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DOI:

[10.1016/j.jmbbm.2018.01.016](https://doi.org/10.1016/j.jmbbm.2018.01.016)

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Document Version

Peer reviewed version

Citation for published version (Harvard):

Majumdar, T, Cooke, M, Lawless, BM, Bellier, F, Hughes, E, Grover, L, Jones, S & Cox, S 2018, 'Formulation and viscoelasticity of mineralised hydrogels for use in bone-cartilage interfacial reconstruction', *Journal of the Mechanical Behavior of Biomedical Materials*, vol. 80, pp. 33-41. <https://doi.org/10.1016/j.jmbbm.2018.01.016>

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Publisher Rights Statement:

Published in Journal of the Mechanical Behaviour of Biomedical Materials on 31/01/2018

DOI: 10.1016/j.jmbbm.2018.01.016

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Formulation and viscoelasticity of mineralised hydrogels for use in bone-cartilage interfacial reconstruction

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Abstract

Articular cartilage is a viscoelastic tissue whose structural integrity is important in maintaining joint health. To restore the functionality of osteoarthritic joints it is vital that regenerative strategies mimic the dynamic loading response of cartilage and bone. Here, a rotating simplex model was employed to optimise the composition of agarose and gellan hydrogel constructs structured with hydroxyapatite (HA) with the aim of obtaining composites mechanically comparable to human cartilage in terms of their ability to dissipate energy. Addition of ceramic particles was found to reinforce both matrices up to a critical concentration (<3 w/v%). Beyond this, larger agglomerates were formed, as evidenced by micro computed tomography data, which acted as stress risers and reduced the ability of composites to dissipate energy demonstrated by a reduction in $\tan \delta$ values. A maximum compressive modulus of 450.7 ± 24.9 kPa was achieved with a composition of 5.8 w/v% agarose and 0.5 w/v% HA. Interestingly, when loaded dynamically (1 – 20 Hz) this optimised formulation did not exhibit the highest complex modulus instead a sample with a higher concentration of mineral was identified (5.8 w/v% agarose and 25 w/v% HA). Thus, demonstrating the importance of examining the mechanical behaviour of biomaterials under conditions representative of physiological environments. While the complex moduli of the optimised gellan (1.0 ± 0.2 MPa at 1 Hz) and agarose (1.7 ± 0.2 MPa at 1 Hz) constructs did not match the complex moduli of healthy human cartilage samples (26.3 ± 6.5 MPa at 1 Hz), similar $\tan \delta$ values were observed between 1 and 5 Hz. This is promising since these frequencies represent the typical heel strike time of the general population. In summary, this study demonstrates the importance of considering more than just the strength of biomaterials since tissues like cartilage play a more complex role.

Keywords; Hydrogels, cartilage, viscoelastic behaviour, dynamic mechanical analysis, hydroxyapatite

1. Introduction

Articular cartilage covers the ends of bones to provide a low-friction joint surface and transfers load to the subchondral bone. The complex organisation of articular cartilage varies through its depth. Within joint spaces four distinct sub-zones are distinguishable: hyaline cartilage, calcified cartilage, sub-chondral bone, and cancellous bone (Costa and Mano, 2015; Liu et al., 2011). Variation in the structure of the extracellular matrix occurs between different layers, specifically glycosaminoglycan (GAG) concentration and the ratio of collagen type II to X. The hyaline cartilage layer has the lowest concentration of GAGs and compressive modulus, and highest ratio of collagen II to collagen X, while the opposite is true for the cancellous bone region (Nguyen et al., 2011). This hierarchical structure is responsible for the viscoelastic response of articular cartilage under deformation. To restore the functionality of osteoarthritic joints it is vital that candidate biomaterials mimic the dynamic loading response of cartilage and bone.

Since the Young's modulus of biological tissues may depend on strain rate, i.e. they are viscoelastic (Hukins et al., 1999), it is important to understand the mechanical behaviour of biomaterials in relation to strain rates. The compressive modulus of cartilage has been shown to be strain rate dependent (Shepherd and Seedhom, 1997). Methods, such as creep and stress relaxation testing, have been used to characterise the viscoelastic response of cartilage and biomaterials, such as hydrogels (Fick and Espino, 2011; Moxon et al., 2017). However, unlike these methods, Dynamic Mechanical Analysis (DMA) is a dynamic testing approach to determine the viscoelastic properties of a material (Barnes et al., 2016; Burton et al., 2017) or multiple component structures (Lawless et al., 2017a, 2016). For DMA, an oscillating force is applied to a specimen and the out-of-phase displacement response is analysed (Menard, 2008). Viscoelastic properties of the material can then be characterised in terms of its storage modulus (ability to store energy for elastic recoil) and loss modulus (ability to dissipate energy); these are the real and imaginary parts of the complex modulus. The storage (E') and loss (E'') moduli are related to the complex modulus (E^*) and the phase lag (δ), of the viscoelastic material (Hukins et al., 1999):

