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Evaluation of alginate hydrogels under in vivo–like bioreactor conditions for cartilage tissue engineering

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Abstract Alginate hydrogels in forms of discs and packed beds of microbeads (~800 μm) were tested in a novel bioreactor at 10% strain using two regimes: at a loading rate of 337.5 μm/s and at sequential increments of 50 μm displacement every 30 min. Compressive strength increased with the increase in alginate concentration (1.5 vs. 2% w/w) and the content of guluronic residues (38.5 vs. 67%). Packed beds of microbeads exhibited significantly higher (~1.5–3.4 fold) compression moduli than the respective discs indicating the effects of gel form and entrapped water. Short-term cultivation of microbeads with immobilized bovine calf chondrocytes (1.5% w/w, 33×10^6 cells/ml) under biomimetic conditions (dynamic compression: 1 h on/1 h off, 0.42 Hz, 10% strain) resulted in cell proliferation and bed compaction, so that the compression modulus slightly increased. Thus, the novel bioreactor demonstrated advantages in evaluation of biomaterial properties and cell-biomaterial interactions under in vivo–like settings.

1 Introduction

Tissue engineering is a promising strategy to treat variety of different clinical problems by aiming to replace damaged or diseased tissues with functional in vitro derived tissue equivalents. This approach is generally based on the integrated use of autologous cells, biodegradable, biocompatible supports, and bioreactor systems that should provide all biochemical and physical signals necessary for regeneration of functional engineered tissues. Biomaterials thus present an indispensable component in tissue engineering as well as in many other applications in regenerative medicine, in which they can be used alone or in conjunction with cells. Last several decades have seen therefore a vast expansion of newly developed biomaterials, natural or synthetic, produced in different forms with controlled properties down to nano-levels [1, 2]. However, clinical use of biomaterials depends on establishment of safe and standardized procedures for characterization and prediction of biomaterial properties and behavior once in contact with cells and upon implantation. Animal studies, although necessary, impose many uncertainties and dilemmas, such as selection of the appropriate model and implantation site, identification of the effects of complex in vivo environment, and development of explicit methods for in vivo biomaterial evaluation [3]. Biomimetic bioreactors developed for tissue engineering with precisely controlled conditions that mimic physical and chemical in vivo environment of the specific tissue and site, may also provide relevant models for biomaterial assessment and studies of human cells and tissues, and thus decrease the extent of animal testing. In this work we have utilized a novel bioreactor with mechanical stimulation for evaluation of alginate hydrogels as cell supports in cartilage tissue engineering.

Alginates are anionic, naturally derived linear copolymers composed of 1,4-linked β-D-mannuronic acid (M-block) and α-L-guluronic acid (G-block) units. Aqueous solutions of alginates are known to form hydrogels in the presence of divalent cations such as Ca^{2+}, via ionic interactions between acid groups on G blocks and the gelating ions. As a result, calcium alginate gels are physically cross-linked polymers with mechanical and structural properties that depend on

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alginate composition [4, 5]. These gels are hydrophilic, biocompatible, biodegradable, and easily processed into different shapes, which made them attractive for immobilization of cells [6, 7]. In addition, it is also possible to produce microporous structures by freeze-drying of alginate or alginate/chitosan solutions, which were shown to be suitable scaffolds for cell attachment and production of extracellular matrix (ECM) components [8]. When implanted in vivo, alginate gels induced only a minimal inflammatory reaction [9, 10]. High water content supports efficient transport of nutrients and gases and provides an aqueous environment comparable to that in soft tissues such as cartilage [11]. When immobilized in alginate matrix, chondrocytes were shown to retain their phenotype and produce cartilaginous components [12–14], while mesenchymal stem cells were shown to proliferate and express chondrogenic differentiation [15]. Alginate gels also supported in vitro neo-cartilage formation based on periosteal explants [16]. Since mass transport rates within alginate matrix to individual cells are governed by diffusion and determined by diffusion lengths (i.e., the size of the construct), particulate cell supports, such as microbeads (<1 mm in diameter) seem especially attractive for tissue engineering applications. Such supports provide not only short diffusion distances, but also possibilities for minimally invasive implantation by injection, environments for uniform cell distribution and ECM regeneration, and structures enabling development of vasculature between individual particles [17]. We have previously shown potentials of alginate microbeads with immobilized chondrogenic cells packed in perfusion bioreactors for cartilage tissue engineering [18, 19]. However, articular cartilage is normally exposed to high compressive stresses during joint motions so that dynamic loading was assumed to be required for functional assembly of cartilaginous ECM and may be the key determinant in skeletal tissue engineering [20]. Thus, bioreactors configured as dishes or wells with plungers positioned on top of disc-shaped cell-polymer constructs and providing controlled dynamic compression were designed and investigated for cartilage tissue engineering aiming to mimic the native biomechanical environment in this tissue [21–23]. Among these, especially attractive is the bioreactor, which enables evaluation of biomechanical properties of tissue engineered constructs over the cultivation time, using unconfined compression mode [21, 24]. Although mass transport is predicted to be enhanced by dynamic loading in this bioreactor system [25], it is still limited to lateral specimen surfaces and may restrict the size of engineered tissues. We have developed a novel bioreactor aimed for skeletal tissue engineering that incorporates dynamic compression, as well as tissue perfusion for mass transport enhancement [26]. The bioreactor conditions may be adjusted to imitate in vivo environment in articular cartilage and expose engineered tissue or a biomaterial specimen to intermittent dynamic loading at physiological frequencies (0.1–1 Hz, 5–10% strain) while providing, in the same time, convective mass transport within the tissue by interstitial medium flow at physiological rates (10–100 μm/s).

In this work, we have utilized the novel bioreactor to evaluate suitability and biomechanical properties of alginate hydrogels for chondrocyte cultivation and cartilage tissue engineering. In specific, the aim was first to validate the bioreactor load measurements by assessment of compression moduli of alginate discs. Then, we utilized the bioreactor to evaluate biomechanical properties of packed beds of alginate microbeads with and without immobilized chondrocytes as well as before and during short-term cultivation under continuous perfusion and repeated cycles of dynamic compression.

