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ABSTRACT

A mechanical-conditioning bioreactor has been developed to provide bi-axial loading to three-dimensional (3D) tissue constructs within a highly controlled environment. The computer-controlled bioreactor is capable of applying axial compressive and shear deformations, individually or simultaneously at various regimes of strain and frequency. The reliability and reproducibility of the system were verified through validation of the spatial and temporal accuracy of platen movement, which was maintained over the operating length of the system. In the presence of actual specimens, the system was verified to be able to deliver precise bi-axial load to the specimens, in which the deformation of every specimen was observed to be relatively homogeneous. The primary use of the bioreactor is in the culture of chondrocytes seeded within an agarose hydrogel while subjected to physiological compressive and shear deformation. The system has been designed specifically to permit the repeatable quantification and characterisation of the biosynthetic activity of cells in response to a wide range of short and long term multi-dimensional loading regimes.

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1. Introduction

Articular cartilage possesses distinctive mechanical properties suited to its function in load distribution at weight bearing interfaces and reducing friction at the articulating surface of synovial joints [1]. Due to a poor intrinsic reparative ability, damage to articular cartilage is often irreversible, and through the mediation of resulting mechanical instability it may progress toward a degenerative joint disease such as osteoarthritis [2]. As conventional therapies provide unsatisfactory long-term results [3], tissue engineering processes have been developed to produce engineered cartilage substitutes providing at least a minimal level of function immediately post implantation, and survival of early joint forces [4]. The development of engineered cartilage substitutes is based upon the three-dimensional (3D) culture of isolated autologous chondrocytes seeded within a scaffold, and developed under the controlled conditions of a tissue bioreactor.

Cartilaginous tissues have been engineered using the common tissue engineering bioreactors such as spinner flask, rotating wall bioreactors and perfusion systems [5]. Besides these multipurpose bioreactors, numerous cartilage bioreactor systems have been developed and custom-made with incorporation of specific type of mechanical stimulation for specific application, which some of them have been commercialized [6–8]. This is due to the fact that articular cartilage is continually exposed to biomechanical environment in vivo that is important for the cells metabolism. Therefore, application of direct physiological stimulation to cartilage constructs is widely considered necessary for the development and function of the engineered tissues. It is for this purpose that bioreactors able to exert precise dynamic load have been designed, which requires not only biological model but also further bioreactor designs with pertinent principles and concepts for the tissue.

Hence, when designing a cartilage bioreactor, the type of mechanical stimulation and its regime of application are the most crucial factors that needed to be considered [5], which in the most favorable cases, must be able to cause similar effects to those found physiologically and at matching conditions. Four main types of force commonly used in culturing and engineering of cartilaginous tissues are; hydrostatic pressures, direct compression, tissue shear forces and fluid-induced shear stresses [9,10]. While most cartilage bioreactor designs incorporate only one type of mechanical force [11,12], some designs integrate more than one type of force [6–8,13] to better reflect the physiological forces that chondrocytes experience in vivo, and to progressively improve the biomimetic functionality of the device as is the future of cartilage bioreactors.

There are significant evidences that controlled application of biophysical stimuli in bioreactor may improve cartilage regenera-
tion in vitro [6,8,12,14–21,26]. Application of dynamic compression, as opposed to static compression, has been shown to modulate various biosynthetic activities of chondrocytes seeded in polymer scaffolds [12,15–18,22,23] and explants [24,25], depending upon specific choice of load amplitude and frequency applied. While the importance of fluid-induced shear stress and its general effect has been widely examined, little is known about the possible effects of macroscopic shear deformation of tissue constructs, nor is the response characterised outside sparse test conditions. Recent studies [14,21,26] show that dynamic shear deformation induces cyclic matrix strain in a nearly uniform manner and improves mechanical properties of engineered constructs. The combined application of axial compression and rotational shear deformation under dynamic loading has also been shown to increase dynamic stiffness of the construct, producing softening effects as well as a significant increase in the metabolism of both proteoglycans and proteins [6,8].

