, Fig. 2.8. Specifically, the inner cylinder (spin filter) with disk (viscous pump) are rotated independently from the outer cylinder vessel wall to create the axial/radial secondary flow, which when added to the primary azimuthal flow, completes the required three-dimensional flow field. The spin filter is covered with a fine filter material to retain the cells on microcarriers within the RWPS, while the media is withdrawn for external conditioning before returning to the vessel at the perfusion inlet. Fresh media enters the vessel from behind the rotating disk near the disk tip and the vessel outer wall.

To achieve the desired large cell number per volume for the RWPS culture, the anchorage dependent cells are grown on porous microcarriers, approximately 170 μm in diameter and coated with extracellular matrix (ECM) proteins. As the cells grow, they spread over the microcarrier surface, forming a confluent unilayer. The individual microcarriers coated with cells then come in contact, allowing cell–cell-junctions to form under low fluid shear conditions, resulting in formation of 3-D tissue-like aggregates over time. Low fluid shear effectively stabilizes the delicate intermediate state required to achieve the more stable and larger 3-D tissue aggregates.

To model the transport of scalars from the perfusion inlet of the RWPS to the surface of the cells, the process was separated into multiple steps as shown schematically in Fig. 2.9. First, the scalar concentration field in the fluid media was computed by numerically solving the transport equations, subject to the assumption that the only effect of the cells was equivalent to a uniform consumption rate of the scalar as a function of position. That is, that the cells were uniformly distributed in space and all cells consumed the scalar at the same rate. The effects of the presence of the cells on the flow field and fluid properties were assumed negligible. Inflight video imaging shows this to be a reasonable assumption.

After the scalar concentration field was computed, the transport of the scalar from the fluid to the surface of the 3-D aggregates was needed. At the very low relative velocities typical of operating conditions in microgravity (low Reynolds number flow), there is a relatively large viscous region of fluid that moves with the cell aggregates (local domain). The scalar transport must traverse this region to reach the aggregate surface. Figure 2.10 shows the model for the solution of mass transport through the local domain. The boundary conditions for the local solution are obtained by solving for the trajectory of the cell aggregate. The relative velocity and concentration value of the scalar at the local domain outer boundary are taken to be the aggregate/fluid relative velocity and the scalar concentration at the aggregate location in the scalar concentration field, respectively. For details of the mass transport calculations in the RWPS geometry, see Rivera-Solorio [53], Kleis and Rivera-Solorio [7], and Rivera-Solorio and Kleis [8].

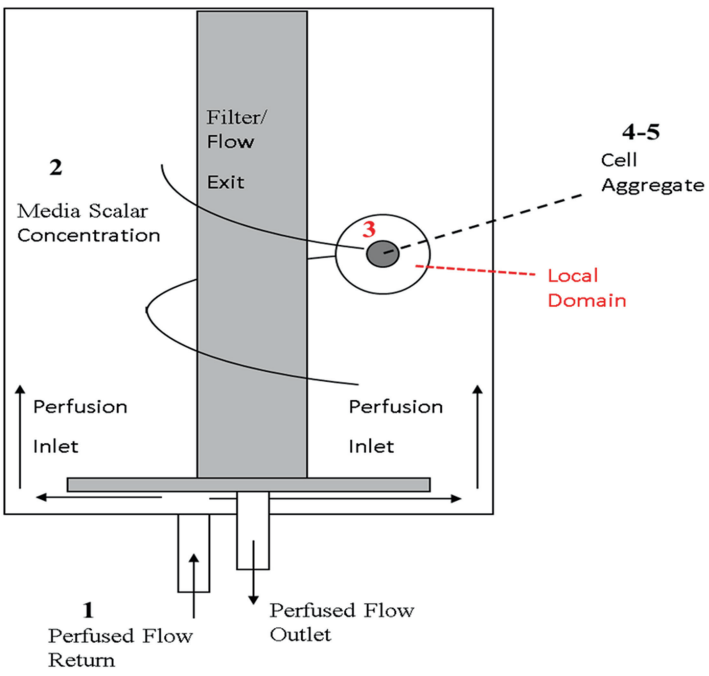


Fig. 2.9 Schematic of mass transport model. Scalar transport from inlet, *1*, through medium, *2*, to the moving local domain, *3*, to the aggregate surface, *4*, to the cells within the aggregate, *5*

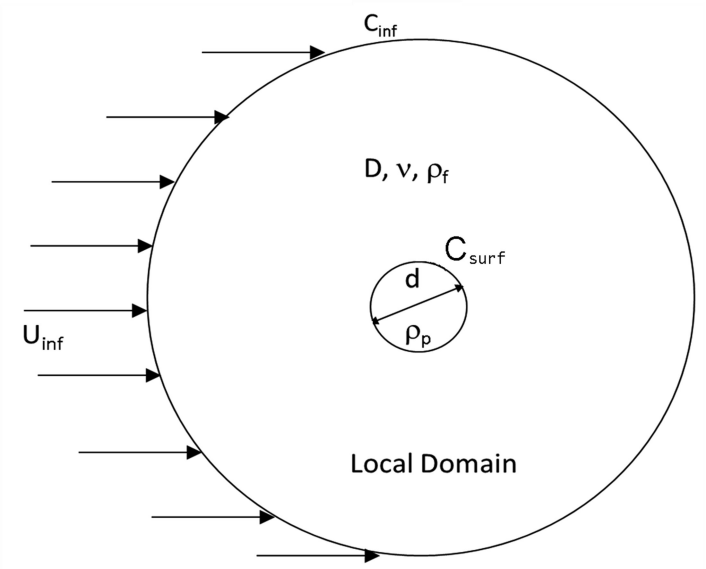


Fig. 2.10 Schematic for local domain scalar concentration solution. Domain size, D, with conditions at outer boundary, U_{inf}, medium viscosity, ν, and density, ρ_f. Inner surface of particle size, d, density, ρ_{p}, and surface concentration, C_{surf}

In calculation of the 3-D aggregate trajectory, when the acceleration of gravity is removed, the dominant forces acting on the aggregate are the Coriolis and centrifugal forces. Since these forces are much smaller than gravity acting on the density differences, the time constant for the response of the aggregate is much longer than the 1 g situation. This means that the implicit assumption of equilibrium conditions at all times generally made in analyses in 1 g may no longer be valid. Specifically, the assumption is often made that steady state drag coefficients based upon the instantaneous velocity difference can be used to compute force balances. When the time constant becomes comparable with the time for one axial orbit of the aggregate, this assumption is no longer valid. The drag and lift forces on the aggregate are then functions of the instantaneous relative velocity and the history of the relative velocity. Also, the large viscous domain moving with the aggregate at these low Reynolds numbers cause a significant increase in the mass being accelerated by the forces. This "added mass" must be accounted for when computing the 3-D aggregate trajectories.

2.5 Fluid Dynamics Results and Discussion

To demonstrate the importance of using a more complete model for the calculations of 3-D tissue aggregate trajectories and local mass transport, a few examples are given with operating conditions typical of cultures of anchorage dependent cells in the RWPS operating in microgravity. Because the basic flow field is axisymmetric,

a stream function can be defined. The secondary flow is then shown by the intersection of the stream function constant surfaces as they intersect a radial/axial plane. Figure 2.11 shows the streamlines for three rotational conditions. The streamline pattern changes with rotation conditions show similar and controllable secondary flows as the inner vessel wall rotation rate is changed. Also, as expected, a strong axial flow is induced by adding a small amount of outer wall rotation. This is a well-known effect in rotating fluid systems often referred to as Taylor columns, after the pioneering work of G. I. Taylor [54]. This relatively strong axial flow is ideal for the RWPS to help distribute inlet fluid throughout the vessel.

Figure 2.12 shows what can happen when the outer vessel wall is held stationary. Three counter rotating fluid cells are formed along the axis. Exchange of scalar concentrations between fluid cells would be limited to diffusion. This situation should be avoided for cell culture in the RWPS. Once the basic flow pattern has been computed, the 3-D aggregate trajectories can be computed from the equations of motion. Figure 2.13 shows a typical single axial orbit and a surface of many axial orbits traversed by a single 3-D cell "aggregate," affected very little by centrifugal

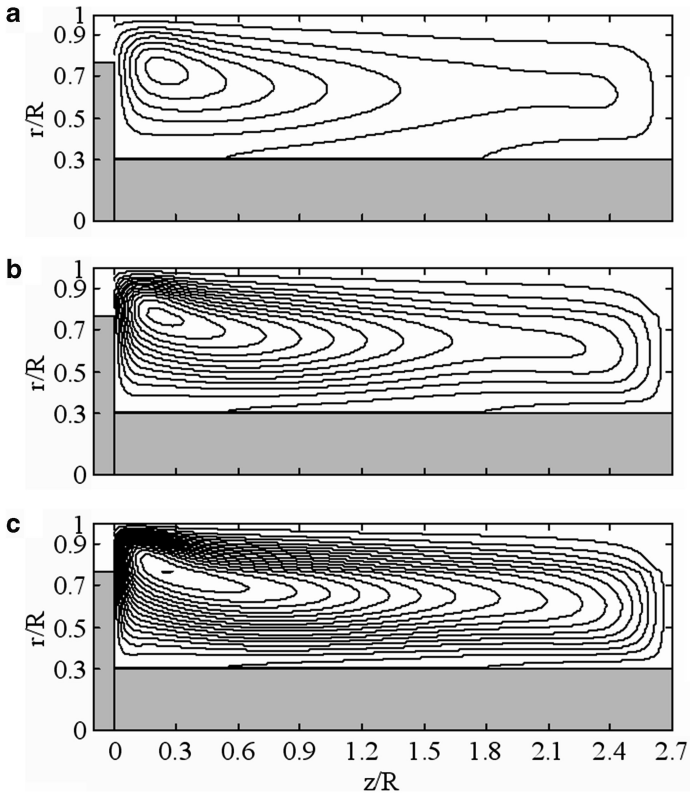


Fig. 2.11 Streamlines in radial/axial planes for 10 cc/min perfusion and outer vessel wall at 1 rpm. The inner wall is varied from (**a**) 5 rpm, (**b**) 10 rpm, and (**c**) 15 rpm. Mass transport is effectively increased by increasing the inner wall rotation rate

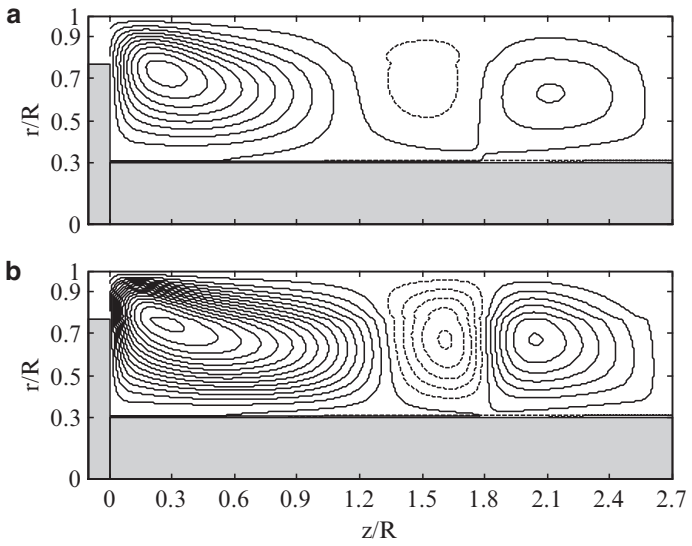


Fig. 2.12 Streamlines in the RWPS for zero outer wall rate and 10 cc/min perfusion. The inner wall is set to (**a**) 6 rpm and (**b**) 11 rpm. *Solid lines* represent clockwise cell rotation and *dashed lines*, counterclockwise. One observes unstable fluid flow breaking up into multiple cells in the absence of outer wall rotation

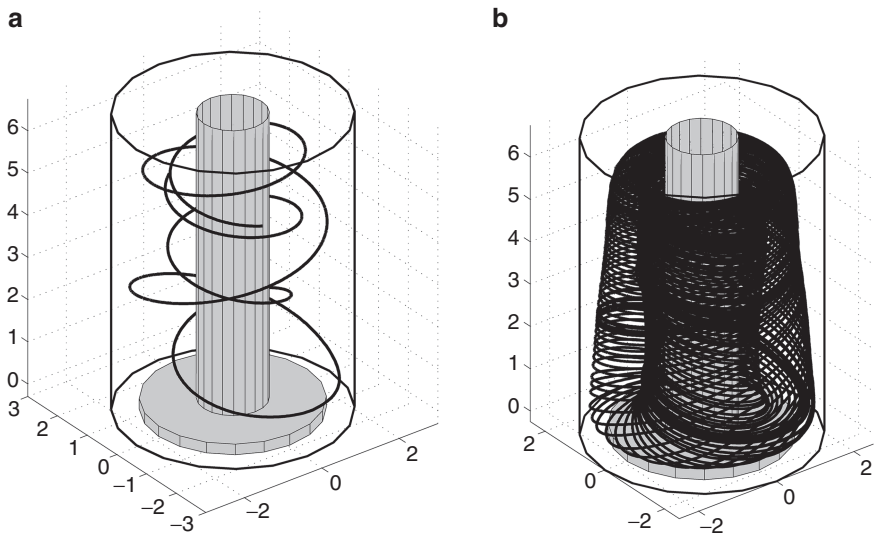


Fig. 2.13 Trajectories for (**a**) one axial orbit and (**b**) multiple axial orbits

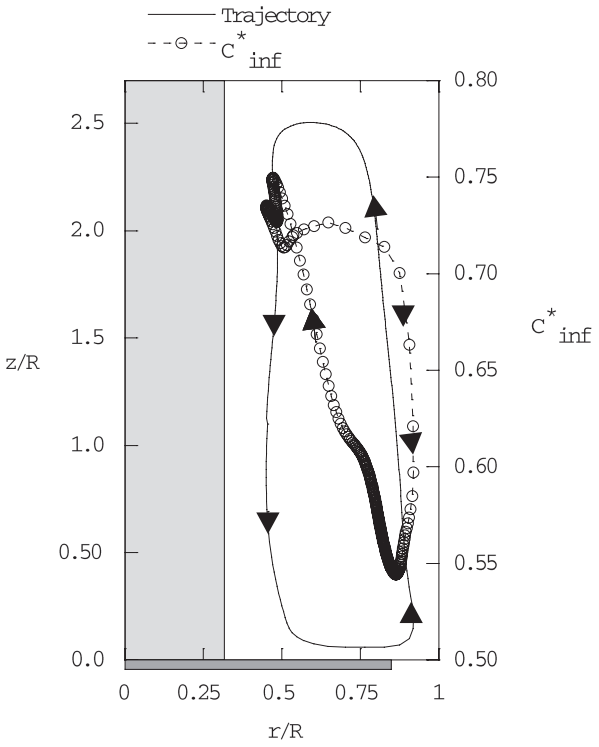


Fig. 2.14 Trajectory and concentration history for a 3 mm aggregate

effects. This allows one to visualize the underlying fluid flow pattern. The basic motion is for the 3-D aggregate to move axially toward the disk at a radial position closer to the axis, followed by (1) a rapid increase in radial position as the aggregate approaches the disk, (2) axial motion away from the disk at the larger radial position, and finally, (3) a decrease in radial position at the axial position farthest from the disk. The aggregate roughly follows the streamline in the radial/axial plane, deviating somewhat due to the centrifugal force.

Once the basic flow field and 3-D tissue aggregate trajectories are computed, the boundary conditions for the local domain mass transport problem are known. Figure 2.14 shows a plot of the aggregate trajectory and resulting normalized oxygen concentration distribution for a 3 mm diameter aggregate. Note that the trajectory and concentration curves are traversed in opposite directions. The concentration numbers are for dissolved oxygen, normalized by the saturated condition at the perfusion inlet. The values are those from the concentration distribution in the media at the center of the aggregate at each time point. This is assumed to be the outer boundary condition for the local domain, which follows the 3-D aggregate position in time.

The results of numerical fluid dynamics analysis highlight that care must be exercised when operating bioreactors in microgravity to ensure sufficient mass transport for the cell culture. Operating conditions must be selected that provide sufficient

transport even at the expense of operating at the minimum fluid shear levels possible. A complete fluid dynamics analysis should be conducted for any bioreactor system to be operated in microgravity in order to properly select the desired conditions and interpret results in a causal manner. RWV culture on Earth, in unit gravity, serves as an analogue to true microgravity but is limited in operating range due to gravity driven sedimentation of the culture components. As microgravity analogues, they are useful for experiment development, selecting high value spaceflight research, anticipating results, and as controls for spaceflight tissue culture.

Gravity introduces fundamental limitations to the adjustable parameters as well as co-mingling critical variables such as fluid mixing required to suspend a 3-D culture versus that required to effect mass transport. Analogue microgravity culture achieves partial relief from such gravity-induced limitations, often achieving state of the art results in terms of establishing 3-D organotypic cell culture models. In particular, the simultaneous achievement of (1) 3-D spatial freedom, (2) low hydrodynamic stress, and (3) co-spatial localization of cells/tissues and their associated scaffolds with differing sedimentation properties is considered to be the primary mechanism of gravity coupling which enables enhanced formation of functional high fidelity 3-D tissue. Analytical and numerical methods, verified by experimental measurements, have been conducted to quantify these simultaneous conditions for true and analogue microgravity RWV culture. The high fidelity 3-D tissue models achieved are central to the emerging field of tissue engineering as it is applied to regenerative medicine, ex-vivo organ development, infectious disease, cancer, pharmaceutical development, and tissue biology basic research.

2.6 Summary

Understanding the effects of microgravity per se on cultured cells is greatly advanced by the use of fluid physics and computational fluid dynamics. It is essential that the design and operation of cell culture systems for application in microgravity and microgravity analogues are accompanied by thorough analysis of the physical environment in the context of gravity and fluid properties. This becomes critically important when assessing whether the cells are responding directly to microgravity as opposed to the indirect cell response to environmental (fluid) conditions created by microgravity. The development of the bioreactor systems described herein was an orderly process of developing and optimizing analytical and numerical models and validation with live cultures and inert measurements. The approach was to deterministically relate gravitational effects to the culture environment and resulting in-vitro tissues. Without this deliberate approach, it would be difficult to advance our understanding of low gravity and low fluid shear environments and their impact on cellular biology and to exploit the advantage of controlled gravitational tissue culture.

In addition to culture of mammalian cells under low fluid shear stress, the RWV bioreactor has also been used to culture microbial pathogens under conditions of

physiological low fluid shear that are relevant to those encountered in the infected host during the natural course of infection. These studies have revealed novel insight into pathogenic mechanisms used by microbes during the infection process, which are not observed during conventional culture, and along with spaceflight studies, are the topic of Chaps. 10–14.

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