2 Materials and methods

2.1 Materials

Two types of sodium alginate with different compositions were used. Low viscosity sodium alginate Protanal LF 20/40 (FMC BioPolymer, Philadelphia, PA) had high content of guluronic (G) residues (67%) resulting in the M/G ratio of 0.49 [27]. GG diad sequences were mostly abundant comprising 55%, while mole fractions of MM, MG, and GM diad sequences were 21, 12, and 12%, respectively [27]. This alginate type, denoted here as "high G", was used for bioreactor assessment of alginate biomechanical properties as well as for cell cultivation. Medium viscosity alginate (A-2033, Sigma, St. Louis, MO), reported to have lower content of G residues and the M/G ratio of 1.6 [28], was used in an additional study to assess the effects of alginate composition. This type of alginate is denoted here as "low G". Calcium chloride dehydrate, Dulbecco's Modified Eagle Medium (DMEM), HEPES, Fetal Bovine Serum, penicillin, streptomycin, proline, trypan blue (0.4%) were purchased from Sigma (St. Louis, MO) and ascorbic acid was purchased from Galenika (Belgrade, Serbia). Sodium citrate was bought from Himedia (Mumbai, India) and papain was from Acros Organics (Geel, Belgium).

2.2 Preparation of calcium alginate discs

Sodium alginate powder was dissolved in WFI water at concentrations of 1.5 and 2.0% w/w. The polysaccharide solution was then poured into a holder made of two glass plates (11 cm × 11 cm) separated by a rectangular rubber gasket (3 mm thick) open at one side. The whole structure was immersed vertically for 2 h in 1.5% w/w $CaCl_2$ solution at room temperature to provide initial alginate gelling. The rubber gasket was then removed and the system was

immersed again into the gelling solution for the following 24 h. The alginate gel sheet was then taken out from the glass holder and placed into a fresh gelling solution for the next 24 h to assure complete gelling. Final alginate gel sheets (9 cm × 5 cm) appeared uniform with flat surfaces and were used to punch out at least six identical alginate discs (13 mm in diameter, 3 mm thick) from each sheet.

2.3 Preparation of calcium alginate microbeads

Sodium alginate solutions in WFI water at concentrations of 1.5 and 2% w/w were used to produce alginate microbeads by electrostatic droplet generation as described previously [29, 30]. In brief, sodium alginate solution was extruded by a syringe pump through a positively charged blunt stainless steel needle (23 gauge, 6 kV applied electrostatic potential) at a constant flow rate of 14 ml/h. The resulting droplets were collected in the gelling bath (1.5% w/w $CaCl_2$). As Na ions were exchanged with Ca ions, alginate droplets hardened and formed insoluble microbeads. The microbeads were left in the gelling bath for 30 min in order to complete gelling and thereafter transferred into the bioreactor with dynamic compression.

2.4 Bioreactor with dynamic compression

The novel bioreactor with dynamic compression and medium perfusion was designed for skeletal tissue engineering and described previously [26]. In brief, the bioreactor is configured as a unit of six cultivation cartridges placed in a holder, which is subsequently secured on a metal base (Fig. 1). The base can be moved vertically by a stepper motor (Surestep STP-MTR-23055, AutomationDirect, Cumming, GA) mounted underneath. Beneath the cartridge holder, a load cell Scaime AL3C3SH5e (Scaime, France) is installed in order to provide measurements of average loads imposed on all six cartridges. Cartridges are cylinders made of polypropylene, supplied with two ports for medium transport and providing space for a biomaterial or tissue specimen up to 16 mm in diameter and up to 3 mm thick. The specimen is placed on a sintered glass plate and secured with an O ring, while the cartridge top is covered by a diaphragm made of Bergaflex (Termoplast DOO, Belgrade, Serbia). The diaphragm can be pressed down by a micrometer screw (11 mm in diameter) providing the exact initial positioning in each cartridge, independently of differences in specimen thicknesses.

Movements of the bioreactor base are controlled using applications developed within the Labview platform (National Instruments, Austin, TX), which send appropriate control signals to the motor drive. The main application is used during cultivation of engineered tissues under intermittent dynamic loading and provides specifications of the amplitude and frequency of the base movements, durations of the working and pause periods and the total number of work/pause cycles. In the same time, measurements of loads imposed on cartridges are recorded upon the user command. In addition to the main application, several other programs were developed, including programs for the initial base positioning, load sensor validation, and controlled movements of the bioreactor base while measuring imposed loads.

2.5 Cell isolation, expansion, immobilization, and bioreactor cultivation

For chondrocyte cultures, full-thickness articular cartilage was harvested aseptically from the femoropatellar grooves of 6 month old bovine calves within 8 h of slaughter. Chondrocytes were isolated using type II collagenase (Worthington, Freehold, NJ) and resuspended in culture medium consisting of DMEM supplemented with 10% Fetal Bovine Serum, 10 mM HEPES, 100 U/ml penicillin, 100 μg/ml streptomycin, 0.4 mM proline, 0.1 mM non-essential amino acids and 50 μg/ml ascorbic acid. The primary chondrocyte culture was propagated in

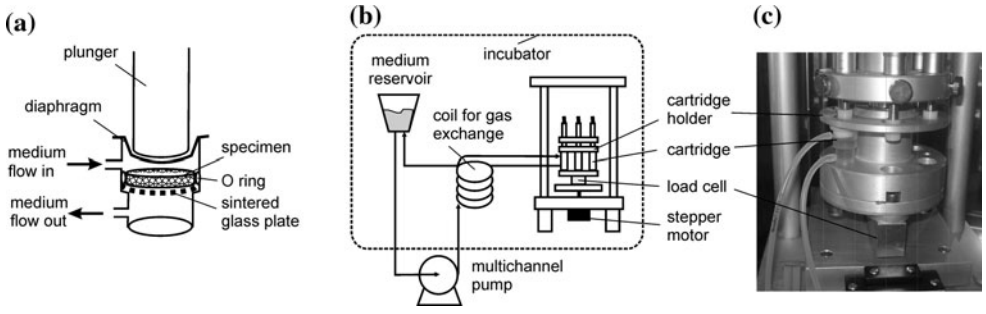

Fig. 1 Bioreactor system: **a** cartridge containing a specimen placed on a sintered glass plate and secured with an O ring; the upper specimen surface can be pressed by a diaphragm forced down by a micrometer screw; **b** bioreactor with the recirculation loop (shown for one cartridge only) consisting of a gas exchanger and a medium reservoir; **c** cartridge holder hosting six cartridges mounted on the bioreactor base

