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Technical note

Design and validation of an *in vitro* loading system for the combined application of cyclic compression and shear to 3D chondrocytes-seeded agarose constructs

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ABSTRACT

Physiological loading is essential for the maintenance of articular cartilage by regulating tissue remodelling, in the form of both catabolic and anabolic processes. To promote the development of tissue engineered cartilage which closely matches the long term functionality of native tissue, bioreactors have been developed to provide a combination of loading modalities, which reflect the nature of normal physiological loads. This study describes the design and validation of an *in vitro* mechanical system for the controlled application of bi-axial loading regimes to chondrocyte-seeded agarose constructs.

The computer-controlled system incorporates a robust gripping system, which ensures the delivery of precise values of cyclic compressive and shear strain to 3D cell-seeded constructs. Sample prototypes were designed, optimised using finite element analysis and validated performing compressive and shear fatigue mechanical tests. The horizontal and vertical displacements within the bioreactor are precisely controlled by a dedicated programme that can be easily implemented. The synchronisation of the orthogonal displacements was shown to be accurate and reproducible.

Constructs were successfully loaded with a combined compressive and shear loading regimen at 1 Hz for up to 48 h with no appreciable loss of cell viability or mechanical integrity. These features along with the demonstrated high consistency make the system ideally suitable for a systematic investigation of the response of chondrocytes to a complex physiologically relevant deformation profile.

1. Introduction

Articular cartilage is a highly organised connective tissue consisting of chondrocytes surrounded by an extracellular matrix (ECM), composed of a proteoglycan gel, enclosed within a complex network of collagen fibres [1]. The interaction of both solid components is critical in providing mechanical competence to support the normal physiological loading experienced within the synovial joints. Changes in the ECM composition can dramatically alter the load bearing capacity of articular cartilage, thereby initiating a process which can eventually result in its total degradation [2,3].

Since articular cartilage is avascular it exhibits a very limited capacity to regenerate and to repair itself into a fully functional tissue [4]. Accordingly, a number of clinical strategies have been established to repair partial thickness cartilage defects, each of which provides additional metabolically active cells to the defect site. However such clinical options rarely provide long term functional stability, thereby motivating considerable tissue engineering research focused on the *in vitro* development of neo-cartilage tissues prior to implantation [5,6].

It is well established that physiological loading is essential for the maintenance of articular cartilage by regulating tissue remodelling, in the form of both catabolic and anabolic processes [7,8]. Indeed a variety of forces are active during joint loading and have been shown to affect cartilage composition and integrity, including compression [9–11], tension [12], shear [13,14], and hydrostatic pressure [15]. This has motivated the design of several bioreactor systems [16,17] to apply mechanical loading at a magnitude and frequency to match the physiological loading conditions [11,18–20].

Several groups have used mechanical conditioning, generally in the form of uniaxial compression, to modulate the response of chondrocytes embedded in polymeric constructs. For example, it has been reported that the application of dynamic compression can regulate a number of biosynthetic activities [19,21,22], which are strongly dependent on both the strain magnitude and frequency.

Most of these studies have revealed a significant up-regulation of proteoglycans while the synthesis of type II collagen has been, at best, modest in comparison [23]. Accordingly few studies have

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addressed this issue by adopting strategies to enhance the presence of type II collagen. These have included the temporal degradation of hydrogel scaffolds [24], the use of mature functional units, incorporating the chondron [25] and the superposition of shear on uniaxial compression [14]. An example of the latter approach used a rotating ball system, which simultaneously applies compression and shear to simulate joint kinematics [26]. Although this system has enabled the application of multi-axial loading on cell-seeded constructs or cartilage explants, the precise levels of stress and/or strain imposed on the specimens have not been quantified. Nonetheless, the combined application of dynamic compression coupled with oscillating sliding contact has been shown to increase the dynamic stiffness of the construct, associated with a significant increase in the synthesis of proteoglycans and proteins [14,23].

It may be hypothesised, therefore, that the superposition of shear on direct compression may up-regulate collagen production in articular chondrocytes seeded in constructs. Accordingly the current study is focused on the design and validation of an *in vitro* loading system for the accurate and reproducible combined application of prescribed magnitudes of cyclic compression and shear to 3D chondrocyte-seeded agarose constructs. The system will apply biaxial loading for 48 h to samples located in independent wells within a culture plate.