$$|E^*| = \sqrt{E'^2 + E''^2} \quad 1$$

$$\delta = \tan^{-1} \left(\frac{E''}{E'} \right) \quad 2$$

Studies have used DMA to quantify the frequency dependent viscoelastic properties of cartilage (Fulcher et al., 2009; Temple et al., 2016) and to understand factors that affect the viscoelastic properties, including resistivity of bone (Lawless et al., 2017b), hydration (Pearson and Espino, 2013), its thickness (Espino et al., 2014) and the induced stress (Lawless et al., 2017b). Also, frequency, independent of load, has been shown to be an important factor in relation to how cartilage damages (Sadeghi et al., 2015) while crack propagation, of articular cartilage, has been shown to be frequency dependent (Sadeghi et al., 2018). Thus, materials intended to replace this tissue should not only exhibit similar mechanical strength but also have comparable frequency response.

Since cartilage is avascular, it is critical that regenerative strategies have the ability to support cellular ingrowth. Generally, hydrogels are the material of choice for the formulation of tissue engineered scaffolds to regenerate cartilage due to the possibility to incorporate cells (Almeida et al., 2016; Balakrishnan et al., 2014; Parmar et al., 2015). However, the frequency dependent viscoelastic response of hydrogels is often overlooked (Cameron et al., 2011). There is also typically a trade-off between the mechanical suitability of hydrogels and cytocompatibility (Bartnikowski et al., 2015; Mironi-Harpaz et al., 2012). The incorporation of a secondary phase to the hydrogel matrix presents the possibility to add complementary properties (Osmalek et al., 2014), such as mechanical response or enhanced cell adhesion. Addition of a mineral phase is an attractive strategy to reinforce the hydrogel matrix and induce osseous tissue growth (D'Este and Eglin, 2013; Hu et al., 2016). This may be particularly useful in hydrogels that display low cell adhesion, such as agarose and gellan gum (Zhang et al., 2012). Many synthetic bone mineral analogues have been explored, including calcium phosphates such as hydroxyapatite ($\text{Ca}_{10}(\text{PO}_4)_6(\text{OH})_2$; HA) (Danoux et al., 2016; Tamaddon and Czernuska, 2013). While the advantages for such an approach are clear, the influence of incorporating these secondary phases on the viscoelasticity of hydrogels is currently limited, that is despite the frequency response of these materials being recognised as critical for the intended application (Bartnikowski et al., 2015; Wands et al., 2008).

In this study, we selected two natural origin hydrogels, gellan gum and agarose, that may be thermally gelled so that suitable specimens for DMA experiments could be produced with relative ease compared with other materials that require more complex gelation mechanisms. This enabled the work to explore the possibility of

structuring gellan gum and agarose, with hydroxyapatite to create cytocompatible constructs with comparable viscoelastic properties to native cartilage. To achieve this, a Doehlert type rotating simplex model was used to systematically formulate mineralised composites. Optimisation of the constructs was performed iteratively by varying hydrogel and mineral concentration. DMA was employed to characterise the viscoelastic response of the hydrogel/mineral composites as well as human cartilage samples. Use of micro-computed tomography allowed for visualisation of mineral distribution and size, which was used to further elucidate the influence of hydroxyapatite on the observed mechanical response. To complement this mechanical analysis, the effect of incorporating HA into agarose and gellan constructs on cell adhesion was also studied *in-vitro*.

2. Materials and methods

2.1 Development of experimental plan

A model to relate the effects of varying the concentration of polymer and mineral on composite mechanical behaviour was developed according to Doehlert's theory of experimental design (Jethara and Patel, 2015; Mennini et al., 2012). This allowed for the resolution of a second degree system to find optimal compositions, whilst allowing for refinement through shifts in experimental field based on initial results (Gabrielsson et al., 2002; Jethara and Patel, 2015; Mennini et al., 2012). While the concentration of gel and mineral were optimised all other processing parameters, including temperature and shear rate, were kept constant. An initial experimental domain for testing hydrogel composite properties was specified: 2.75-6.75 w/v% agarose to 0-25 w/v% hydroxyapatite (HA), and 1.5-6 w/v% gellan to 0-15 w/v% HA. Details of all compositions tested can be found in Supplementary Information 1. At least 5 repeats were conducted for model validation. Coefficients and optimal compositions were obtained through polynomial regression and optimisation was completed for rupture stress. The model was validated by Fisher tests and any significance of coefficients were determined using student t-tests. A p value less than 0.05 was deemed significant.