two passages according to the protocol described previously [31]. The 2nd passage cells were mixed with 2.2% w/w solution of sodium alginate (high G) in WFI water to obtain final concentrations of 1.5% w/w alginate and 33×10^6 cells/ml. The alginate-cell suspension was then extruded using the same electrostatic droplet generation technique as described in the Sect. 2.3, to form alginate microbeads with immobilized chondrocytes. The obtained beads were loaded into three bioreactor cartridges (0.6 g in each) connected to separate recirculation loops consisting of a medium reservoir, silicone tubing, and an extra tubing coil serving as a gas exchanger (Fig. 1b). Micrometer screws were positioned so to provide a small gap between the diaphragm and the upper surface of the packed bed in order to allow unobstructed medium flow. The bioreactor system was placed in a humidified 5% CO_2 incubator at 37°C and continuously recirculated by a multichannel peristaltic pump at the medium flowrate of 0.28 ml/min. The total medium volume in each loop was 15 ml while 4 ml of medium was exchanged three times per week. Dynamic compression was performed at the regime 1 h on/1 h off, at the rate of 337.5 μm/s and 0.4 mm total displacement, corresponding to frequency of 0.42 Hz and approximately 10% strain of the packed bed. In a control study, packed beds of equivalent alginate microbeads (1.5% w/w, high G) without immobilized cells were cultured under the same conditions (medium flowrate of 0.28 ml/min, dynamic compression regime: 1 h on/1 h off, at the rate of 337.5 μm/s, 0.3 mm absolute displacement corresponding to 10% strain and frequency of 0.56 Hz). Alginate microbeads with and without immobilized cells were cultured for up to 14 days and tested at 10% strain at a loading rate of 337.5 μm/s in timed intervals (every 2–4 days).

2.6 Characterization of alginate microbeads

Diameters of microbeads were measured using an optical microscope. The average microbead diameter was calculated from the measured data of at least 10 microbeads.

Cell concentrations and viabilities were determined following dissolution of microbeads in 0.05 M sodium citrate (1 g of microbeads in 5 ml of sodium citrate solution). Chondrocyte concentration was estimated by using a Thoma counting chamber and cell viability was assessed by means of trypan blue staining technique.

Alginate microbeads sampled for biochemical analysis were frozen, lyophilized and digested with 125 μg/ml papain solution [32]. Glycosaminoglycan (GAG) content was determined spectrophotometrically using modified dimethylmethylene blue (DMMB) assay [33] and bovine chondroitin sulfate in phosphate buffer as a standard.

Alginate microbeads sampled for histological analysis were stained according to the protocol initially proposed by Stevens et al. [16] and modified by Obradovic et al. [19]. Beads were fixed and stained simultaneously for 48 h in a solution of 2.5% glutaraldehyde, 0.05% alcian blue 8GX, with 25 mM acetic acid (pH 1.5) and 0.4 M $CaCl_2$. The beads were then washed sequentially in 3% acetic acid, 3% acetic acid and 25% ethanol, 3% acetic acid and 50% ethanol, and finally in 70% ethanol.

2.7 Bioreactor validation

Validation of the bioreactor load sensor was performed as previously described [26]. Outputs of the load sensor were calibrated by measuring voltage signals as a function of weights (0–900 g) placed on the bioreactor base. Tests were performed in several series with different cartridge loadings and repeated 50 times each. Next, load signals due to deformation of cartridge diaphragms were determined by testing all six empty cartridges. Diaphragms were loaded (i) at the absolute displacement of the bioreactor base of 375 μm at a loading rate of 337.5 μm/s and (ii) at sequential increments of 50 μm displacement every 30 min. The load sensor outputs were measured as a function of the base displacement.

2.8 Compression tests

The novel bioreactor was used to determine mechanical properties of alginate discs (13 mm diameter and 3 mm thick), packed beds of alginate microbeads (3 mm initial bed height) and packed beds of alginate microbeads with immobilized chondrocytes (3 mm initial bed height) before and after cultivation under continuous medium perfusion and dynamic compression cycles. In general, the compression tests were performed simultaneously in three loaded cartridges. Tests of alginate specimens without immobilized cells were performed in cartridges filled with WFI water while the tests during cultivation studies of alginate microbeads with immobilized chondrocytes and control microbeads were performed in cartridges filled with cultivation medium.

Before each measurement, the plungers were positioned so that the diaphragm touched the upper surface of the specimen as determined visually as well as by the onset of the increase of the load sensor output.

Specimens were tested at the 10% strain (absolute displacement of 300 μm) in two regimes: (i) at a loading rate of 337.5 μm/s and (ii) at sequential increments of 50 μm displacement at the rate of 337.5 μm/s with a pause of 30 min to allow stress relaxation and reach equilibrium values. All measurements were repeated with a minimum of $N = 3$. Values of stress were calculated using measured

outputs of the load sensor, subtracting the outputs due to the diaphragm deformation, and normalizing the obtained values of the force to the area of the plunger. Compression and equilibrium unconfined compression moduli were determined from the slopes of the best linear fits of the stress plotted against the applied strain.

3 Results

3.1 Bioreactor validation

Validation of the bioreactor load sensor was performed as previously described [26]. The sensor output (O, mV) was correlated as a linear function ($r^2 = 0.99$) of the applied load (m, g):

$$O = O_o + 7.89 \cdot 10^{-3} m$$

where O_o is the initial sensor output depending on the initial weight put on the base and amounted to 26.7 mV when the cartridge holder was not attached.

In order to distinguish loads owing to mechanical deformations of cartridge diaphragms and the loads due to strains of the specimens, the sensor outputs were next measured while the empty cartridges were loaded in two, the above specified regimes: (i) at a loading rate of 337.5 μm/s and (ii) at sequential increments of 50 μm displacement every 30 min. The outputs in both cases were almost linear functions of the base displacements i.e., diaphragm deformations (Fig. 2).

3.2 Compression of alginate discs

Alginate discs were tested at 10% strain in the same above specified regimes and the results for both alginate concentrations tested (1.5 and 2% w/w) for high G alginate are presented in Figs. 3 and 4. In all cases, stress was a linear function of the applied strain. Compression moduli

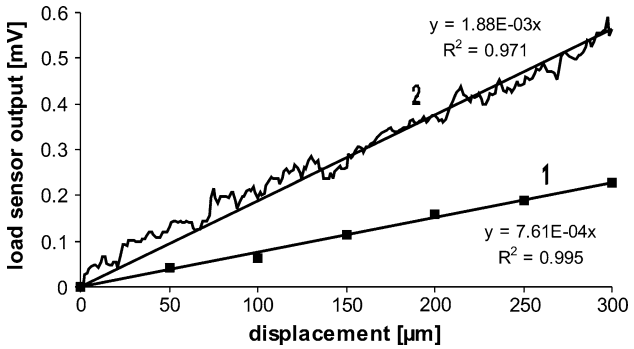

Fig. 2 Load sensor outputs due to diaphragm deformation as a function of displacement; *curve 1* at sequential increments of 50 μm displacement every 30 min; *curve 2* at a loading rate of 337.5 μm/s; (data represent average of $n = 3$)