2. Materials and methods

2.1. Bi-axial loading bioreactor

A bioreactor was designed to feature a controlled loading system (ElectroForce 5500, BOSE, Minnesota, USA), placed in a tissue culture incubator (BINDER, D-78532, Tuttingen, Germany) equipped with a 225 N load cell (BOSE ElectroForce System Group, Minnesota, USA). The loading system provides strain *via* a vertical actuator, which is connected by a central shaft to a mounting plate located within a Perspex chamber. The chamber was aligned to the loading system and its bottom surface was secured to the system platform.

The pin holder plate includes 12 holes which restrain the vertical movement of each of the loading pins (Fig. 1C). The loading pins consist of a 316L stainless steel rod (⌀3 mm, 30 mm length) connected to PTFE cylindrical cups (⌀12 mm, 10 mm deep). This arrangement permits the simultaneous testing of 12 constructs, centred individually in the outer wells of a 24 well culture plate, whilst being maintained in aseptic culture conditions (Fig. 1). Control constructs prepared from the same batch of chondrocytes, were left either in a free swelling state or under tare load equivalent to a static strain of 0.2%.

The internal lateral surfaces of the cylindrical cups were designed with a chamfered surface to facilitate the positioning of the loading pins on each construct. Each loading pin can be locked in the pin holder.

It is well reported that viscoelastic behaviour of the agarose constructs during dynamic compression leads to permanent deformation of each construct [27], resulting in "lift-off", the loss of contact between loading systems and the top surface of the construct. To provide shear deformation even with lift-off, the cups on the loading pins were designed with sufficient depth to accommodate a permanent deformation of 2 mm (equivalent to 40% strain).

Shear strain was applied using a linear positioning motorised stage (Zaber Technologies, T-LSM025A-KT03 Miniature Linear Slide, Laser 2000 Ltd., UK) with built-in controller, accuracy of ±4 μm and adequate speed capabilities (Maximum Speed 7 mm/s, Speed Resolution 0.00022 mm/s).

The sample holder, connected to the linear stage, located a 24-well plate (Corning Costar culture plate, Sigma–Aldrich, Poole, UK). Each sample was designed to be positioned immediately beneath a corresponding loading pin. All elements of the rig were easy to dismantle and sterilise in an autoclave.

2.2. Control system

The linear stage was connected to the Bose system PCI output channel through a multi-function Analogue I/O Device for Data Acquisition (14-Bit, 48 kS/s Multifunction DAQ, National Instruments Corp, Newbury, UK) which provides high-speed ADC.

A Labview (LabVIEW 8.2, National Instruments, Newbury, UK) routine was implemented to synchronise the vertical and horizontal displacements in the bioreactor. The time delay between the movement of the stage and the vertical actuator was adjusted by using a closed loop control system with a Linear Variable Differential Transformer (LVDT DG 2.5 Guided, Solartron Metrology, RS Components Ltd, Corby, UK) to provide feedback of the position of the stage. The moving tip of the LVDT was positioned in contact with a deflection plate mounted on the stage platform (Fig. 1C). Dedicated programmes enabled a range of waveforms, strain amplitudes and frequencies to be selected.

2.3. Validation protocol

To ensure the consistency and reproducibility of the system a validation protocol examined the synchronisation of the vertical and horizontal movements. The time delay between the two distinct waveforms and the efficacy of the compensation system was monitored. The vertical displacements of the actuator were compared with the displacements of the stage. The vertical actuator was prescribed to move sinusoidally between 0 and 0.75 mm at a frequency of 1 Hz, while a triangular waveform (0.5 mm, 1 Hz) was employed for the lateral translation of the linear stage. The data collected over 48 h were processed and the time delay determined between the peaks.

2.4. Design and characterisation of the construct

A cylindrical construct was developed, which could be gripped in the bioreactor using nylon endplates (component a, Fig. 2A). These endplates, with their inherent material porosity were designed to minimise the static preload applied to the cell-seeded constructs and ensure hydration of the constructs. To increase the contact between the sample and the endplates, improving the interlock between the two materials and minimising the sliding between their surfaces, a thread (ISO STD M8X0.5 mm) was introduced onto the internal surface (Fig. 2B) of the nylon endplate.