Based on these studies revealing the effect of individual and combined application of dynamic compressive and shear loading, a bi-axial loading bioreactor, specifically for chondrocyte-seeded hydrogels, has been developed. Existing systems have been reported which are able to produce dynamic shear loading without dynamic compression [6]; further, a device which applies dynamic forces in compressive and shear directions to cartilage explants has been previously described [8]. In both of these cases the sample is manipulated by a friction-based/sliding coupling, and no mechanism is provided to ensure a return to initial geometry within each cycle. To the knowledge of the authors this is the first system reported able to provide cyclic loading in both compressive and shear modes; in addition, it ensures that tissue geometry is recovered within each cycle by reliably coupling both faces and applying tensile forces as required to return the sample to its full size. Previous designs [6,8] provide for mechanical stimulation on cartilage explants [6], and tissue constructs [8], while the present design not only incorporates mechanical stimulation, but serves as a tissue bioreactor in which 3D tissue constructs can be cultured and grown while subjected to mechanical loadings to form a functional engineered tissue. The device includes the essential features of a bioreactor, including a continuous supply of culture medium, as well as providing a wide range of mechanical loading profiles to the constructs. The present paper describes the design and validation of the device.

2. Bi-axial loading bioreactor

2.1. Finite element modeling

Finite element modeling (FEM) was used to predict the spatial stress distributions throughout the cell constructs, in response to the applied loading. This is necessary as it serves as a practical tool to facilitate the system characterisation and preparation of research protocols. In addition, the finite element analysis was conducted to numerically verify the effectiveness of bi-axial loading as claimed by some studies [6,8,26], in comparison to uni-axial loading in terms of stress distribution patterns throughout the construct and its overall mechanical properties.

In this study, a 3D solid model of a chondrocyte-seeded agarose cube, with dimensions of 5 mm × 5 mm × 5 mm was constructed using a commercial software package, ABAQUS/Standard 6.5-1. Meshes contained 1000 elements and 1331 nodes and the elements were defined as C3D8R type, an 8-node linear brick, reduced integration and hourglass-control type [27]. For the analysis, a custom FEM model incorporating the single phase model was used as the model provides assessment on flow-independent properties of cartilage, thus excluding the effect of fluid-induced forces in the analysis. Hence, the solid was assumed to be homogenous, with a linear isotropic elasticity and nearly incompressible solid matrix, and assigned with mechanical properties of agarose (3% Agar type VII), with Young’s modulus, EY of 114 kPa [18], and Poisson’s ratio, ν of 0.42, representing an almost incompressible material.

In the pre-processing modules, the solid model was configured to apply 5–25% strain. Relative displacements with the percentage strain in 1-direction represent compression and in 3-direction represent shear deformation of the solid model. In response to the strain applications, stress fields were generated throughout the model and can be analyzed in the post-processing modules. Using linear elastic equation, the resulting stresses with the applied strains can be calculated. It theoretically corresponds to the definite magnitude of stress that cells within the construct should experience under the same level of applied strain. To predict how many cells would experience the resulting stress, an assumption was made in which the percentage of area with the resulting stress (affected area) over the total area is considered equal to the percentage of cells experiencing the resulting stress.

When comparing stress distribution patterns induced under uni- and bi-axial loadings with the same magnitude strain, it has been observed that the resulting stresses under bi-axial loading are distributed homogeneously throughout the central region of the construct, as opposed to those under uni-axial loading, which are largely distributed at the edges of the construct (Fig. 1). This observation hypothetically indicates that application of bi-axial loading may improve the overall construct properties as it is demonstrated to be able to distribute the resulting stresses uniformly throughout the construct.

2.2. Design and description

The specifically designed bioreactor is comprised of a Perspex tray that is able to hold in culture up to six cuboid tissue constructs, having been cubes of tissue constructs prepared using a custom made mould. The culture tray is attached to a large aluminum base to ensure the stability of the bioreactor.

Using a bespoke mould, 5 mm × 5 mm × 5 mm cuboid constructs are prepared in such a way that two opposite sides of the constructs are readily attached to two separate sintered glass endplates. Two Perspex brackets hold each piece of sintered glass inside the culture tray and work together as individual platen in contact with the constructs. The platen-constructs structure is then held down in position by stainless steel screw shafts that connect the platen to 2-phase stepper motors (Vexta® PK256-02A). By employing a power screw mechanism, the connecting screw shafts were able to convert the rotational motion of the stepper motor into linear
Fig. 2. (i) The bi-axial loading bioreactor: (a) Top view: A – base, B – culture tray, C – Perspex platens, Ds – compression shafts, E – shear shaft, Fs – stepper motors for compression, G – stepper motor for shear, and H – cell-seeded scaffolds. Two stepper motor will move a platen perpendicular to the attached constructs to give compression and one stepper motor will move another platen parallel to the construct to give shear, and (b) side view: (ii) An overall configuration of the incubator-housed and PLC-controlled cartilage bioreactor system; A – standard incubator, B – culture tray, C – peristaltic pump, D – medium reservoir, Es – medium inlet and outlet ports, Fs – stepper motors that are connected to the platens through screw shafts; G – PLC control box; Hs – stepper motor drivers; I – power supply.
motion of the platen. Two stepper motors are used to move a platen perpendicular to the attached constructs to give compressive deformation, and one motor is used to move another platen parallel to the constructs to give shear deformation (Fig. 2(i)). The heat released from the motors is cooled by a water cooling system.

The culture is covered by a transparence Perspex lid, tightened with screws, permitting aseptic monitoring of tissue development during the culture and stimulation processes. The lid is also coupled with a small filter cap (BugStopper™, Whatman® Filter Device) to allow aeration and release of pressure, while keeping the culture protected from bacterial and viral contamination.

The bioreactor as a whole is small enough (7 cm high × 26 cm × 36 cm) (Fig. 2(i)) to be placed within a standard laboratory incubator to allow long-term culture within a temperature controlled environment, taking advantage of standard buffering systems for the maintenance of pH. The entire system and the associated mould may be dismantled easily into individual components for sterilization, in which they are designed to be assembled within a Class II laminar flow bio-safety hood while maintaining the sterility. Nevertheless, sterility in transport is limited to the internal compartment in which the tissue culture medium and cell-seeded constructs are found.

As an ideal bioreactor, the developed cartilage bioreactor also has been implemented with other fundamental features of a culture system such as feeding pump, medium vessel and most importantly, the control strategies. Fig. 2(ii) illustrates the overall bioreactor system configuration.

2.2.1. Medium transfer

Using a peristaltic pump (Watson-Marlow Bredel Pumps, UK), culture medium may be supplied continuously into the culture tray without the need to open the lid and manually change the media, thus preventing the culture from being contaminated. The medium is pumped from a ready-made medium reservoir to the constructs through an inlet port on the culture tray lid and drawn out to the same reservoir through an outlet port on the culture tray wall, which, the level of the outlet port on the wall is intended to be three times higher than the construct thickness. This is an important design consideration as it serves as a level marker, applying an overflow concept, to maintain the level of medium in the tray throughout the culture process, thus keeping the constructs constantly supplied with optimum volume of culture media.

The transfer of medium is intended to be slow enough to ensure the flow will not induce any hydrostatic pressure and shear effects on the construct samples. This is important as it allows research studies to characterise explicit effects of direct compression and/or shear loadings in isolated from other variables such as hydrostatic pressure and fluid-flow induced shear variations. Thus, tubing with small bore (1.6 mm) is used to transfer medium in and out of the culture tray, transporting only small volume of medium, in the range of 0.81–110 ml/min at speed of 3–400 rpm provided by the pump. The tubing used is platinum-cured silicone tubing (Watson-Marlow Pumpsil Tubing, UK) as it is highly permeable to both carbon dioxide and oxygen, permitting proper gas exchange for maintenance of optimum pH of culture media. The platinum curing silicone offers low protein binding and prevents leakage of chemical, which are important considerations in tissue culture work.

2.2.2. Control system

In the present system, the displacement of platen is regulated as an input, rather than the load pattern, which is accurately controlled by a dedicated program, developed within a Programmable Logic Controller (PLC) platform that is based on the step size of the stepper motor used. The stepper motor is controlled such that the position of its shaft is accurately defined at each point, to a resolution of ±0.05° of rotation. Theoretically, as step size of the motor is 1.8° per step, a full revolution of the motor shaft will give 200 steps. A high number of steps per revolution provided by the motor increases its accuracy, thus assuring the accuracy of platen movement. Since the thread pitch of the screw shaft is designed to be 1.25 mm, 200 steps or one revolution of the motor shaft will therefore, displace the platen in the distance of 1.25 mm from its initial position. Therefore, one step will move the platen about 0.00625 mm in distance, which gives percentage strain of 0.125% on a sample size of 5 mm. Further, the stepper motors have been selected for the design application based on a holding torque 50 times greater than the maximum predicted load, thus ensuring that the motors are always able to maintain the requisite positions during loading, and permitting them to be used reliably and repetitively over a long period of time.