2.2 Preparations of gels and composites

Agarose and gellan gum were selected for this study since both may be set via thermal gelation hence samples suitable for the proposed mechanical testing (section 2.3) could be produced with relative ease and high batch reproducibility. To prepare the gels, agarose (genetic analysis grade, Fisher Scientific, UK) and gellan (food grade, Kelcogel, CP Kelco, UK) powders were dissolved in deionised water (pre-heated to 100°C for gellan) while stirring on a magnetic hot plate at 200 rpm and 100°C resulting in transparent solutions. HA ($\geq 90\%$, Sigma-Aldrich, UK) was added if necessary, and the solution stirred for a further 10 minutes at 200 rpm. Gels were cast into sealed cylindrical moulds (diameter 6 cm and height 10 cm), and allowed to cool at room temperature overnight prior to testing. Following testing, optimal compositions were chosen as determined, on the basis of rupture stress, through the model and used for all following tests. A full list of all compositions tested can be viewed in Supplementary Information 1. Notably, incorporation of HA particles within the two matrices exhibited different behaviour and hence the concentrations investigated for each material group were not the same.

2.3 Mechanical testing

All hydrogel/mineral specimens (diameter = 14 ± 1 mm; height = 6 ± 1 mm; $n = 4$) were mechanically tested by using a Bose ElectroForce 5500 controlled by WinTest 7 software (TA Instruments, New Castle, DE USA). Quasi-static mechanical testing was performed at a rate of 0.1 mm/min until specimen failure. The loads were applied using a flat compression platen with a diameter of 25 mm. For each group, the Young's modulus and rupture stress were calculated and reported as mean $\pm$ standard deviation (SD) ($n=6$). Viscoelastic testing was performed, with the WinTest 7 DMA software, on the optimal and boundary conditions for both matrices. DMA was performed with a sinusoidal compressive strain between 15 and 20% of the specimen height. Following pre-conditioning cycles at 0.2 and 0.5 Hz, each specimen was tested at 1, 5, 10 and 20 Hz. For each frequency, the complex (E^*) and the dissipation factor ($\tan \delta$), also known as loss factor, were quantified. Further information on the calculation of these properties can be found elsewhere (Barnes et al., 2016; Fulcher et al., 2009; Lawless et al., 2016). For each composition, four repeats were completed.

All cartilage explants were taken from human femoral condyles. The study was approved by the United Kingdom National Research Ethics Service (Nottingham Research Ethics Committee 1 (05/Q2403/24) and Derby Research Ethics Committee 1 (11/H0405/2)). Informed consent was provided patients next of kin. The cartilage explants (diameter = 5.2 mm; height = 1.47 ± 0.36 mm; $n=3$ healthy) were tested using a Bose ElectroForce 3200 controlled by 4.1 WinTest software (TA Instruments, New Castle, DE, USA). In accordance with previous studies, the cartilage explants were saturated with Ringer's solution prior to the frequency sweep (Temple et al., 2016). Following preconditioning cycles at 25 and 50 Hz (Espino et al., 2014; Temple et al., 2016), compressive load was applied between 16 and 36 N at 1, 8, 10, 12, 29 Hz to fully hydrated healthy (post-

mortem cartilage from individuals with no history of joint pain) cartilage explants. All mechanical tests of the hydrogel/mineral specimens and cartilage explants were performed under unconfined conditions, in air at room temperature.

2.4 Visualisation of hydroxyapatite distribution in selected composites

Optimal hydrogel and mineral compositions for both agarose and gellan with HA were determined using Doehrlert's model. For these two selected formulations the distribution of HA particles within the matrix was visualised in 3-dimensions (3D) using a Skyscan1172 micro-computed tomography (micro-CT) system (Bruker, Belgium). Composite cylinders ($n = 1$) were scanned with a 64 kV maximum X-ray energy, 6.4 W beam power, 1150 ms exposure per projection, 0.5 mm aluminium filter, and 9.87 μm pixel size. Data was reconstructed using *NRecon* (1.6.10.2, Bruker), binarised by applying a global threshold using *CTAn* (version 1.15.4.0, Bruker) and grey scale data was visualised in 3D using *CTVox* (version 3.0, Bruker).