2.4.1. Optimisation of the sample profile by finite element analysis (FEA)

Finite element analysis (FEA) was performed to optimise the sample profile and ensure the design of an experimental model with a well characterised and uniform strain distribution across the construct. This would avoid the presence of strain concentrations, leading to premature construct failure during the dynamic loading experiments.

Models of agarose cylindrical construct and nylon endplates were achieved using a commercial software package (ABAQUS/CAE standard 6.9). The hydrogel was idealistically modelled as a single phase, homogenous, linear isotropic and elastic material [28], with $E = 100$ kPa [21], $\nu = 0.32$ [29] and mass density $= 1.64 \cdot 10^{-6}$ g/cm^3. The 3D solid models of endplates and constructs, produced with representative dimensions, were meshed using tetragonal shaped elements (C3D4: 4-node linear tetrahedron) [28] with a global

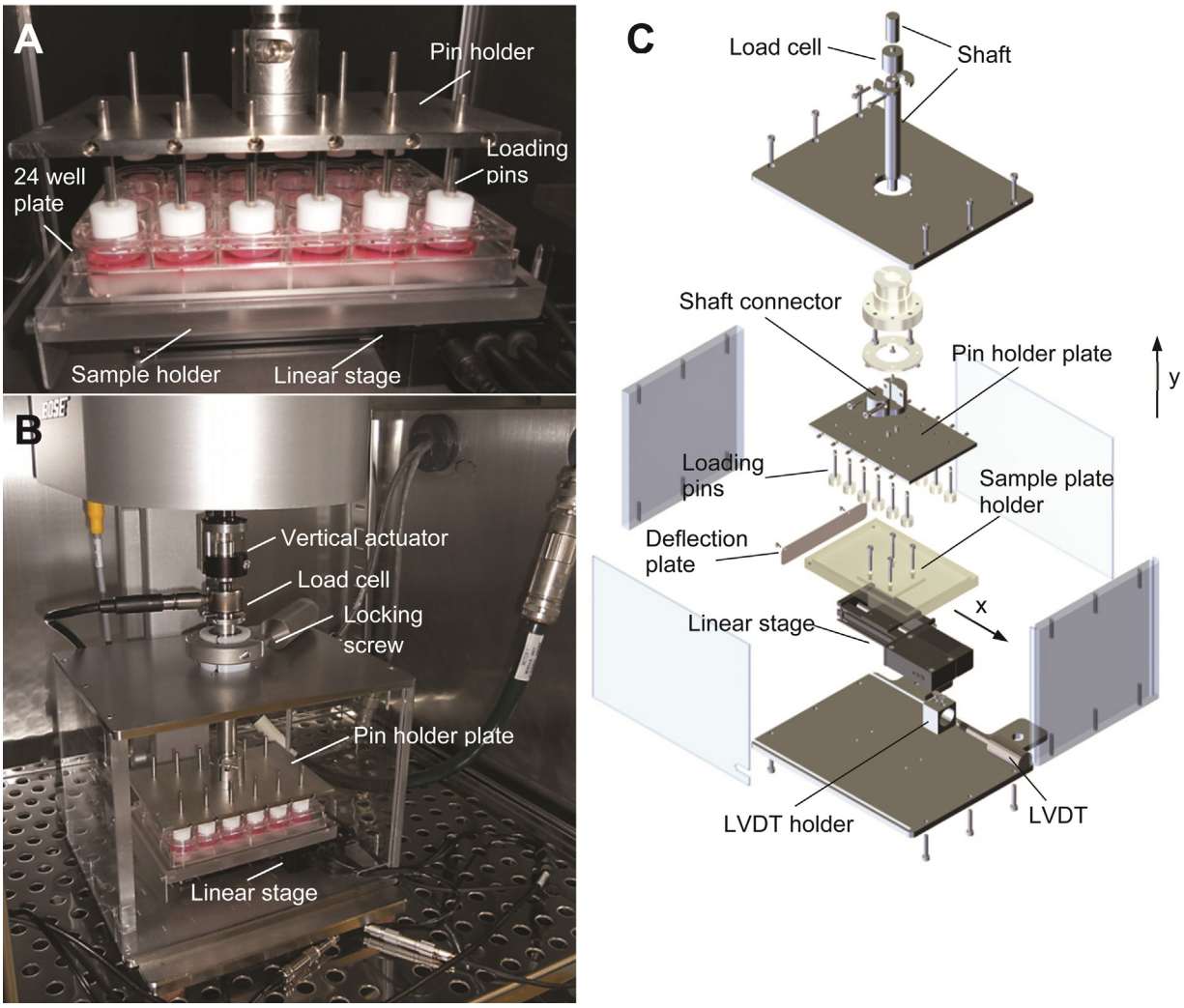

Fig. 1. Images of the assembled tissue culture sterile chamber positioned in the incubator (A and B). Schematic of the bi-axial loading system (C).