The prompt-driven PLC software allows for configuration of waveforms (sinusoidal and trapezoidal), strain amplitude in terms of platen displacement and frequency, and then controls the movement of the stepper motors through external motor drivers (Vexta® CSK256AP). Static loading can also be operated by manipulating parameters in the trapezoidal waveform as it allows the platen to be held for certain period of time before returning to its initial position. The feature as well allows configuration of intermittent loading, which is an essential measures that closely reflects the physiological joints loading.

3. Validation tests

The efficacy of the device is determined by how accurately the load is transmitted to the constructs, which in turn requires accuracy of platen movement in response to the system controller. Validation was carried out to confirm the spatial and temporal accuracy of platen movement in the absence of actual cell-seeded specimen to determine whether or not it is maintained over the operating period of the system. In the presence of agarose specimens, a further study was conducted to examine the ability of the system to exert precise bi-axial load to the specimens. These are necessary to ensure the reliability and reproducibility of the system.

3.1. Platen displacement

Practically, the accuracy of platen displacement was assessed by comparing expected results with those obtained. To monitor the displacement of the platen, a sheet of 1 mm ruled graph paper was placed at the bottom of the tray which then served as a reference scale to measure the displacement. Different magnitudes of displacement in millimeters were configured for the individual application of compressive and shear loadings, ranging from 0.25 mm to 1.25 mm, corresponding to compressions of between 5% and 25% deformation strain for 5 mm long specimens. A frequency of 1 Hz with trapezoidal waveform was constant throughout the experiment. A trapezoidal waveform was adopted as it provided dwell times, thus allowing images of platen displacement to be captured. Accordingly, digital photographs before and after the movement were then taken to measure the platen displacement. The distance between initial (before) and final (after) positions was calculated, where graph paper was used as the reference scale and precise measurement made by pixel interpolation and each measurement was repeated five times. The applied displacements were then plotted against the measured displacements to assess the correlation between these variables. The measured displacements showed a very good correlation with the applied displacements (Fig. 3), thus confirming the spatial accuracy of the platen movement.
3.2. Platen frequency

The accuracy of platen displacement with respect to time was validated by operating the system at frequencies in the range of 0.1–1.0 Hz of both sinusoidal and trapezoid waveforms while capturing a digital recording of the motion. A displacement of 1.0 mm, equivalent to 10% cyclic strain for 5 mm long specimen was configured and kept constant throughout the experiment. Three cycles of displacement were examined at each frequency.

Similar to the displacement validation methods, graph paper was used to monitor the platen movement. A video of platen movement at every respective frequency was taken and the time taken to complete the loading cycles was recorded. Using computer software, a few frames (still pictures) were then cut from the full video. The platen position in every frame was noted and the difference between its initial position (first frame) was calculated to get the platen displacement with respect to time.

Analysis showed that the times taken to complete three cycles of 1 mm compression at two different frequencies were accurate within 100% of the configured frequency with comparatively fine shape sine and trapezoid wave (Fig. 4). This, therefore, confirms the temporal accuracy of the platen movement.

3.3. Consistency of platen movement and stability of long-term operation

The platen movement was observed over long periods to verify its ability to maintain accuracy over a longer period of operation.

In this experiment, distilled water was used within the culture medium reservoir, and continuously circulated throughout the process at a flow rate of 70 rpm. Similar to the frequency validation methods, live video of platen movement in the first, intermittent, and last cycles were recorded. Four frames which represent the four stages of a trapezoid cycle (compress, hold, decompress and rest) were then cut from each video and served as way-markers for the spatial and temporal characterisation of platen loading in each cycle. The characteristics of platen loading in the intermittent and last cycles were examined and compared with those of the first cycle to verify the ability of the system to maintain its accuracy over the operation period.