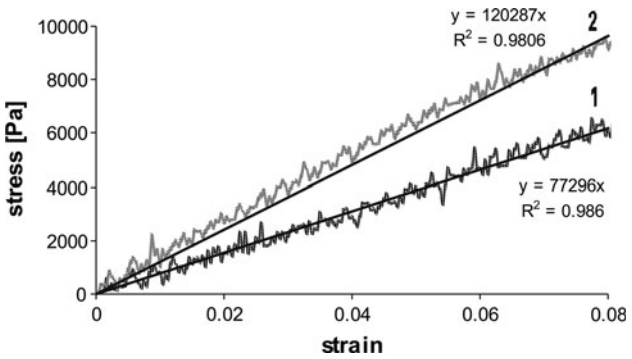

Fig. 3 Stress–strain relationships for 1.5% w/w (*curve 1*) and 2% w/w (*curve 2*) high G alginate discs at a loading rate of 337.5 μm/s; (data represent average of $n = 3$)

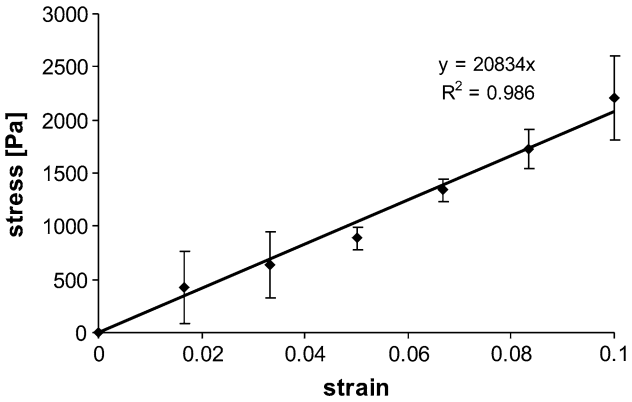

Fig. 4 Average stress–strain relationships for 1.5 and 2% w/w high G alginate discs at sequential increments of 50 μm displacement every 30 min; (data represent average of $n = 6$)

determined from the slopes of stress–strain functions obtained at the loading rate of 337.5 μm/s were 77.3 and 120.3 kPa for 1.5 and 2% w/w alginate discs, respectively (Fig. 3). However, equilibrium stresses determined at sequential strains after 30 min pauses were not statistically different for the two alginate concentrations tested (Fig. 4) and the equilibrium unconfined compression modulus was determined as 20.9 kPa.

In order to assess the effects of different alginate composition, discs of low G alginate at 2% w/w were also tested in both regimes. Stress-strain functions were similar to those measured for high G alginate and followed linear trends ($r^2 > 0.97$, data not shown) with the compression modulus of 70.2 kPa and equilibrium unconfined compression modulus of 9.4 kPa.

3.3 Compression of packed beds of alginate microbeads

In this study, we have investigated biomechanical properties of packed beds of alginate microbeads of two

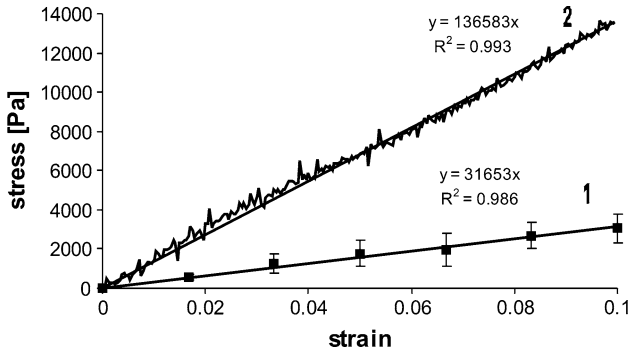

Fig. 5 Stress–strain relationships for packed beds of 1.5% w/w high G alginate microbeads; *curve 1* at sequential increments of 50 μm displacement every 30 min; *curve 2* at a loading rate of 337.5 μm/s; (data represent average of $n = 3$)

concentrations and two alginate types (1.5% w/w, high G and 2% w/w, low G). The microbeads were spherical, transparent, and uniform in size (diameters 822 ± 11 μm and 783 ± 26 μm for high G and low G alginates, respectively). For the imaging purposes, the beads were stained with alcian blue stain, which colored only lightly the alginate gel (Fig. 6a).

Packed beds of the microbeads were tested at 10% strain in the two above specified regimes and in all cases, experimentally determined stresses yielded linear functions of the imposed strains ($r^2 > 0.98$). In Fig. 5, representative results of 1.5% w/w high G alginate are presented. Biomechanical properties of the investigated packed beds of alginate microbeads (1.5% w/w high G and 2% w/w low G) were comparable so that the compression moduli were calculated as 136.6 and 110.0 kPa, respectively, while the equilibrium unconfined compression moduli were estimated to be 31.6 kPa for both cases.

Results of all biomechanical tests described above are summarized in Table 1.

Table 1 Summary of biomechanical properties of alginate hydrogels determined in the novel bioreactor with dynamic compression

Form of the specimen, alginate concentration and type	Compression modulus (kPa)	Equilibrium unconfined compression (Young's) modulus (kPa)
Disc, 1.5% w/w, high G	77.3 ± 1.1	20.9 ± 1.6
Disc, 2% w/w, high G	120.3 ± 2.8	
Disc, 2% w/w, low G	70.2 ± 3.9	9.4 ± 0.4
Packed bed, 1.5% w/w, high G	136.6 ± 2.8	31.6 ± 8.3
Packed bed, 2% w/w, low G	111.0 ± 8.1	31.6 ± 0.4

3.4 Cultivation of alginate microbeads with immobilized chondrocytes

Alginate microbeads (1.5% w/w, high G) with immobilized chondrocytes (33×10^6 cells/ml) were initially spherical and uniform in size (810 ± 13 μm in diameter). After 2 weeks of bioreactor cultivation under continuous medium perfusion and repeated cycles of dynamic compression at about 10% strain, packed beds of alginate microbeads compressed for 850 μm that is approximately 30% of the initial bed height. Microbeads slightly deformed and increased in size (962 μm ± 103 μm in diameter, Fig. 6b) while retaining compactness. During the short-term cultivation, cells proliferated and were metabolically active reaching the cell concentration of 86×10^6 cells/ml on day 14. The cells also started to produce GAG as revealed from alcian blue staining but the overall synthesized amounts were small (<10 μg/g of alginate gel). Packed beds were tested at 10% strain at a loading rate of 337.5 μm/s at the beginning of cultivation and at day 11 (Fig. 7). Experimentally determined stresses yielded again approximately linear functions of the applied strains with compression moduli of 114.3 and 137.5 kPa before and after 11 days of cultivation, respectively.