size of 0.48 mm. A local seed with an approximate element size of 0.3 mm was applied to the edge of the thread to provide a higher mesh density. The constructs contained a maximum number of nodes and elements equal to 64,470 and 38,735 respectively while, both top and bottom endplates were characterised by a maximum of 37,634 nodes and 22,293 elements.

A range of simulations were generated using different internal thread designs (M8, pitch 0.5, 0.75 and 1 mm) and using a range of inclinations of a chamfered surface (90, 121, 129, 135, 141 and 149°), defined as M8-pitch-chamfered inclination.

A two stage process was initiated. Initially each model was configured such that a compression strain was applied along the y-direction, followed by a shear strain in the x-direction. The interaction between sample and endplates was modelled as "general contact" characterised by isotropic frictional behaviour. A sensitivity analysis was performed in which the friction was varied between 0.25 and 1.00 under the conditions of 15% compression strain and shear strains of 5– 15%. The maximum Principal Stress value was minimally affected (approximately 2% at 15% shear strain) by the change of friction coefficient. Consequently a value of 0.3 was selected for all subsequent analyses.

During the FE simulations, the numerical problems were solved using the standard implicit direct equation solver. The numerical simulations were performed using the Static/General step. A full Newton solution technique with unsymmetric matrix storage was adopted [28].

The maximum Principal stresses and their distributions were determined in response to the applied compression and shear loading regimes.

2.4.2. Sample validation under dynamic shear

The sample integrity and functionality under dynamic shear was estimated using a bespoke rig, accommodating two constructs. The rig body was composed of three blocks, two sample locators, placed laterally and connected together through their bottom surface and a moveable grip (Fig. 4). One endplate of each sample was located in the sample locator block, while the other was located in the loading pin. 15% static compression strain was applied to the each of samples using precision length punches (Fig. 4). When the rig was assembled, it was fixed in a sterile chamber with culture medium and transferred to an incubator (BINDER, D-78532, Tuttingen, Germany). The rig was coupled by a shaft to a mechanical loading system (BOSE ElectroForce System Group, Minnesota, USA). During set up the loading pin was supported; this support was removed immediately prior to testing so that the loading pin was free to move vertically and apply dynamic shear strain to the samples. Each construct was tested under static compression and a cyclic shear strain of 10%, equivalent to a maximum