2.5 *In vitro* adhesion and viability of MC3T3 pre-osteoblast cells

Since both agarose and gellan gum hydrogels are known to be non-cell adhesive an initial assessment of the effect of HA incorporation on the ability of native cells to adhere and survive on the composite samples was warranted. Given the focus of this study is the whole osteochondral unit pre-osteoblasts were selected as a native cell population for this initial *in-vitro* work due to their relatively rapid growth rate and robustness. Alpha Minimum Essential Media (α -MEM) with sodium bicarbonate, ribonucleosides and deoxyribonucleosides (M426, Sigma, UK) was used as a base medium. Complete growth media was made by supplementing α -MEM media with a final concentration of 10% fetal bovine serum (F7524, Sigma, UK), 2.4% l-glutamine (G7513, Sigma, UK) and 1% penicillin-streptomycin (F4333, Sigma, UK). MC3T3-E1 preosteoblast cells (Subclone 4, CRL-2593, ATCC, USA) were cultured in complete growth media as per supplier instructions. Prior to *in vitro* experiments, hydrogel samples (5.8 w/v% agarose with and without 0.5 w/v% HA, gellan 5.3 w/v% with and without 3.0 w/v% HA) prepared as described in section 2.2, were washed with 100% ethanol, and sterilised for 24 hours under ultra-violet light immersed in complete growth media. Following, aliquots of MC3T3-E1 cells (2×10^4 cells/cm²) were seeded on to the surface of hydrogel samples and tissue culture plastic as a control. Cells were allowed to adhere for 2 hours and after this time complete growth media was added to each well to cover the sample surface. Cultures were incubated in 5% CO₂ atmosphere maintained at 37°C. The viability of cells after culturing for 1, 3, and 7 days were analysed by staining with calcein-AM (1 mg/mL, Molecular Probes, UK) and propidium iodide (1 mg/mL, Invitrogen, UK), respectively in the dark. Stained cultures were visualised ($n=3$) using a scanning confocal microscope (Olympus FV1000, Multiple Ar laser, Germany).

2.6 Statistical analysis

All data was analysed and figures generated using Prism software (version 6 for Windows, GraphPad Software, USA). All figures are presented as mean $\pm$ SD of $n \geq 5$. One-way analysis of variance (ANOVA) was used for single factor testing, while two-way ANOVA was used for analyses with two variables. Both tests were followed by post-hoc Tukey's (when comparing to a control group) or Dunnett's tests (when comparing multiple groups). P values < 0.05 were considered significant.

3. Results

3.1 Optimising hydrogel and HA composite ratios

For both matrices, the concentration of gel was shown to affect both compressive modulus and maximum stress (Figure 1). Increasing the gellan concentration for composites containing 12.8 w/v% HA, from 1.5 to 4 w/v% showed a three-fold increase in the maximum stress from 10.4 kPa (1.5GG 12.8HA) to 37.2 kPa (4GG 12.8HA). Similarly for agarose samples containing 0.5 w/v% HA, an increase in the concentration of matrix from 2.8 to 5.8 w/v% caused a 3-fold increase in both rupture stress ($p < 0.0001$) and compressive modulus ($p < 0.0001$).

Increasing the amount of HA in 5.8 w/v% agarose from 0 to 0.5 w/v% caused an increase in average rupture stress from 120.2 ± 6.7 to 156.3 ± 16.5 kPa. Further increases in HA concentration for this matrix reduced rupture stress; 25 w/v% HA resulted in a rupture stress of 92.1 ± 3.5 kPa. For mineralised gellan composites both rupture stress and compression modulus increased up to a critical point of HA incorporation (4GG 0.5HA) and beyond this deterioration occurred (4GG 12.8HA). Using the Doehrlert model, 5.8AG 0.5HA and 5.3GG 3.0HA were determined to be the optimal concentrations with respect to maximum stress. Boundary conditions for agarose were found to be: 2.8AG 0.5HA, 2.8AG 25HA, and 5.8AG 25HA. For gellan boundary conditions were 1.5GG 12.8HA, 4GG 0.5HA, and 4GG 12.8HA.

Analysis of viscoelastic behaviour up to 20 Hz revealed that hydrogel composites exhibited a tunable frequency response (**Error! Reference source not found.**). Similar to the quasi-static testing, the viscoelastic behaviour of the boundary compositions determined from the model demonstrated that agarose/HA constructs generally exhibited a higher complex modulus than the gellan/HA composites (**Error! Reference source not found.a and b**). The complex modulus, of the AG optimal and boundary conditions, gradually increased within the examined range of 1 to 20 Hz ($R^2 > 0.90$, $p < 0.05$). In comparison, for the gellan gum composites similar trends were observed ($R^2 > 0.73$, $p < 0.05$). For both matrices, the highest complex modulus across all frequencies for the model boundary conditions was 5.8AG 25HA (2.3 ± 0.5 MPa at 1 Hz) and 5.3GG 3HA (1.0 ± 0.1 MPa at 1 Hz). Notably, for the agarose samples this result did not agree with the optimal formulation determined from quasi-static testing (5.8AG 0.5HA).

The dissipation factor ($\tan \delta$), also known as loss factor, of composites was quantified (**Error! Reference source not found.c and d**). Between 1 - 20 Hz, a gradual increase in loss tangent was observed, which revealed that more energy was stored by constructs than dissipated. The lowest loss tangent values were observed for 4GG 12.8HA ($\tan \delta = 0.21 \pm 0.02$ at 1 Hz) and 5.8 AG 25HA ($\tan \delta = 0.12 \pm 0.03$ at 1 Hz). For agarose samples, $\tan \delta$ values were lower at all frequencies for constructs containing more HA.

3.2 Comparison of the dynamic mechanical behaviour of human cartilage and mineralised hydrogel composites

The complex moduli of the mineralised hydrogel samples were clearly shown to be lower than human cartilage isolated from healthy patients at all tested frequencies ($p < 0.05$) (**Figure 3a**). The optimised agarose (5.8AG 0.5HA) and gellan (5.3GG 3.0HA) formulations exhibited complex moduli of 1.7 ± 0.1 and 1.0 ± 0.2 MPa, respectively, at 1 Hz while healthy cartilage (HC) was 26.3 ± 6.5 MPa. Cartilage was shown to be substantially higher than these hydrogel composites (26.3 ± 6.5 MPa at 1 Hz). The complex modulus of human cartilage and agarose samples with, as well as without, mineral was shown to increase logarithmically with increasing frequency ($R^2 > 0.9$). When gellan was used as the composite matrix this relationship was found to fit a second order polynomial.

The dissipation factor of gellan samples up to 5 Hz were found to have comparable $\tan \delta$ values to HC (**Figure 3b**). Above this frequency the gellan samples exhibited an increase in the ability to store energy as evidenced by a significant increase in $\tan \delta$ values ($y = 0.008$, $R^2 > 0.98$, $p < 0.01$). At 1 Hz, the loss factor of the 5.8AG 0.5HA was found to be almost double the value for HC; 0.19 ± 0.00 compared with 0.11 ± 0.02 (**Figure 3b**).

Micro-CT was employed to visualise the agglomeration behaviour of HA particles within optimised AG and GG composites to identify any differences in particle size and homogeneity, which may have influenced mechanical response (**Figure 4**). HA particles (white) were distinguishable from both matrices (grey) through differences in X-Ray attenuation (**Figure 4a**). A sample of human articular cartilage isolated from femoral condyles allowed for visualisation of the bone to cartilage interface, however, it was not possible to resolve any variation between sub-zones of the cartilage structure (**Figure 4b**). Agglomeration of individual HA particles was identified in both AG and GG structures and 3D visualisation revealed that the calcium phosphate was not entirely homogeneous throughout the matrices. The majority of HA clusters were found to be less than $110 \mu\text{m}$ for both hydrogels, however notably a higher proportion $> 150 \mu\text{m}$ was observed in 5.8AG 0.5HA compared with 5.3GG 3.0HA (**Figure 4c**).

3.5 Cytotoxicity studies

In addition to assessing the mechanical suitability of the mineralised hydrogel constructs it is important to also consider the ability of native cells to survive and proliferate at the biomaterial/tissue interface, in particular for an avascular tissue such as cartilage. The viability and adherence of MC3T3 pre-osteoblasts on optimised formulations with and without HA was visualised (**Figure 5**). As expected, very few cells were found to be viable on AG and GG constructs without HA after 1 day of culture. The addition of HA to both matrices was shown to facilitate attachment of MC3T3 pre-osteoblast cells. After 3 days of culture, the distribution of cells across mineralised hydrogel surfaces was generally less dense compared with the control (tissue culture plastic). Notably, cells appeared to preferentially adhere in localised regions on 5.8AG 0.5HA and 5.3GG 3.0HA samples at days 3 and 7. The number of MC3T3s on the mineralised composites appears to increase over 7 days and cells were observed to spread across the surface, however compared with the control there was clearly fewer adherent cells.

4. Discussion

The use of a simplified Doehrlert model to optimise the composition of mineralised hydrogel constructs allowed for the determination of a domain and predicted composite behaviour on the basis of experimental data. A limitation of the Doehrlert model is that it does not represent physical or chemical phenomena and therefore, predictions may not fit all circumstances. Thus, experimental quasi-static testing was performed to validate the model. The compositions determined by the model to be optimal (5.8AG 0.5HA and 5.3GG 3.0HA) were confirmed to be consistent with the quasi-static mechanical testing results (**Figure 1**). The composition which had the highest average compressive modulus was 5.8AG 0.5HA (450.7 ± 24.9 kPa). In relation to the viscoelastic properties quantified by DMA, the 5.3GG 3.0 HA composition had the highest complex modulus of the GG hydrogels (**Figure 2b**). However, for the optimal agarose sample (5.8AG 0.5HA) this was the second stiffest for every frequency tested (**Figure 2a**). This may highlight a potential limitation of the optimisation technique as the prediction may not fit all circumstances of mechanical loading.

Here a reduction in temperature was the only mechanism used to induce gellan gelation, which is known to cause a random coil-helix transition to occur and further aggregation of helices leads to the formation of junction zones (Miyoshi et al., 1996). However, since the sol-gel transition of gellan is ionotropic the presence of cations is necessary for the formation of a more stable hydrogel, which may explain why gellan samples were generally less stiff compared with agarose constructs (**Figure 1**). Coutinho *et al.* demonstrated that it is possible to formulate both physically and chemically crosslinked gellan hydrogels by incorporating methacrylate groups into the polymer chain (Coutinho et al., 2010). These methacrylated gellan hydrogels exhibited highly tunable quasi-static properties, for example ultimate stress varied from 11.7 to 889.5 kPa and ultimate strain 34.2 to 96.1%, however Coutinho *et al.* did not consider viscoelastic response. In this study, gellan samples exhibited maximum stress values below 100 kPa and ultimate strain of up to 40% (**Figure 1b**).

Matrix and HA concentration were both found to have an effect on the quasi-static mechanical properties of gellan and agarose composites (**Figure 1**). For both hydrogel matrices a critical concentration at which reinforcement with ceramic particles led to a reduction in rupture stress and compressive modulus was observed. Jamshidi *et al.* noted a similar phenomenon when incorporating HA into gellan, however, notably this critical concentration was found to be substantially higher for nano-sized HA sol compared with microcrystalline HA (Jamshidi et al., 2012). A maximum compressive strength of 162.5 ± 9.3 kPa was achieved for a 2.5 w/v% gellan hydrogel reinforced with 50 w/v% nano-sized HA (Jamshidi et al., 2012). Here a significantly lower critical concentration of HA (3 w/v%) was found to have a similar effect. This may be attributed to the method of particle incorporation; following mixing some sedimentation was observed, which lead to agglomeration as demonstrated in micro-CT images (**Figure 4**). Particle size has been demonstrated to impact rupture stress and compressive modulus (D'Este and Eglin, 2013). If the ceramic is not incorporated into the matrix homogeneously the particles may behave as sites of stress concentration. While small amounts of HA may act as brittle networks, allowing for the dissipation of stress through the breaking of bonds locally around the particles, which explains the initial increase in quasi-static properties, as the amount of HA is increased these sites begin to overlap leading to earlier failure. The stress rising effect of the HA particles was particularly evident for agarose samples, which exhibited lower $\tan \delta$ values at all frequencies for composites containing increased concentrations of ceramic powder (**Figure 2c**). This may be linked with the higher proportion of HA particles $>150 \mu\text{m}$ in agarose samples as determined by particle size analysis conducted on micro-CT data (**Figure 4c**). However, to confirm this further scanning would need to be conducted and statistical significance demonstrated. The greater propensity for HA to agglomerate in AG compared with GG may be due to difference in gelation kinetics and gel chemistry. The gelation mechanism of both AG and GG proceeds through a thermal transition, occurring when heated gel solutions are cooled (section 2.2). Unlike agarose, the molecular structure of gellan gum is known to possess acyl groups that facilitate crosslinking in the presence of cations (e.g. Mg^{2+} , Ca^{2+} , etc.). If such cations are present during cooling, GG can undergo a more efficient gelation. Therefore, it is postulated that Ca^{2+} associated with HA particulate surfaces becomes liberated during composite processing, in turn enhancing gelation efficiency. Ultimately, this stabilises the distribution of HA particulates in the gelling GG matrix. Agarose does not have an affinity for Ca^{2+} , thus particulates of HA in AG/HA formulations may not be stabilised as gelling proceeds, potentially allowing for the greater extent of agglomeration as demonstrated by microCT analysis (**Figure 4**).

As cartilage is a frequency-dependent viscoelastic material (Fulcher et al., 2009; Temple et al., 2016), comparing the compressive modulus, obtained through quasi-static testing, does not provide all the information required to compare synthetic and natural cartilage viscoelastic behaviour. In this present study, a general understanding of the composites compression moduli were obtained through quasi-static mechanical testing, but it should be noted that as hydrogels are viscoelastic, their behaviour is significantly affected by the frequency of loading (Vincent, 2012). Similarly to other studies (Espino et al., 2014; Lawless et al., 2017b; Temple et al.,

2016), the viscoelastic properties of both synthetic and natural cartilage was observed to be frequency-dependent. Mineralised agarose compositions displayed higher complex moduli than the mineralised gellan compositions between 5-20 Hz ($p < 0.05$), but were lower compared to native cartilage explants for all tested frequencies ($p < 0.05$) (**Figure 3**). At 1 Hz the complex moduli of the optimised gellan (5.3GG 3.0HA) and agarose (5.8AG 0.5HA) constructs were 1.0 ± 0.2 MPa and 1.7 ± 0.2 MPa, respectively, compared with 26.3 ± 6.5 MPa for healthy human cartilage.

The specific organisation and morphological arrangement of collagen fibrils throughout the superficial, transitional and deep zones of articular cartilage structure enables the tissue to resist forces imposed upon it during articulation. Tensile, shear and compressive forces can be countered effectively by collagen fibrils of the superficial zone due to being highly aligned parallel to the surface in contact with synovial fluid. In contrast, AG and GG are absent of such specific alignment, therefore requiring the addition of a mineral phase to reinforce physical properties. In healthy tissue, HA and the extracellular matrix (ECM) interact in calcified regions of articular cartilage, contributing to an interface between cartilage and bone. Natural ECM structure facilitates strong binding interactions with hydroxyapatite, with collagen providing sites for mineral deposition, and non-collagenous proteins possessing a high affinity for hydroxyapatite mineral. Mineral formation is tightly regulated in such tissues in terms of mineral distribution, as well as the size and shape of crystallites. The mineral component of the composites analysed in this study is formed in the absence of the matrix component, such that interactions between HA with AG and GG phases are reliant on the development of mechanical interlocking between hydrogel chains and mineral particulates. Taken together, differences in the viscoelastic behaviour between the mineralised composites and natural cartilage are likely due to a combination of differences in composition, molecular organisation and mineral/hydrogel interactions. However, between 1-5 Hz, the gellan compositions displayed a comparable dissipation factor to healthy human cartilage. In general, the rise time of the heel strike force is approximately 100-150 ms (Shepherd and Seedhom, 1997). At 5 Hz, the rise time of the heel strike is 100 ms, thus the optimised gellan composition (5.3GG 3.0HA) formulated in the present study, has a similar loss-rate of energy to human healthy cartilage at a heel strike rise time of the general population. Therefore, further mechanical evaluation of this hydrogel will be conducted at physiological temperatures, and under different cyclical loading conditions.

The axial frequency dependent viscoelasticity of calcium alginate with HA have been previously quantified up to 20 Hz (Bouropoulos et al., 2010; Wands et al., 2008); this maximum frequency is comparable to the present study. Similar to Bourpoulos et al., 2010 and Wands et al., 2008, the present study demonstrates that the alginate hydrogels can be stiffen by HA and that the viscoelastic properties are frequency dependent (up to 20 Hz). Unlike the previous studies (Bouropoulos et al., 2010; Wands et al., 2008), the present study compared the results of the hydrogels to healthy cartilage and discovered that the agarose composition (5.8 AG 0.5 HA) dissipation factor was up to two times greater than healthy cartilage. Furthermore, the frequency dependent viscoelastic properties of gelatin methacrylamide (GelMA), Gellan Gum (GG), gellan gum methacrylate (GGMA) and combinations of GelMA/GG and combinations of GelMA/GGMA were quantified 0.01 to 5.12 Hz (Bartnikowski et al., 2015). Similar to the present study, Bartnikowski *et al.* (Bartnikowski et al., 2015) found that the fabrication of the hydrogels resulted in frequency-dependent viscoelastic properties and discovered that the $\tan \delta$ values of 1.0GG was most comparable to human donor cartilage. However, in the present study it was identified that the optimum gellan composition (5.3GG 3.0HA) displayed a similar dissipation factor to healthy human cartilage between 1-5 Hz. Extrapolatory to the frequency range used by Bartnikowski et al. (Bartnikowski et al., 2015), this present study demonstrates that at frequencies greater than 5 Hz, the dissipation factor of the GG hydrogels were larger than the dissipation factor of healthy human cartilage. Further investigations of hydrogel frequency-dependency viscoelastic properties should evaluate the material at frequencies greater than 20 Hz as Radin et al. (Radin et al., 1991) identified a subset of the population to have a heel-strike rise time of around 5-25 ms.

The final part of this study examined the effect of HA particle inclusions in gellan and agarose composites on cell adhesion and viability. While agarose and gellan can support cell encapsulation these hydrogels do not facilitate cell adhesion due to a lack of ligands as seen here in confocal live/dead images (**Figure 5**). It has been hypothesised that the addition of HA and/or other bioceramics may provide building blocks or signposts for cell migration and attachment (D'Este and Eglin, 2013; Suzawa et al., 2015). Here incorporation of 0.5 and 3.0 w/v% HA into agarose and gellan composites, respectively was shown to facilitate adherence of pre-osteoblast cells up to 7 days of culture. While there was some degree of cell spreading observed, generally live cells on these samples were seen in clusters (**Figure 5**). This behaviour may be because cells were only able to adhere where HA agglomerations were near the surface and acted as anchors for initial attachment. To confirm this suggestion further work could be conducted to analyse the co-localisation efficiency between cells and HA,

which could be labelled with Alizarin red (Moriguchi et al., 2003). Furthermore, it would be of interest to assess the ability of all native osteochondral cell types, including chondrocytes, seeded on the surface to infiltrate the composites. Overall, this initial cytotoxicity assessment demonstrates that potentially there is a trade-off between the mechanical influence of HA particles and the positive effect inclusions may have on controlling cellular responses.

5. Conclusion

The frequency-dependent viscoelastic properties of gellan and agarose hydrogels were found to be significantly affected by the addition of HA. While small amounts of HA reinforced the composite matrix, concentrations over a critical amount resulted in higher degrees of agglomeration and acted as stress risers, which was evidenced by micro-CT analysis and $\tan \delta$ values. A Doehlert model was used in combination with quasi-static mechanical testing to iteratively optimise formulations; 5.8 AG 0.5HA and 5.3GG 3.0HA were found to exhibit the highest maximum stresses. Interestingly, the agarose sample which was the most promising based on quasi-static tests did not exhibit the highest complex modulus between 1 – 20 Hz, instead a sample with increased mineral (5.3 AG 25HA) was found to be more comparable to human cartilage.

Generally the complex moduli of the optimised gellan (1.0 ± 0.2 MPa at 1 Hz) and agarose (1.7 ± 0.2 MPa at 1 Hz) constructs did not match the complex moduli of healthy human cartilage samples (26.3 ± 6.5 MPa at 1 Hz). However, the dissipation factors of this gellan composition was found to be comparable to human cartilage between 1 – 5 Hz. Since 5 Hz is representative of the heel strike rise time for the general population, further mechanical evaluation of this formulation is suggested.

In summary, this study demonstrates the advantages of using a model to systematically optimise hydrogel composites as well as the importance of examining the mechanical behaviour of biomaterials under conditions representative of the native environment. Furthermore, this approach needs to be balanced with a biological assessment of the material being developed.

Acknowledgements

The Bose ElectroForce 3200 used in this study was funded by Arthritis Research UK (Grant number H0671). The authors would like to thank the University of Birmingham for receiving TM and FB as visiting researchers from their home institutions. TM would also like to thank her supervisor Nick Birbilis for providing partial funding for this trip.

Funding

Not applicable.

References

- Almeida, H.V., Eswaramoorthy, R., Cunniffe, G.M., Buckley, C.T., O'Brien, F.J., Kelly, D.J., 2016. Fibrin hydrogels functionalized with cartilage extracellular matrix and incorporating freshly isolated stromal cells as an injectable for cartilage regeneration. *Acta Biomater.* 36, 55–62. doi:10.1016/j.actbio.2016.03.008
- Balakrishnan, B., Joshi, N., Jayakrishnan, A., