Increased Rate of Chondrocyte Aggregation in a Wavy-Walled Bioreactor

Ericka M. Bueno, Bahar Bilgen, Rebecca L. Carrier, Gilda A. Barabino

Abstract: A novel wavy-walled bioreactor designed to enhance mixing at controlled shear stress levels was used to culture chondrocytes in suspension. Chondrocyte aggregation in suspensions mixed at 30, 50, and 80 rpm was characterized in the wavy-walled bioreactor and compared with that in conventional smooth-walled and baffled-walled spinner flask bioreactors. Aggregation was characterized in terms of the percentage of cells that aggregated over time, and aggregate size changes over time. The kinetics of chondrocyte aggregation observed in the bioreactors was composed of two phases: early aggregation between 0 and 2 h of culture, and late aggregation between 3 and 24 h of culture. At 50 rpm, the kinetics of early aggregation in the wavy-walled bioreactor was approximately 25% and 65% faster, respectively, than those in the smooth-walled and baffled-walled spinner flask bioreactors. During the late aggregation phase, the kinetics of aggregation in the wavy-walled bioreactor were approximately 45% and 65% faster, respectively, than in the smooth-walled and baffled-walled spinner flasks. The observed improved kinetics of chondrocyte aggregation was obtained at no cost to the cell survival rate. Results of computerized image analysis suggest that chondrocyte aggregation occurred initially by the formation of new aggregates via cell–cell interactions and later by the joining of small aggregates into larger cell clumps. Aggregates appeared to grow for only a couple of hours in culture before reaching a steady size, possibly determined by limitations imposed by the hydrodynamic environment. These results suggest that the novel geometry of the wavy-walled bioreactor generates a hydrodynamic environment distinct from those traditionally used to culture engineered cartilage. Such differences may be useful in studies aimed at distinguishing the effects of the hydrodynamic environment on tissue-engineered cartilage. Characterizing the wavy-walled bioreactor’s hydrodynamic environment and its effects on cartilage cell/tissue culture can help establish direct relationships between hydrodynamic forces and engineered tissue properties.

KEYWORDS: cartilage tissue engineering; bioreactor; chondrocyte aggregation; aggregation kinetics; residence time distribution

INTRODUCTION

Engineered tissues not only provide an alternative approach to the treatment of patients who suffer from tissue damage, but can also serve as models for studies of tissue development and pathology. Cartilage’s self-repair abilities are very limited, mostly because of its avascular nature and the low metabolic activity of its mature cells. Tissue-engineered constructs matching the structural and functional properties of native articular cartilage would thus be a promising therapy for patients suffering from irreversible cartilage damage.

To develop cartilage tissue-engineered constructs, investigators have employed a cell–polymer scaffold–bioreactor system. Isolated chondrocytes are cultivated on highly porous, biocompatible polymer scaffolds within the controlled hydrodynamic environment of a bioreactor (Freed and Vunjak-Novakovic, 2000). After several weeks of culture, the resulting tissue constructs resemble natural cartilage in terms of histology, cell density, and tissue composition (Aigner et al., 1998; Freed et al., 1993a; LeBaron et al., 2000; Martin et al., 2002; Peel et al., 1998; Saini and Wick, 2003).

Cartilage tissue–engineering bioreactors play a crucial role in the final properties of engineered cartilage. The bioreactor environment directly affects the uniformity of cell seeding into three-dimensional scaffolds as well as the maintenance of the chondrocyte phenotype and, therefore, the cartilaginous characteristics of the tissue. Bioreactors are responsible for providing the right mass transfer conditions for nutrients and waste to be exchanged between the culture medium and the chondrocytes, and for scaffolds to degrade simultaneously with tissue production. Ideally, the bioreactor itself provides in vivo–like physical stimulation to the growing tissues, either by mechanical or hydrodynamic loading, enhancing the secretion of extracellular matrix and tissue formation. Bioreactors that have been used for the tissue engineering of cartilage include conventional spinner flasks (Freed et al., 1993b), rotating wall vessels (Freed and Vunjak-Novakovic, 1995), concentric cylinders (Saini and Wick, 2003), and perfusion bioreactors (Carver and Heath, 1999; Davisson et al., 2002). Each of these bioreactors
provides a different hydrodynamic environment, which results in variations in tissue properties. Differences in flow and mixing conditions around the growing cartilage constructs affect tissue structure by inducing changes in cell shape and function as well as in mass transfer rates of nutrients and metabolites (Vunjak-Novakovic et al., 1996).

At the macromolecular level, cartilage formation starts with the aggregation of mesenchymal cells. The aggregation of chondrocytes in suspension cultures leads to other biological reactions beneficial to cartilage tissue engineering, such as cell differentiation and secretion of extracellular matrix molecules (Abbott and Holtzer, 1966; Lavietes, 1970). Aggregation of chondrocytes in suspension in well-mixed smooth-walled spinner flasks has been described as a steady increase in the fraction of aggregated cells with time, along with a limitation on the size of forming aggregates (Vunjak-Novakovic et al., 1998). Chondrocyte aggregation also appears to be beneficial to the cell seeding of polymer scaffolds. In a study conducted in smooth-walled spinner flasks mixed at 50 rpm, the rate of cell seeding of polyglycolic acid scaffolds was higher for chondrocyte aggregates than for single cells (Vunjak-Novakovic et al., 1998). It can thus be hypothesized that a bioreactor environment that favors improved chondrocyte aggregation under mixed conditions will also favor improvements in the tissue engineering of cartilage constructs. These improvements will be in terms of kinetic rate of chondrocyte seeding of polymer scaffolds and the rate at which extracellular matrix components are secreted and deposited into the matrix network to form tissue.

One of the factors influencing chondrocyte aggregation and engineered cartilage tissue formation is the mixing environment. Mixing was shown to improve the cell seeding of polymer scaffolds and the morphology of cartilaginous tissue constructs. However, it also induced the formation of an outer fibrous capsule in cartilage constructs grown in regular spinner flasks and Petri dishes (Freed et al., 1994). It is known that, although most mammalian cells are damaged and respond negatively to the high shear stresses introduced at high agitation rates (Croughan and Wang, 1989; Kunas and Papoutsakis, 1990), methods to protect the cells from shear damage usually affect the quality of mixing ( Chattopadhyay et al., 1997; Wu, 1999). A low-shear-stress fluid regime helps prevent cell damage and detachment from aggregates or scaffolds (Saini and Wick, 2003). A combination of low-shear-stress regimes and appropriate mixing can be achieved using bioreactor design.

The wavy-walled bioreactor was designed to enhance mixing at controlled shear stress levels by the introduction of smooth waves that mimic baffles (Natarajan et al., 1992). The internal radius, $R$, of the wavy-walled bioreactor as a function of the angle, $\phi$, is governed by the equation:

$$R = R_{avg} + A \sin(N\phi)$$  \hspace{1cm} (1)

where $R_{avg}$ is the average radius ($R_{avg} = 3.35$ cm), $A$ is the magnitude of peak amplitude at the node ($A = 0.45$ cm), and $N$ is the number of grooves ($N = 6$) (Fig. 1). In mammalian microcarrier cell cultures, higher cell density (i.e., higher cell proliferation) was observed in the wavy-walled bioreactor when compared with a regular spinner flask (Barabino et al., 1993).

By introducing a novel geometry, the wavy-walled bioreactor may be a useful vessel in studies aimed at distinguishing the effects of the hydrodynamic environment on tissue-engineered cartilage. Characterizing the wavy-walled bioreactor’s hydrodynamic environment and its performance in cartilage cell and tissue cultures will help establish direct relationships between hydrodynamic forces and engineered tissue properties.

As an initial step toward this characterization, this study compares chondrocyte aggregate formation in the wavy-walled bioreactor to that in conventional smooth-walled and baffled-walled spinner flask bioreactors. Mixing in the wavy-walled bioreactor was investigated using residence time distribution (RTD) and dissolved oxygen concentration data and compared with the mixing inside the regular spinner flask. Because all other culture parameters were equal, the differences in chondrocyte aggregation patterns were only related to the differences in the mixing environments of these three vessels.

**MATERIALS AND METHODS**

**Materials**

Freshly explanted knee joints from 2- to 6-week-old bovine calves were obtained from John Larusso Research 87

![Figure 1](image-url)
Type II collagenase enzyme was obtained from Worthington Biochemical (Lakewood, NY). Gibco (Grand Island, NY) was the source of Dulbecco’s modified Eagle medium (DMEM), phosphate-buffered saline (PBS), N-(2-hydroxyethyl)piperazine N’-2-ethanesulfonic acid (HEPES), and fetal bovine serum (FBS). Penicillin/streptomycin, ethylene-diamine tetraacetic acid (EDTA), nonessential amino acids (NEAA), proline, ascorbic acid, Trypan blue solution, fluorescent Hoechst 33258 tracer dye, and agarose were from Sigma (St. Louis, MO). Nytex mesh (150 μm) was from Sefar America (Depew, NY). Regular smooth-walled spinner flasks (125-mL capacity, 6.7-cm average diameter, with a nonsuspended stir bar of 0.8-cm diameter × 3.8-cm length) were custom-made to design by Finkenbeiner Glass Blowers (Waltham, MA). Wavy-walled bioreactors (125-mL capacity, 6.7-cm average diameter, with a nonsuspended stir bar of 0.8-cm diameter × 3.8-cm length) were fixed to an orbital shaker placed inside a 37°C, 5% CO₂ incubator and stirred overnight (16 to 20 h) at 50 rpm. The bioreactors were filled with 120 mL of chondrocyte culture medium and mixed at one of three speeds (30, 50, and 80 rpm) inside a humidified, 37°C, 5% CO₂ incubator for 12 to 18 h with the sidearm caps loosened to allow for gas exchange.

At time zero, an aliquot of freshly isolated chondrocytes was injected into the bioreactors while keeping the medium volume at 120 mL. The cell concentration inside the bioreactors was fixed at 120 cells/μL, which corresponds to a total of 1.4 × 10⁷ cells. This value is within the range of cell seeding densities reported in cartilage-tissue engineering literature for a 5-mm-diameter and 2-mm-thick scaffold: 1 to 10 × 10⁶ cells/scaffold, where usually there are 4 to 12 scaffolds/bioreactor (Freed et al., 1998).

**Sample Analyses**

At timed intervals (1, 2, 3, 4, 6, 8, 10, 12, 20, and 24 h), 0.5-mL aliquots of cell suspension were extracted from each vessel under uninterrupted mixing and aseptic conditions. Aliquots were extracted in triplicate using a regular-tipped 1-mL pipette, centrifuged, and resuspended to assess single cell concentration by hemocytometer counting and viability by Trypan blue exclusion. Single-cell viability was monitored only for the initial 12 h of culture. After this timepoint, most of the cells (>50%) existed as aggregates. Accurate estimation of the number of viable cells inside aggregates was not attempted because of the effects of aggregate dissociation methods on cell viability.

Aggregation was defined as the percentage of chondrocytes existing in aggregates with respect to the total number of chondrocytes in suspension. It was calculated using an assumed constant total cell number in the flasks (120 cells/μL) over the 24-h culture period, as has been done previously in cell aggregation studies (Morris, 1976; Moscona, 1961; van Kampen and Veldhuijzen, 1982). This assumption was confirmed by experiments that included trypsinization of aggregates and counting of chondrocytes (data not shown). Aggregation percentage at a given time, t, was calculated according to the formula:

\[
\text{Aggregation percentage} \,(t) = 100 \times \frac{C_{s0} - C_s(t)}{C_{s0}}
\]  

where \(C_{s0}\) and \(C_s\) are the concentration of single cells (single cells/microliter) at seeding time and time t, respectively. Cell concentration was verified by use of computerized image analysis employing a computer macro.

A simple model was used to look at the kinetics of cell aggregation, based on the disappearance of single chondrocytes from the suspension (Morris, 1976). The model assumes uniform concentration of single chondrocytes throughout the bioreactor at any specific timepoint, no cell attachment to the bioreactor’s walls, and first-order kinetics of chondrocyte attachment to aggregates. The change in
single cell concentration in the well-mixed suspension is then:

$$-\frac{\partial C_s}{\partial t} = k \times C_s$$

which becomes:

$$\frac{C_s}{C_{s0}} = e^{-kt}$$

where $k$ ($h^{-1}$) is the kinetic constant of cell attachment to aggregates, \( t \) (h) is the time elapsed since the start of the cell culture, $C_s$ is the concentration of total single cells in suspension at time \( t \), and $C_{s0}$ is the initial concentration of single cells in suspension, 120 cells/\( \mu \)L.

The size distribution of aggregates and single cells was determined at the specified timepoints by computerized image analysis. For this purpose, 0.5-mL samples were extracted from the bioreactors in triplicate using a wide-mouth (5-mm-diameter) 1-mL pipette, transferred into individual 5-cm-diameter, agarose-coated Petri dishes for analysis, as described in what follows.

### Image Analysis

Phase-contrast images of cell suspension samples inside agarose-coated Petri dishes were acquired by a CCD camera (XC-75, Sony) mounted on an inverted microscope (ausJENA, Telaval 3). The images were digitized by a Scion LG-3 frame-grabber installed in a Dell Optiplex computer and analyzed using the SCION IMAGE for Windows (Scion Corp.) software. Aggregate and cell size distribution were determined using a computer macro generously provided by Dr. Ivan Martin (Martin et al., 1997). By randomly acquiring 15 images per dish, 3% of the total sample area was subject to analysis. Briefly, each image was segmented according to an automatically determined threshold of optical density, where objects were defined as being lighter than the background. Aggregates and single cells were classified according to size and geometry and automatically counted. Aggregates were modeled as ellipsoids and cells as spheres.

To better understand how the size of chondrocyte aggregates is affected by the choice of bioreactor geometry and agitation rate used, the aggregate population at any given timepoint was divided into two subpopulations: (i) small aggregates with a projected area of $<2500 \, \mu m^2$; and (ii) large aggregates with a projected area of $>2500 \, \mu m^2$.

According to Vunjak-Novakovic et al. (1998), chondrocytes in small aggregates can be considered as packed in a manner conforming to solid-sphere packing theory, where the average packing factor is 0.62. The diameter of an average bovine chondrocyte is 11 \( \mu \)m; therefore, an aggregate of 2500-\( \mu \)m$^2$ projected area contains approximately 16 chondrocytes. The size limit of 2500 \( \mu \)m$^2$ dividing the two aggregate subpopulations was chosen because it constitutes the size threshold at which significant differences between bioreactors were first observed.

### Statistical Analyses

Primary chondrocytes explanted from the same knee joint were used in four to six different bioreactors. Each experimental group was performed at least three separate times, and within each group a minimum of one bioreactor contained chondrocytes from a different animal. Individual samples from the same experiment were taken and analyzed in triplicate (\( n = 3 \)). For each data set, the values presented are sample means calculated using Student’s \( t \) distribution with a 95% confidence level. Statistical analyses were performed using full factorial one-way analysis of variance (ANOVA). Statistical significance was determined by \( P \)-values ($P < 0.05$).

### Mixing Studies

To characterize mixing in the wavy-walled and spinner flask bioreactors, residence time distribution (RTD) and dissolved oxygen (DO) studies were performed. RTD data were obtained using tracer-response experiments. The vessels were initially filled with 120 mL of water and stirred at 50 rpm. Water was continuously infused (Model 956, Harvard Apparatus) into one side-arm of the bioreactor and simultaneously withdrawn out of the other side arm at a constant flow rate of 2.0 mL/min. Using a 1-mL syringe, a 1-mL bolus of 360 \( \mu \)g/mL fluorescent dye was injected via the inlet stream at \( t = 0.5 \) min. Approximately 30 data points were taken for 90 min at 0.5- to 5-min time intervals. The outlet concentration of the fluorescent dye was assessed using a spectrofluorometer (Perkin Elmer LS45 Luminescence Spectrometer) with water as blank and background at 365-nm excitation and 468-nm emission wavelengths. The dispersion model and the tanks-in-series model for closed systems were applied to the tracer response measurements to determine the dimensionless dispersion number ($D/uL$) and the number of tanks in series ($N_i$) as defined by Levenspiel (1972). Dissolved oxygen measurements were made at five different positions in wavy-walled bioreactors and regular spinner flasks containing a suspension of chondrocytes in culture media (Fig. 7). Oxygen transfer in the culture took place via surface aeration and mixing in the bioreactors, and therefore the axial distribution of dissolved oxygen concentration depended on the axial mixing in the bioreactors. An oxygen probe (MI-730, Microelectrodes, Inc.) and an oxygen meter (OM-4, Microelectrodes, Inc.) were used for these measurements.

### RESULTS

#### Cell Survival

For the purpose of comparison, all cell viability values were normalized to a mean initial value of 95% (i.e., 95 viable cells out of 100 cells at the time of bioreactor
seeding). In all experiments, the viability of cells in the seeding suspension was between 90% and 98%.

The WWB showed a significant drop in single cell viability over time at 30 rpm, whereas the RSF and the BSF maintained similar and acceptable viability (Fig. 2A). Based on this observation, a decision was made against further analysis of the behavior of chondrocytes in the WWB at 30 rpm and its comparison to the other vessels. At 50 and 80 rpm, cell survival was similarly high ($P > 0.05$) within the three bioreactors (Fig. 2B and C). In all cases, except the WWB at 30 rpm, cell viability remained $>80\%$ after 12 h of culture. Viability in the WWB improved with increasing agitation rate, whereas, in the RSF and the BSF, viability was unaffected by the level of agitation ($P > 0.05$).

Chondrocyte Aggregation

Aggregation percentages calculated using Eq. (2) showed that, in all experimental groups, $>60\%$ of chondrocytes had become part of aggregates after 24 h of culture (Fig. 3A and B), and there were significant differences in the rate of aggregation with respect to bioreactor geometry and agitation rate.

Although final aggregation percentages were similar in all bioreactors at 50 rpm (Fig. 3A), the WWB supported higher cell aggregation throughout the culture period (chondrocytes in the WWB aggregated at a faster rate than in the other two bioreactors). At 80 rpm, aggregation fluctuated in the RSF and the BSF, but consistently increased with time in the WWB (Fig. 3B). Initially, higher aggregation in the RSF and the BSF in comparison to the WWB was observed at 80 rpm. This behavior shifted between 6 and 8 h of culture and, from this point through the end of culture, more chondrocytes were aggregated in the WWB than in the RSF or the BSF. Differences in chondrocyte aggregation between the RSF and the BSF were noticeable only at 80 rpm: the BSF demonstrated lower aggregation percentages throughout most of the culture period ($10\%$ to $30\%$ less than the RSF after 10 h). Within single bioreactor types, aggregation percentages attained at 80 rpm were significantly lower ($P < 0.05$) than those at 50 rpm.

Kinetics of Cell Aggregation

Suspended single chondrocytes attached to each other, generating aggregates of two or more cells. The attachment rate was dependent on the type of bioreactor and agitation rate used. Time profiles of concentration of single cells in suspension were used to assess the kinetics of cell aggregation using least-squares regression of single-cell
concentration data, $\ln(C_s/C_{0s})$ vs. time (Fig. 4). As seen in Figure 4, aggregation behavior was best described by a two-phase kinetic model, in which both phases can be described by Eq. (4). The early phase took place between 0 and 2 h of culture and was characterized by rapid chondrocyte aggregation. Decreased rates of aggregation were observed during the late phase (3 to 24 h). This decrease was approximately four- to fivefold and as much as ninefold in the RSF at 80 rpm. The two-phase kinetic model fit the data at 50 and 80 rpm for the WWB ($R^2 > 0.90$) and the RSF ($R^2 > 0.85$), and only at 50 rpm for the BSF ($R^2 > 0.80$). The coefficient of correlation ($R^2$) was <0.80 for BSF at 80 rpm, suggesting that aggregation was disturbed by events related to the fluid environment.

For all acceptable correlations ($R^2 > 0.80$) based on Eq. (4), the bioreactors exhibited decreasing $k$-values (slower aggregation) with increasing agitation rate. There were also differences related to the bioreactors: early aggregation kinetics at 50 rpm were approximately 25% and 65% faster in the WWB than in the RSF and the BSF, respectively. At 80 rpm, the rate of early aggregation was the same for both the WWB and the RSF ($k = 0.30$ h$^{-1}$). During the late phase, aggregation was faster in the WWB than in the RSF and the BSF (WWB > RSF > BSF) for both agitation rates; at 50 rpm, $k$ was approximately 45% and 65% higher in the WWB than in the RSF and the BSF, respectively; whereas, at 80 rpm, $k$ was 79% higher in the WWB than in the RSF. These results suggest that, for the conditions studied, the wavy-walled bioreactor hydrodynamic environment promotes faster aggregation of chondrocytes with no effect on cell survival when compared with the environments of the RSF and the BSF.

**Aggregate Size Distribution**

The increase in percentage of aggregated chondrocytes with time in culture (Fig. 3) was not accompanied by a clear and proportional increase in the size of the average aggregate at 50 rpm (Fig. 5). After 4 to 10 h of culture at 50 rpm, the size of the average aggregate reached a steady value in the WWB and the BSF, whereas the area of the average cell aggregate in the RSF continued to increase over 24 h of culture. The rapid initial increase in $A_{avg}$ (0 to 2 h) was consistent with the rapid kinetics of early aggregation presented in Table II. At 80 rpm, $A_{avg}$ fluctuated with time (data not shown), possibly due to the high mixing environment disrupting aggregate conformation.

Differences in the concentration of aggregates of a particular size group were related to bioreactor geometry and

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**Figure 4.** Kinetics of chondrocyte aggregation. The concentration of single chondrocytes in suspension decreased exponentially with time. Data were divided into two aggregation phases: early aggregation and late aggregation, and plotted according to first-order kinetics: (A) early phase at 50 rpm; (B) early phase at 80 rpm; (C) late phase at 50 rpm; and (D) late phase at 80 rpm. Values of the first-order kinetic constant of aggregation, $k$ (h$^{-1}$) are presented in Table II. Data represent the average ± SD of $\ln(C_s/C_{0s})$ calculated using three independent measurements of $C_s$.

**Figure 5.** Area of the average aggregate at 50 rpm. Images of cell suspension aliquots were computer analyzed using a macro (Martin et al., 1997). Three percent of the total sample area was subject to analysis. Objects were classified as either cells or aggregates depending on their size and geometrical shape of their projection on the image plane. Aggregates were modeled as ellipsoids and their area measured from their projected profile. The area of the average aggregate is calculated as the sum of all measured ellipsoid projections over the number of such projections. Data represent the average ± SD of three independent measurements. Each measurement comprised 10 to 15 images.
agitation rate (Fig. 6). At both 50 and 80 rpm, the WWB produced the highest number of large aggregates in suspension. In the WWB, the differences between the 50- and 80-rpm aggregate size distributions were significant: from low to high agitation rates the concentration of large aggregates decreased by half, whereas the concentration of small aggregates increased by a factor of three or four. The fluid environment within the WWB at 50 rpm may have allowed small aggregates to join and form large aggregates, whereas higher mixing at 80 rpm possibly limited this event. The RSF and the BSF demonstrated similar aggregate size profiles with no significant effects due to agitation rates. As seen in Figure 6, the number of aggregates per milliliter in all bioreactors increased with time, peaking at around 8 h of culture and either remaining constant or decreasing from then on.

At any given timepoint during culture at 50 rpm, there were approximately 50 to 200 small aggregates/mL in the RSF and the BSF, compared with 50 to 100 in the WWB. On the other hand, at the same agitation rate, the concentration of large aggregates went from 0 to 5 or 6 aggregates/mL in the WWB, approximately twice the large aggregate concentrations in the RSF and the BSF.