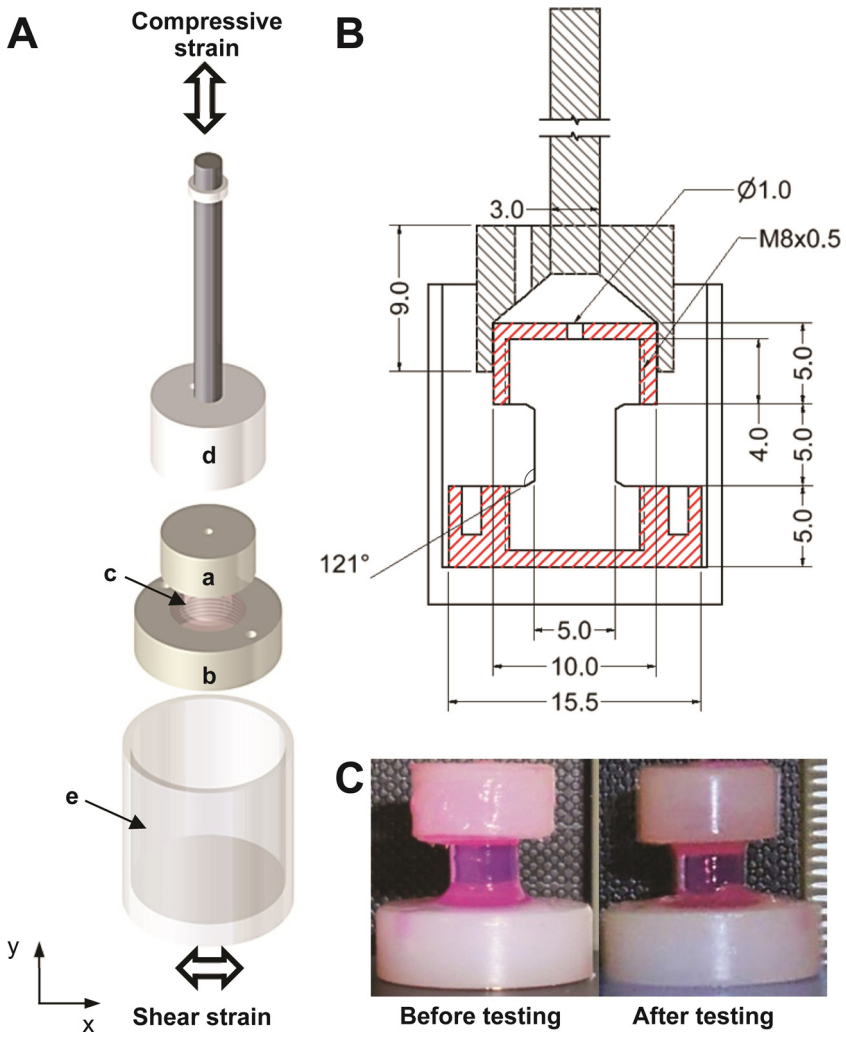

Fig. 2. (A and B) Schematic of agarose construct (c: ⌀5 mm × height 5 mm) located in custom designed nylon endplates (a: ⌀10 mm × 5 mm, b: ⌀15.5 mm × 5 mm), loaded through a cup shaped pin (d), within a single well of a 24 well tissue culture plate (e). (C) Construct before and after shear fatigue test.

amplitude of 0.5 mm, using a sinusoidal waveform at a frequency of 1 Hz for 48 h (172,800 cycles) [27]. Storage (G') and loss moduli (G'') were calculated over 40 cycles at three times over the test period.

2.5. Cell viability

Different loading regimes over 48 h were applied to bovine chondrocytes seeded in 3% w/v agarose gel (Low gelling, Type VII, Sigma–Aldrich, Poole, UK), representing a well-established model system in the host laboratory [30]. Both free swelling constructs and constructs subjected to a tare compressive load acted as controls. These regimes were applied to constructs pre-cultured in low glucose Dulbecco's modified Eagle's medium (DMEM, Sigma–Aldrich) with 16.1% (w/v) foetal calf serum (FCS, all from Sigma–Aldrich, Dorset, UK) for 24 h.

At the end of each experiment, cell viability was assessed by removing vertical slices from the centre of the agarose constructs and incubating them in 5 μM per ml of calcein AM and Ethidium homodimer-1 (both Life Technologies Ltd, Paisley, UK) for 30 minutes. The cells were viewed on an epifluorescence microscope (Leica Microsystems GmbH), and the percentage of viable cells was determined.

2.6. Statistical analysis

All results were expressed as the mean ± standard deviation (SD). Mechanical characterisation and cellular viability results were analysed using a one-way ANOVA. A level of 5% ($p < 0.05$) was considered statistically significant for each test.

3. Results

Mean values of chondrocyte viability were maintained above 90% for all test conditions (Table 1). Close examination revealed no

Table 1
Viability of agarose-chondrocyte constructs subjected to different loading regimes. At the 0.05 level the population variance are not significantly different.

Conditions	Cells viability (%)
24 h FS (control)	93 ± 3
72 h FS	90 ± 1
24 h FS + 48 h TL	92 ± 2
24 h FS + 48 h DC	92 ± 4
24 h FS + 48 h DC & DS	91 ± 1

FS, free swelling; TL, tare load; DC, 15% dynamic compression (1 Hz); DS, 10% dynamic shear (1 Hz).

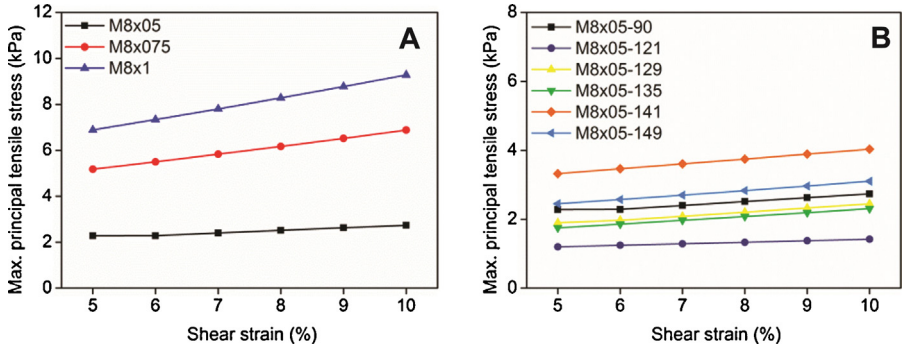

Fig. 3. The relationship between maximum principal tensile stress and shear stress as predicted from the simulation of 3D-models for (A) three different models and (B) one model (M8×05) with five different chamfer angles, under an applied static compression of 15% and shear strains ranging from 5% to 10%.

statistically significant differences in chondrocyte viability when compared to control constructs, defined as free swelling constructs cultured for 24 and 72 h, respectively (Table 1).

From the validation protocols, the mean time delay between the sinusoidal and the triangular waveforms was estimated to be 0.014 ± 0.013 s over the 48 h period. There was no systematic increase in the delay, which strongly indicated minimal drift in the triangular waveform with respect to the sinusoidal waveform.

The FE analyses for each model demonstrated similar patterns of internal stresses within the constructs under different biaxial loading regimes. The simulations indicated that the maximum principal tensile stresses were mainly located along the chamfered surfaces. Indeed, from the sensitivity analysis, each of the three models exhibited comparable trends of maximum Principal tensile stress (σ_{1max}) (Fig. 3). However, σ_{1max} values were considerably higher for models M8X1 and M8X075 when compared to the corresponding values for model M8X05 (Fig. 3A). Accordingly, the 3D-model M8X05 was modified to introduce a chamfered surface with an angle, ranging from 90° to 149°. The results, Fig. 3B, clearly demonstrated that lower values of σ_{1max} correspond to a chamfered angle of 121°. Therefore to minimise the risk of sample failure, this angle was selected for subsequent testing in the system. Due to manufacturing limitations, chamfered surfaces of less than 121° were not considered.

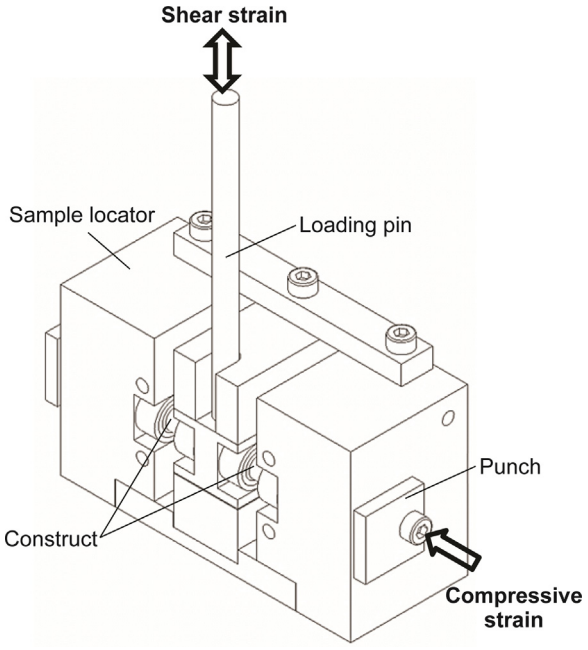

Fig. 4. Schematic of the sample validation rig.

The fatigue test revealed no evidence of fracture or excessive deformations, for each of the six samples tested under cyclic shear and static compression over the 48 h test period (Fig. 2C). The resulting dynamic parameters (G' and G'') decreased approximately 3.7% during the first 24 h and up to 6.9% at 48 h due to the viscoelastic nature of the hydrogel. Nonetheless, there was no evidence of reduction in load-bearing capacity of the sample over the duration of the test.