In the control study, equivalent alginate microbeads (1.5% w/w, high G, 923 ± 28 μm in diameter) without immobilized cells, cultured under the same conditions for 14 days, retained compactness but slightly deformed in shape similarly to the beads with immobilized cells. The stress–strain functions followed linear trends during the first 7 days of cultivation with the approximately constant compression modulus of 131.6 ± 4.6 kPa. However, after that time point, in contrary to the beads with immobilized cells, the stress response of control microbeads slightly decreased in magnitude and significantly deviated from linearity toward a parabolic form (data not shown).

Results of biomechanical tests performed in cultivation studies are summarized in Table 2.

4 Discussion

One of the benefits of tissue engineering is development of sophisticated culture systems that provide controlled studies of biomaterials, cells and tissues under normal and pathological conditions [20]. These studies can contribute to fundamental knowledge in cell biology as well as to practical advancements in pharmacy and medicine by establishment of standardized procedures and products potentially transferable to clinics and thus may possibly decrease the need for animal tests.

In this work, we have evaluated alginate hydrogels in a novel bioreactor with mechanical stimulation and perfusion

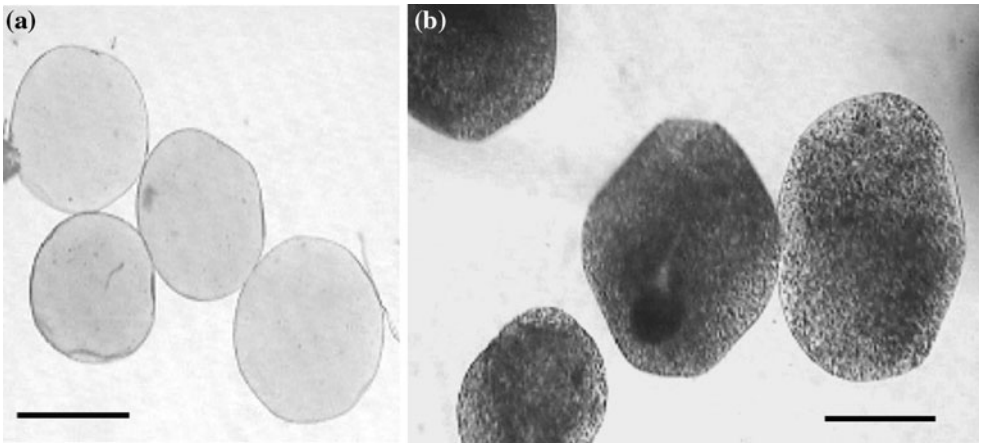

Fig. 6 Alginate microbeads, high G (alcian blue stain): **a** control microbeads without immobilized cells; **b** microbeads with immobilized cells after 14 days of cultivation under continuous perfusion and repeated cycles of dynamic compression: beads are somewhat larger and deformed with visible immobilized cells; darker spots appear due to the deformed bead shape and represent areas with higher cell densities and some ECM accumulation as revealed by dark blue staining; (scale bar = 500 μm)

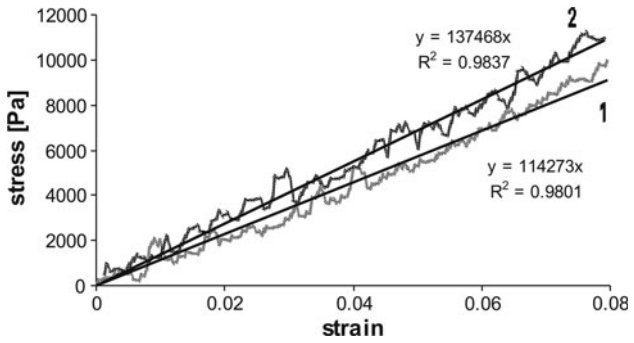

Fig. 7 Stress–strain relationships for packed beds of 1.5% w/w high G alginate microbeads with immobilized cells at a loading rate of 337.5 μm/s at the beginning of cultivation (*curve 1*) and at day 11 of cultivation (*curve 2*), (data represent average of $n = 3$)

Table 2 Effects of immobilized cells and cultivation time on the compression moduli of packed beds of alginate microbeads (1.5% w/w, high G)

Cultivation time	Compression modulus (kPa)
No cells, period of 7 days	131.6 ± 4.6
Cells, day 1	114.3 ± 1.0
Cells, day 11	137.5 ± 2.9

aimed for cartilage tissue engineering under conditions that imitate native in vivo environment. The bioreactor was developed to enable skeletal tissue engineering under precisely controlled conditions while providing in the same time, determination and monitoring of average biomechanical properties of the developing tissue [26]. In the present study we have first validated the bioreactor load measurements by evaluating biomechanical properties of calcium alginate gels in the form of discs and then evaluated the same hydrogel in the form of microbeads for short-term chondrocyte cultivation under repeated cycles of dynamic compression and continuous medium perfusion.

We have focused primarily on alginate with the M/G ratio of 0.49 and the gel concentration most suitable for production of uniform microbeads and mammalian cell cultivation (i.e., 1.5% w/w). Increase in alginate concentration over 2.5% w/w was shown previously to result in larger and non-spherical microbeads formed by electrostatic extrusion [34]. In addition, alginate concentration of 1.5% w/w was successfully used previously for production and prolonged bioreactor cultivation of uniform and stable microbeads with immobilized murine bone marrow stromal cells [19]. However, in order to validate the bioreactor measurements, in the present work we have also included studies of alginate with the higher M/G ratio of 1.6 at a concentration of 2% w/w that was possible to extrude under the same conditions to produce uniform microbeads of the comparable size.

Biomechanical tests of alginate discs confirmed viscoelastic behavior of alginate hydrogels as reported in literature [35, 36]. Compression at the rate of 337.5 μm/s as well as ramp-and-hold displacement to 10% strain yielded almost linear stress–strain functions for both investigated concentrations and both alginate types. Obtained compression moduli increased with the increase in alginate concentration, as expected and attributed to the increase in the number of junction zones between alginate chains in the polymer network. In addition, alginate with higher content of G residues exhibited higher compression and equilibrium unconfined compression moduli than the alginate of the same concentration but with lower G content (Table 1). These results support previous findings that high G alginates yielded stronger and more ductile hydrogels as compared to weaker and more brittle high M alginates [4, 5, 37].