At 80 rpm there was a shift in the patterns of aggregate size distribution: the WWB had the highest concentration of small aggregates, ranging from 150 to 350 aggregates/mL, compared with 70 to 170 aggregates/mL in the RSF and the BSF. These results suggest that the increased shear levels accompanying the increased agitation rate in bioreactors limited the growth of aggregates and prevented small aggregates from forming bigger structures.

Mixing
Table I summarizes the RTD and the non-ideal flow parameters. The dispersion number and the number of tanks in series were similar in the WWB and the RSF, indicating an equivalent extent of mixing. The mean residence time and the variance were 15% and 22%, respectively, higher on average in the WWB than in the RSF, suggesting backmixing and recirculation in the WWB, possibly due to the presence of the grooves.

The dissolved oxygen (DO) concentration was highest (>90%) at the top fluid level in both bioreactors, because oxygen transfer was carried out by surface aeration. The DO concentrations decreased axially from the fluid surface to the bottom of the bioreactor, as expected. DO in the bottom of the reactors ranged from 78% to 92% depending on the experimental conditions. There was no significant change in the DO concentrations with radial positions (i.e., center and wall). The WWB provided lower DO concentrations for the chondrocyte suspension cultures. Figure 7 shows how increasing the agitation rate from 50 to 80 rpm seemed to close the gap in the DO concentrations between the WWB and the RSF.

DISCUSSION
The formation of cell aggregates is an important first step in cartilage tissue engineering. Aggregation of chondrocytes promotes faster attachment to polymer scaffolds (Vunjak-Novakovic et al., 1998) and stimulates cell proliferation and synthesis of extracellular matrix proteins (Abbott and Holtzer, 1966; Lavietes, 1970). In suspension cultures, ag-
aggregation of chondrocytes occurs as a result of cell–cell and cell–aggregate encounters. Such encounters are promoted by the fluid environment provided by bioreactors. A number of studies have addressed the effect of the hydrodynamic environment on properties of engineered cartilage (Gooch et al., 2001; Vunjak-Novakovic et al., 1996, 1999). However, the impact of flow environment on early events (i.e., cell aggregation) crucial to the development of engineered cartilage is not fully understood. In this study, the performance of a novel bioreactor with a unique hydrodynamic environment has been characterized in terms of the formation of chondrocyte aggregates in suspension. Important differences in chondrocyte interactions, as reflected by cell-to-cell and cell-to-aggregate attachment, were observed in the wavy-walled bioreactor in comparison to smooth-walled and baffled-walled spinner flasks. These differences are believed to be due to differences in shear stresses and mixing characteristics.

Chondrocytes aggregated according to a two-phase kinetics model, which can be explained by a mechanism in which rapid single-cell–to–single-cell attachment occurred at the beginning of the culture period, leading to prompt formation of aggregate structures and disappearance of single chondrocytes. After 2 h of culture, the concentration of aggregates in suspension was significant, and the concentration of single chondrocytes had decreased. These events combined to cause a decrease in the rate of chondrocyte aggregation, such that a second, slower phase of aggregation was evident. Given that (a) chondrocyte aggregates seed onto polymer scaffolds at a faster rate than single chondrocytes and (b) chondrocyte aggregates attach to polymer scaffolds as soon as they are formed in suspension (Vunjak-Novakovic et al., 1998), it follows that, in an environment wherein kinetics of early chondrocyte aggregation are high, the rate of chondrocyte attachment to scaffolds is increased. The WWB, as demonstrated by this study, provides such an environment, especially at 50 rpm (Table II).

Although a two-phase kinetics model described chondrocyte aggregation in most of the reactors and operating conditions used in this study, it was not a good fit for the BSF at 80 rpm. In this hydrodynamic environment, cells aggregated at a significantly lower rate than in either the WWB or the RSF. Baffles in the BSF may have introduced high fluid shear-stress regimes that appeared to become more pronounced as agitation rates increased. At 80 rpm, high shear forces imposed by liquid flow around cells and aggregates may have overcome cell–cell adhesion mechanisms and disrupted cell aggregates. The hydrodynamic environment in the BSF at 80 rpm is hypothesized to represent an upper extreme of the mixing environments studied. The lower extreme is the WWB at 30 rpm. At these extremes, the effects of fluid shear on the cultured cells’ behavior and interactions were manifested as negative responses (cell death and poor aggregation). Although fluid shear is a form of hydrodynamic stimulation that can be beneficial to growing cells, there is a delicate balance between the risks and benefits associated with this form of stimulus. The successful culture of chondrocytes and, ultimately, of engineered cartilage cannot be achieved in either extreme: Within the low-shear regime, chondrocytes undergo insufficient mass transport of metabolites, resulting in changes in phenotype that can bring about undesirable changes in tissue properties. Within the high-shear regime, cells are prevented from participating in the necessary interactions that stimulate them to secrete extracellular matrix and form complex, functional tissue.

Differences in the mixing environments afforded by the various bioreactors also influenced aggregate size. The area of the average aggregate was calculated as the sum of all aggregate areas divided by the number of existing aggregates. After 6 h of culture, both the number of aggregates per milliliter (Fig. 6) and $A_{avg}$ (Fig. 5) remained, for the most part, unchanged in a particular bioreactor, whereas the percentage of aggregated cells increased (Fig. 3). These simultaneous observations can be explained by a mechanism of late aggregation in which the predominant events were: (i) single cells attaching mostly to existing aggregates rather than to other single cells; and (ii) small aggregates joining already existing large aggregates.

**Table II.** Results of least-squares regression of ln ($C/C_{o}$) data.

<table>
<thead>
<tr>
<th>Agitation and phase</th>
<th>Early aggregation, $k$ (h$^{-1}$)</th>
<th>Late aggregation, $k$ (h$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>50 rpm</td>
<td>80 rpm</td>
</tr>
<tr>
<td></td>
<td>50 rpm</td>
<td>80 rpm</td>
</tr>
<tr>
<td>Wavy-walled</td>
<td>0.5128 ± 0.0004</td>
<td>0.2978 ± 0.0283</td>
</tr>
<tr>
<td></td>
<td>$R^2 = 1.00$</td>
<td>$R^2 = 0.95$</td>
</tr>
<tr>
<td>Regular spinner</td>
<td>0.4106 ± 0.0094</td>
<td>0.2978 ± 0.0073</td>
</tr>
<tr>
<td></td>
<td>$R^2 = 1.00$</td>
<td>$R^2 = 1.00$</td>
</tr>
<tr>
<td>Baffled spinner</td>
<td>0.3081 ± 0.0519</td>
<td>N/A</td>
</tr>
<tr>
<td></td>
<td>$R^2 = 0.84$</td>
<td></td>
</tr>
</tbody>
</table>

The culture period comprised between 0 and 2 h is called “early aggregation” phase, and the period between 3 and 24 h is the “late aggregation” phase. The kinetic constants of chondrocyte aggregation are presented as $k$, and the fit of the regression is described by $R^2$ values.
This mechanism would also be consistent with slow late-phase aggregation kinetics, where single-cell-to-single-cell fusion occurs more rarely with time and thus the formation of new aggregates is a rare event. In a mixed environment, aggregate-to-aggregate fusion requires greater forces than cell-to-cell or cell-to-aggregate fusion (Morris, 1976). The hydrodynamic environment in the bioreactors used in this study appears to have supported aggregate-to-aggregate fusion. Although new small aggregates were rapidly formed (as shown by early aggregation kinetics) by cell-to-cell interactions in the early phase, large aggregates were formed by small aggregates joining a larger structure throughout the culture period.

The size of chondrocyte aggregates may be a factor in their ability to attach to polymer scaffolds, most of which are 80% to 97% porous. Aggregates may either attach to the surface of these scaffolds or they may migrate through the pores toward the center of the scaffolds. Although the optimal size of aggregates for enhanced attachment is not known, Vunjak-Novakovic et al. (1998) report improved kinetics of chondrocyte attachment to 97% porous polyglycolic acid scaffolds with an aggregate population ranging from 4 to 16 chondrocytes per aggregate. The wavy-walled bioreactor promoted the formation of aggregates within this size range as well as much larger aggregates. In this study, because scaffolds were not seeded with the obtained aggregates, it is not possible to predict an optimal aggregate size for this application. Nevertheless, the attachment of large aggregates to polymer scaffolds may be irrelevant because aggregates are known to attach to scaffolds as soon as they form (i.e., while they are still relatively small).

The bioreactors in this study performed satisfactorily in terms of maintaining cell viability and promoting cell–cell interactions, both of which are desirable for cartilage tissue engineering. However, at 30 rpm, the lowest agitation rate investigated, massive cell death was observed in the WWB, indicating that this agitation rate may not be feasible for the development of engineered cartilage tissue. Lack of nutrients as a result of insufficient mixing is not a plausible explanation for the observed chondrocyte death, because chondrocytes can survive even when cultured under static conditions. A more likely reason, and one supported by visual observations, is that, at this low agitation rate, chondrocytes settled to the bottom of the vessel, where they were physically damaged by the stir bar. Another effect of agitation rate was limited chondrocyte aggregation in the BSF, caused by increased mixing and shear stresses introduced by the baffles. Taken in aggregate, these observations suggest that the effects of different hydrodynamic environments on cell and tissue culture can be either radical, as in massive cell death, or subtle, as in decreased cell aggregation. The importance of these effects depends on the aim of the cell or tissue culture.

In terms of chondrocyte interactions, consistent patterns of aggregation were observed over time in culture. Aggregation of chondrocytes occurred in response to a number of forces acting inside the bioreactors. These include the flow of the culture medium, which promoted cell-to-cell encounter and clustering, and shear stresses introduced by liquid circulation, which promoted cell dispersion. These combined effects differentially influenced the cells’ tendency to adhere to each other in distinct hydrodynamic environments.

Clear differences in the mixing environments generated by the WWB, the RSF, and the BSF were observed. Longer residence time of particles in the WWB with respect to the RSF during RTD studies suggests that recirculation occurs in certain “zones” (such as inside the grooves) in the wavy-walled bioreactor. Large fluctuations in pH prior to steady state during acid–base neutralization experiments in the WWB (data not shown) further confirmed the existence of such recirculation pathways. A particle following these pathways inside the WWB is analogous to a cell or an aggregate. Compared with the same particle in the RSF, the WWB particle has increased chances to interact with other particles, because it spends a longer time in a certain region. These increased interactions result in faster formation of new aggregates, and growth of existing ones.

In contradiction to the observation of higher residence times and decreased oxygen concentrations in the WWB, the dispersion and tanks-in-series model results indicated equivalent mixing in the WWB and the RSF (Table I). Although these models have previously been used for tissue-engineering bioreactors (Freed and Vunjak-Novakovic, 1997), they may not sufficiently characterize fluid flow that deviates from plug flow because of the presence of fluid patterns such as recirculation and eddies in odd corners (Levenspiel, 1972). These types of fluid patterns are present in the WWB and could account for the observed deviation from the model. Lower dissolved oxygen concentration in chondrocyte cultures implies a lower mixing intensity (Gooch et al., 2001), and hence the WWB did not provide increased mixing when compared with the RSF. This finding contradicts previous studies in which the WWB was reported to enhance mixing when compared with the RSF, as assessed by mixing times measured using a pH probe, and flow-field investigation via ultrasound Doppler velocimetry (Natarajan et al., 1992). Natarajan observed shorter mixing times in the WWB, whereas, in the present study, longer mixing times were observed when compared with the RSF (data not shown). This discrepancy in observed mixing times could be due to the different WWB configurations used in the two studies. The 250-mL WWB used by Natarajan was designed for microcarrier cultures, whereas the WWB in the present study was scaled down to 120 mL for its use in cartilage tissue engineering. Design considerations caused the downscaled WWB to have a smaller stir bar diameter to tank diameter ratio ($D_s/T$) (reduced from $D_s/T = 0.71$ to $D_s/T = 0.57$) and a smaller amplitude of the grooves ($A$) (reduced from $A = 0.65$ cm to $A = 0.45$ cm). The combination of these factors potentially influenced the mixing and the hydrodynamic environment in the new WWB.
This study has demonstrated that the wavy-walled bioreactor provides a culture environment that supports enhanced chondrocyte aggregation and viability. The 25% faster kinetics of early chondrocyte aggregation observed in the WWB at 50 rpm is expected to translate into faster chondrocyte attachment to polymer scaffolds. The results of the applied dispersion model, tanks-in-series model, and high cell viability presented in this work show that the WWB provides sufficient mixing for chondrocyte cultures. Aggregation results are also satisfactory for tissue-engineering purposes. Further observation revealed that chondrocyte morphology (and thus phenotype) was also preserved in all bioreactors used in this study (data not shown). The presence of the grooves in the WWB provides a unique environment, which can be a useful tool in cartilage-tissue engineering using chondrocytes seeded on polymer scaffolds. The aim of future studies will be characterization of hydrodynamic environment effects on the growth and function of engineered cartilage in the WWB and the RSF.

**NOMENCLATURE**

- $A_{avg}$: amplitude of the grooves in the wavy-walled bioreactor (cm)
- $A_{p}$: projected area of the average cell aggregate ($\mu m^2$)
- $C_i$: concentration of single cells in suspension at $t$ (cells/µL)
- $C_{0i}$: initial concentration of single cells in suspension (cells/µL)
- $D$: axial dispersion coefficient (cm$^2$/s)
- $D_s$: stir bar diameter (cm)
- $(Dtu_L)$: dispersion coefficient
- $k$: kinetic constant of aggregation (h$^{-1}$)
- $L$: length of the reactor (cm)
- $N$: number of grooves in the wavy-walled bioreactor
- $N_{t}$: number of tanks in series necessary to achieve mixing
- $R$: radius of the bioreactor (cm)
- $R_{avg}$: average radius of the bioreactor (cm)
- $t$: time (h)
- $T$: tank (vessel) diameter (cm)
- $u$: fluid velocity (cms$^{-1}$)

**Greek letter**

- $\sigma^2$: variance (min$^{-2}$)

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**References**