4. Discussion

The current study presents the design, manufacture and characterisation of a biaxial loading system to enable cell seeded constructs to be dynamically loaded under compression and shear deformations, separately or simultaneously, using a wide range of precisely defined strain and frequency regimes.

Many research groups have used chondrocytes seeded in agarose gel to study the influence of compressive strain on the metabolism of chondrocytes [11]. This homogeneous model system provides mechanical stability and reproducibility as well as the capacity to be subjected to physiological levels of strain. Accordingly it is appropriate to examine the effect of shear strain on biosynthetic activity. However, due to the inherent viscoelastic properties of the hydrogel, in order to apply dynamic shear strain to the constructs it was necessary to design an appropriate gripping mechanism.

Previous studies [27] performed in the host laboratories employed sintered glass endplates combined with agarose gel in order to apply static shear strain. Although the bond between the two interface materials was considered to be strong enough to support the application of static strain [27] its performance, which was monitored under different loading conditions including 15% dynamic compressive strain, was not adequate to support the application of dynamic loads for 48 h [30]. Indeed the constructs showed detachment of the porous glass endplates from the hydrogel even during their insertion in the rig. Accordingly, we developed the novel gripping mechanism and employed custom-made nylon endplates which resolved this issue of maintaining construct integrity during mechanical conditioning.

These nylon endplates were designed so that their light weight would minimise the static preload applied to the cell-seeded constructs and also facilitate its hydration, and not affect the diffusion of nutrients within the hydrogel. Furthermore, the thread introduced onto the internal surface of the nylon endplate increases the contact surface between the sample and the endplates and ensures mechanical stability with the construct. The use of the nylon endplates provided further advantages namely to allow the easy insertion and removal of the construct from the 24 well plate, to avoid direct contact with the relatively delicate agarose,

as well as to allow the accurate positioning of the sample in the centre of the well. Furthermore, coupling the endplates with cup-shaped loading pins overcame the problems associated with lift-off between pin and the construct under dynamic compression. The improved pin shape ensured that the prescribed shear strain was precisely transmitted to the specimen during the 48 h of dynamic loading. The construct characteristics were further improved by introducing chamfered ends to minimise stress concentrations, and hence reduce the risk of premature failure during dynamic loading.

Shear fatigue tests, employed to validate the construct performance, confirmed the efficacy of the designed experimental model. The biaxial bioreactor combined with the optimised sample design has been shown to enable precise levels of gross dynamic shear strain to be applied to the construct with no apparent failure. Given the importance of delivering well defined strains to the relatively small constructs it was critical to utilise two precision-made commercial actuators for compression and shear. The former was provided by a system (Bose) well established in the host laboratory [31,32]. Although, several biaxial loading systems have been previously reported in literature, few have provided a system for accurate application of shear deformation [30,33,34] on tissue engineered constructs. The direct contribution of the applied shear load is not easily quantifiable in rotating culture bioreactors [35], where cell seeded scaffolds are exposed to complex mechanical loading patterns incorporating fluid-induced shear.

A requirement of any bioreactor is the maintenance of cell viability and the potential for active metabolism of cell seeded constructs within the culture system. It is evident that loading regimens may alter transport of metabolites into the constructs. However, the present tests confirmed that the mean chondrocyte viability was maintained above 90% for all test conditions. These results suggest that the culture conditions and the applied loading regimes did not affect cell viability or mechanical integrity of the constructs (Table 1). Additionally, the present design can be used to test simultaneously twelve samples located in separate wells of a culture plate, under highly controlled environment, providing an adequate number of replicates for each batch of chondrocytes. Furthermore, the system enables the culture and the application of the different loading regimes to individual constructs, as opposed to bioreactors in which the samples are all contained within the same culture chamber [30]. Thus unforeseen failure of a single construct would not compromise the entire experiment. The bioreactor can also been easily adapted to incorporate a system for the automated change of culture medium during the conditioning process, which can enable long term (weeks rather than days) culture experiments to be performed.

Accordingly, these features and the high consistency make this bioreactor system ideally suited to enable a systematic investigation of the biosynthetic response of chondrocytes to dynamic macroscopic shear deformation. In particular, we can examine the temporal nature of the synthesised ECM components, its organisation and how it matches the functionality of native cartilage tissue.

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Ethical approval

Not required.

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Conflict of interest statement

None declared.

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