The results confirmed that the system is capable of applying dynamic loading in a reproducible manner as the platen displacement of the first, 50th, 500th, 5000th, and 99,999th cycles with respect to time is significantly similar, with only 0.01901 ± 0.00625 mm and 0.01597 ± 0.00625 mm displacement errors for compression and shear, respectively (Fig. 5).

Following extended operation within the tissue culture incubator, the device maintained operation and was sufficiently cooled such that the incubator was able to maintain a physiological temperature profile.

3.4. Validation of agarose deformation

The system was assembled and assessed to confirm the ability of the system to exert precise bi-axial load to three-dimensional constructs (agarose gels). Low gelling agarose (Type VII, Sigma-Aldrich, Kuala Lumpur, MY) was added to distilled water at 4% (w/v) and autoclaved to melt. Once melted, the gel was placed on rollers in a 37°C oven for 15 minutes until the gel tempera-
ture had reached approximately 37 °C. The agarose solution was plated into the mould to produce six cubic agarose gels of 5 mm length, attached to platens to be directly transferred into the culture tray. Distilled water was continuously supplied throughout the experiment, which was circulated at a flow rate of 70 rpm.

The specimens were dynamically loaded with 1 mm compressive or shear displacement, which is equivalent to a 20% strain magnitude for a 5 mm long specimen, using a 0.1 Hz trapezoidal waveform. The gross deformation of every specimen in response to the applied displacement, in the first and the last cycles, was assessed by using a similar method to that used in platen displacement validation and the experiment was repeated three times. The assessment of specimen deformation was only performed for the first and the last cycles, in view of the demonstrated ability of the device to relatively maintain the accuracy of platen movement over a long period of time, as validated in Section 3.3.

Result showed that every specimen had been loaded homogeneously in the first as well as in the last cycles, and underwent relatively comparable gross deformation to the applied displacement for both compression and shear, with an average error of only 0.01774 ± 0.00625 mm and 0.01648 ± 0.00625 mm, respectively (Fig. 6). This represents a system able to deliver precise amounts of strain to the specimens, which has been maintained over the operating length.

4. Discussion

One of the main architectural specifications of the bioreactor is being a closed system as this criterion is very crucial in keeping...
the culture in asptic condition during the operation of the device. Therefore, the developed bioreactor, as a whole, is a closed system, having been assembled from individual parts made of biocompatible materials that can be sterilised prior to the operation. Further, during the operation of the device, the shafts that connect to the platens which transmit motion from the stepper motors outside the compartment, turn rather than slide to move the platens, thus keeping the culture in asptic condition as there is no part which moves from the outside of the compartment to the inside. Nevertheless, there remains a dependence upon individual operating technique in handling and assembling the device in order to maintain long term sterility.

The FEM studies has been conducted to numerically predict the stress distribution patterns throughout the tissue construct upon the application of uni- and bi-axial loadings. To take account the potential degradation of the material and the firmer coupling produced by the modeling restraints, the model has been assigned mechanical properties equivalent to of agarose of 3% (w/v). Increasing the weight percentage of agarose increases the modulus of the model while keep other variables constant. As indicated in Fig. 7, a simulation using agarose of twice the modulus did not alter the stress distribution patterns.

5. Conclusion

We have developed a novel tissue bioreactor capable of applying various regimes of bi-axial compression and shear loadings under highly controlled environment over extended periods of time. The accuracy of the platen movement throughout the operating length and its ability to deliver precise load to specimens have allowed the device to be used for carrying out reliable and reproducible experiments. Furthermore, the physical design of the bioreactor also encompasses other general design criteria such as ease of handling and assembling, while lowering the risk of contamination, sterilizability, fit into an incubator and operates at the given conditions, facilitate monitoring of tissue development as well as ability to produce several constructs at the same time, thus making it an ideal culture system. Current studies are aimed at using the developed bioreactor to stimulate the synthesis of extracellular matrix (ECM) proteins by chondrocytes seeded in agarose.

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Conflict of interest

We, the authors, declare that there have been no involvements that might raise the question of bias in the work reported or in the conclusions, implications, or opinions stated.

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