The obtained values of 70.2 and 120.3 kPa for compression moduli of alginate discs (2% w/w, 3 mm thick) with M/G ratios of 1.6 and 0.49, respectively, and hardened for 48 h in 1.5% w/w $CaCl_2$ solution are in the order of magnitude but somewhat lower than the values reported in literature. Compression moduli in the range of 115.8–162.9 kPa determined at the compression rate of 42 μm/s were reported for alginate discs (2% w/v, 2.5 mm thick) with M/G ratios of 1.61 and 1.71 and hardened for 2 h in $CaCl_2$ solutions at concentrations in the range of 50–300 mM (~0.6–3.3% w/w) [36]. Similarly, the compression modulus of 163 kPa was determined for quite thicker alginate discs (2% w/v, 7 mm thick) with M/G ratios in the range 0.33–0.54 and hardened for 12 h in 100 mM (~1.1% w/w) $CaCl_2$ solution at the compression rate of 83.3 μm/s to a deformation of 15% [16].

Equilibrium unconfined compression moduli of alginate discs were determined in the present study by measurements of loads at ramp-and-hold surface displacements every 30 min. This time period was provided for stress relaxation i.e., to relax the alginate polymer network and was chosen according to previous studies of alginate [37] and agarose gels (typically 1000–2000 s) [21, 38]. This relaxation period was also confirmed experimentally in the present study to be long enough to reach equilibrium stress values since sensor outputs measured after 30 min and after 24 h differed for about 5% (data not shown). Equilibrium unconfined compression moduli were not statistically different for the two concentrations tested of high G alginate. These results are in agreement with reported low dependences of equilibrium unconfined compression modulus on alginate concentration in the range of 1.25–1.75% w/v [39] as well as of the confined compression aggregate modulus on alginate concentration in the range of 2–5% w/v [21]. Furthermore, it is hypothesized that viscoelastic response of alginate hydrogels is at least in part governed by interstitial fluid pressurization and associated flow effects [21] and at higher compression speeds the water trapped in the gel pores does not have time to flow out and thus contributes to the compression modulus [40]. Therefore, the results obtained in our study indicate structural differences between 1.5 and 2% w/w alginate gels, which induced different dynamic properties but when given the time to relax, insignificantly differed in mechanical strengths.

Values of equilibrium moduli of 9.4 and 20.9 kPa obtained in this study (Table 1) for alginate discs with M/G ratios of 1.6 and 0.49, respectively, are in agreement with the data reported in literature. Equilibrium compressive modulus of 9.6 kPa was determined for 2% w/v alginate discs (12 mm diameter, 1.5 mm thick) with the M/G ratio of 1.67 and hardened for 1.5 h in 0.05 M (~0.6% w/w) $CaCl_2$ in unconfined compression with a ramp speed of 8 μm/s at sequential increments every 30 min until reaching 16% strain [37]. Similarly, equilibrium aggregate modulus of ~7 kPa was determined for 2% w/v alginate discs (6.76 mm diameter, 1.7 mm thick) with the M/G ratio of 1.6 and hardened for 30 min in 100 mM (~1.1% w/w) $CaCl_2$ in confined compression stress-relaxation tests with a ramp speed of 2 μm/s until reaching 10% strain and measuring the equilibrium value [21]. In addition, Young's moduli in the range of 14–29 kPa were determined for alginate membranes at concentrations in the range 1.25–1.75% w/v and with the M/G ratio of 0.23 and hardened for 10 min in 0.5 M (~5.6% w/w) $CaCl_2$ using indentation method [39].

Overall, results of the biomechanical tests of alginate discs have shown suitability of the novel bioreactor for evaluation of average biomechanical properties of biomaterial and tissue specimens. In the next experimental series we have utilized the bioreactor to evaluate properties of alginate microbeads in a packed bed as a model scaffold attractive for cartilage tissue engineering. Alginate microbeads (1.5% w/w high G and 2% w/w low G) were spherical, about 800 μm in diameter, appropriate for efficient mass transport by diffusion. Alginate microbeads were packed with the porosity of about 30%. We have first evaluated biomechanical properties of packed beds of microbeads without immobilized cells in the same manner as of the alginate discs i.e., at 10% strain in two regimes: (i) at a loading rate of 337.5 μm/s and (ii) at sequential increments of 50 μm displacement every 30 min. Both compression and equilibrium unconfined compression moduli for both types of microbeads were significantly higher (~1.5–3.4 fold) than the respective values for alginate discs of the same concentrations (Table 1). These results could be attributed to better gelling of microbeads, which were significantly smaller than the discs, providing faster diffusion of Ca^{2+} ions. In addition, the packed beds of microbeads contained water, entrapped in the interstitial channels. When the force was applied on the packed bed, the trapped water most probably influenced the overall resistance against the deflection. On the other hand, mechanical testing and mathematical modeling of microcompression of a single alginate microbead (2 w/v %, 102 μm in diameter) at a rate of 500 μm/s up to a deformation of 28.5% followed by a relaxation period of 2 s resulted in estimation of the compression modulus of 490 kPa and the long-term modulus of 68.6 kPa [40, 41]. Thus, the values of moduli obtained in our study for the packed beds of microbeads are lower than those reported for a single microbead while higher than those of alginate discs. These results indicate that biomechanical behavior of packed beds of hydrogels under dynamic compression depends on both, hydrogel structural properties itself and the shape and packing arrangement of particles.

Consequently, evaluation of biomechanical properties becomes even more important in tissue engineering applications where significant changes could be expected due to ECM synthesis, biomaterial degradation, and particle deformation and bonding over the cultivation time and/or upon implantation.

We have next evaluated packed beds of alginate microbeads with immobilized chondrocytes in bioreactor cultivation under continuous medium perfusion and repeated cycles of dynamic compression imitating in vivo conditions. Superficial medium velocity of about 25 μm/s as well as the interstitial medium velocity of about 80 μm/s, based on the initial bed porosity, corresponded to the range of blood velocities found in capillaries and provided convective mass transport within the packed bed while not affecting chondrocytes entrapped and protected within the alginate gel. Dynamic compression in the physiological regime (10% strain, 0.42 Hz) was applied in repeated cycles of 1 h on/1 h off. Control study of alginate microbeads without cells has shown structural stability of the beads under the bioreactor conditions over 2 weeks of cultivation. Compression modulus stayed approximately constant for 7 days but stress–strain curves adopted a parabolic form in the second week of experiment while the beads still retained compactness. In both cultivation studies of alginate microbeads with and without cells, packed beds compressed for about 30% over 2 weeks of cultivation, indicating almost complete bed compaction and inducing slight deformation of microbeads (Fig. 6b). The compression modulus of the packed bed with immobilized cells determined at the start of cultivation was about 13–16% lower than the initial modulus determined for packed beds of corresponding alginate microbeads without immobilized cells. This difference is expected since immobilization of chondrocytes [21] as well as of microorganisms in alginate gels was previously reported to decrease the gel strength due to cell interference in the gelling process causing irregularities in the network structure [36, 42]. However, in contrary to the control beads, the stress–strain response of alginate beads with immobilized cells remained linear over 2 weeks of cultivation so that the compression modulus even slightly increased and at day 11 reached the initial value of the bed of microbeads without immobilized cells. This result is probably owed to the significant increase in cell density ($\sim$2.6 fold) and some production of ECM. It is also consistent with the reports that mechanical properties of agarose and alginate gels with immobilized chondrocytes improve over the cultivation time [21, 43]. It could be further expected that in longer bioreactor cultivation under dynamic compression and interstitial flow of medium possibly supplemented with additional growth factors, the synergistic action of deformational loading and biochemical signals could lead to structurally and mechanically competent engineered tissue constructs [38].

5 Conclusion

Biomaterial evaluation under strictly controlled in vitro conditions that mimic those found in vivo could provide relevant models to determine and predict the biomaterial behavior upon implantation and physiological loading. Alginate hydrogels are widely investigated for immobilization of cells and bioactive molecules as well as for applications in tissue engineering, due to biocompatibility, biodegradability, and ease of processing. Hydrogels are generally mechanically weak materials and exhibit viscoelastic properties. In addition, particulate forms of the cell support, attractive for providing short diffusion distances and simple administration procedure, when packed in a bed, may exhibit different biomechanical properties than a continuous scaffold. In this study, we have utilized a novel bioreactor with dynamic compression and medium perfusion to evaluate alginate hydrogels under in vivo-like conditions for cartilage tissue engineering. Packed beds of alginate microbeads exhibited significantly higher compression and equilibrium unconfined compression moduli than alginate discs, the result that could be explained by better gelling of microbeads as well as by the influence of trapped water inside the packed bed contributing to the overall mechanical properties. Immobilization of chondrocytes slightly decreased initial mechanical properties of the packed bed of alginate microbeads. However, during 2 weeks of cultivation under continuous medium perfusion and repeated cycles of dynamic compression in physiological regime, cells proliferated and started to produce ECM while the bed compressed to almost a solid phase, inducing a slight increase in compressive stiffness. Results of this study show potentials of alginate microbeads as cell carriers in cartilage tissue engineering. Moreover, with the caution that loading regimes utilized in vitro can only remotely mimic the complexity of joint loading in vivo, our findings suggest that the novel bioreactor with mechanical stimulation and interstitial medium flow is well suited for studies of the effects of dynamic compression on in vitro regeneration of skeletal tissues, as well as for biomaterial evaluation and monitoring over the cultivation under biomimetic conditions.

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References

1. Seal BL, Otero TC, Panitch A. Polimeric biomaterials for tissue and organ regeneration. Mater Sci Eng. 2001;34:147–230.
2. Lakshmi SN, Cato TL. Biodegradable polymers as biomaterials. Prog Polym Sci. 2007;32:762–98.
3. Bianco W, Grehan JF, Grubbs BC, Mracher JP, Schoeder EL, Schumacher CW, Svedsen CA, Lahti M. Large animal models in cardiac and vascular biomaterials research and testing. In: Dratner BD, Hoffman AS, Lemons JE, Schoen FJ, editors. Biomaterial science: an introduction to materials in medicine. New York: Academic press; 2004. p. 379–86.
4. Martinsen A, Skjak-Braek G, Smidsrod O. Alginate as immobilization material; I. Correlation between chemical and physical properties of alginate gel beads. Biotechnol Bioeng. 1989;33: 79–89.
5. Drury JL, Dennis RG, Mooney DJ. The tensile properties of alginate hydrogels. Biomaterials. 2004;25:3187–99.
6. Melvik JE, Dornish M. Alginate as a carrier for cell immobilisation. In: Nedović V, Willaert RG, editors. Focus on biotechnology: fundamentals of cell immobilisation biotechnology. Dordrecht: Kluwer Academic Publishers; 2005. p. 33–53.
7. Augst AD, Kong HJ, Mooney DJ. Alginate hydrogels as biomaterials. Macromol Biosci. 2006;6:623–33.
8. Tıgh RS, Gumusderelioglu M. Evaluation of alginate-chitosan semi IPNs as cartilage scaffolds. J Mater Sci: Mater Med. 2009;20: 699–709.
9. Atala A, Kim W, Paige KT, Vacanti CA, Retik AB. Endoscopic treatment of vesicoureteral reflux with a chondrocyte-alginate suspension (discussion 644). J Urol. 1994;152:641–3.
10. Marijnissen WJCM, van Osch GJVM, Aigner J, van der Veen SW, Hollander AP, Verwoerd-Verhoef HL, Verhaar JAN. Alginate as a chondrocyte-delivery substance in combination with a non-woven scaffold for cartilage tissue engineering. Biomaterials. 2002;23:1511–7.
11. Lum L, Elisseeff J. Injectable hydrogels for cartilage tissue engineering. In: Ashammakhi N, Ferretti P, editors. Topics in tissue engineering. Oulu: University of Oulu; 2003. p. 1–25.
12. Masuda K, Sah R, Hejna M, Thonar EJ-MA. A novel two–step method for the formation of tissue–engineered cartilage by mature bovine chondrocytes: the alginate–recovered–chondrocyte (ARC) method. J Orthop Res. 2003;21:139–48.
13. Sharma B, Elisseeff J. Engineering structurally organized cartilage and bone tissues. Ann Biomed Eng. 2004;32:148–59.
14. Xu X, Urbanz JPG, Tirlapury UK, Cui Z. Osmolarity effects on bovine articular chondrocytes during three-dimensional culture in alginate beads. Osteoarthr Cartil. 2010;18:433–9.
15. Weber M, Steinert A, Jork A, Dimmler A, Thurmer F, Schutze N, Hendrich C, Zimmermann U. Formation of cartilage matrix proteins by BMP-transfected murine mesenchymal stem cells encapsulated in a novel class of alginates. Biomaterials. 2002;23: 2003–13.
16. Stevens MM, Qanadilo HF, Langer R, Shastri VP. A rapid-curing alginate gel system: utility in periosteum-derived cartilage tissue engineering. Biomaterials. 2004;25:887–94.
17. Orr DE, Burg KJL. Design of a modular bioreactor to incorporate both perfusion flow and hydrostatic compression for tissue engineering applications. Ann Biomed Eng. 2008;36:1228–41.
18. Osmokrovic A, Obradovic B, Bugarski D, Bugarski B, Vunjak-Novakovic G. Development of a packed bed bioreactor system aimed for cartilage tissue engineering. FME Trans. 2006;34:65–70.
19. Obradovic B, Osmokrovic A, Bugarski B, Bugarski D, Vunjak-Novakovic G. Alginate microbeads as cell support for cartilage tissue engineering: bioreactor studies. Mater Sci Forum: Prog Adv Mater Process. 2007;555:417–22.
20. Freshney RI, Obradovic B, Grayson W, Cannizzaro C, Vunjak-Novakovic G. Principles of tissue culture and bioreactor design. In: Lanza RP, Langer R, Vacanti J, editors. Principles of tissue engineering. 3rd ed. New York: Elsevier, Inc.; 2007. p. 155–83.
21. Mauck RL, Soltz MA, Wang CCB, Wong DD, Chao PHG, Wilmot B, Valhmu WB, Hung CT, Ateshian GA. Functional tissue engineering of articular cartilage through dynamic loading of chondrocyte-seeded agarose gels. J Biomech Eng. 2000;122: 252–60.
22. Demarteau O, Jakob M, Schafer D, Heberer M, Martin I. Development and validation of a bioreactor for physical stimulation of engineered cartilage. Biorheology. 2003;40:331–6.
23. Seidel JO, Pei M, Gray ML, Langer R, Freed LE, Vunjak-Novakovic G. Long term culture of tissue engeneering cartilage in a perfused chamber with mechanical stimulation. Biorheology. 2004;41:445–58.
24. Mauck RL, Byers BA, Yuan X, Tuan RS. Regulation of cartilaginous ECM gene transcription by chondrocytes and MSCS in three-dimensional culture in response to dynamic loading. Biomech Model Mechanobiol. 2007;6:113–25.
25. Mauck RL, Hung CT, Ateshian GA. Modeling of neutral solute transport in a dynamically loaded porous permeable gel: Implications for articular cartilage biosynthesis and tissue engineering. J Biomech Eng. 2003;125:602–14.
26. Petrovic M, Mitrakovic D, Bugarski B, Vonwil D, Martin I, Obradovic B. A novel bioreactor with mechanical stimulation for skeletal tissue engineering. CI&CEQ. 2009;15:41–4.
27. Manojlovic V, Rajic N, Djonlagic J, Obradovic B, Nedovic V, Bugarski B. Application of electrostatic extrusion–flavour encapsulation and controlled release. Sensors. 2008;8:1488–96.
28. Lawrie G, Keen I, Drew B, Chandler-Temple A, Rintoul L, Fredericks P, Grøndahl L. Interactions between alginate and chitosan biopolymers characterized using FTIR and XPS. Biomacromolecules. 2007;8:2533–41.
29. Nedović VA, Obradović B, Leskošek CI, Trifunović O, Pešić R, Bugarski B. Electrostatic generation of alginate microbeads loaded with brewing yeast. Process Biochem. 2001;37:17–22.
30. Obradovic B, Bugarski D, Petakov M, Jovcic G, Stojanovic N, Bugarski B, Vunjak-Novakovic G. Cell support studies aimed for cartilage tissue engineering in perfused bioreactors. Mater Sci Forum. 2004;453–454:549–54.
31. Barbero A, Ploegert S, Heberer M, Martin I. Plasticity of clonal populations of dedifferentiated adult human articular chondrocytes. Arthriris Rheu. 2003;48:1315–25.
32. Freed LE, Marquis JC, Nohria A, Emmanual J, Mikos AG, Langer R. Neocartilage formation in vitro and in vivo using cells cultured on synthetic biodegradable polymers. J Biomed Mater Res. 1993;27:11–23.
33. Enobakhare BO, Bader DL, Lee DA. Quantification of sulfated glycosaminoglycans in chondrocyte/alginate cultures, by use of 1,9-dimethylmethylene blue. Anal Biochem. 1996;243:189–91.
34. Manojlovic V, Djonlagic J, Obradovic B, Nedovic V, Bugarski B. Investigation of cell immobilization in alginate: rheological and electrostatic extrusion studies. J Chem Technol Biotechol. 2006; 81:505–10.
35. Mancini M, Moresi M, Rancini R. Mechanical properties of alginate gels: empirical characterization. J Food Eng. 1999;39: 369–78.
36. Junter GA, Vinet F. Compressive properties of yeast cell-loaded Ca-alginate hydrogel layers: comparison with alginate–$CaCO_3$ microparticle composite gel structures. Chem Eng J. 2009;145: 514–21.
37. LeRoux MA, Guilak F, Setton LA. Compressive and shear properties of alginate gel: effects of sodium ions and alginate concentration. J Biomed Mater Res. 1999;47:46–53.

38. Mauck RL, Nicoll SB, Seyhan SL, Ateshian GA, Hung CT. Synergistic action of growth factors and dynamic loading for articular cartilage tissue engineering. Tissue Eng. 2003;9: 597–611.
39. Ahearne M, Yang Y, El Haj AJ, Then KY, Liu K-K. Characterizing the viscoelastic properties of thin hydrogel-based constructs for tissue engineering applications. J R Soc Interface. 2005;2:455–63.
40. Wang CX, Cowen C, Zhang Z, Thomas CR. High-speed compression of single alginate microspheres. Chem Eng Sci. 2005;60: 6649–57.
41. Nguyen VB, Wang CX, Thomas CR, Zhang Z. Mechanical properties of single alginate microspheres determined by micro-compression and finite element modeling. Chem Eng Sci. 2009; 64:821–9.
42. Gilson CD, Thomas A, Hawkes FR. Gelling mechanism of alginate beads with and without immobilized yeast. Process Biochem. 1990;25:104–8.
43. Williams GM, Klein TJ, Sah RL. Cell density alters matrix accumulation in two distinct fractions and the mechanical integrity of alginate–chondrocyte constructs. Acta Biomater. 2005;1:625–33.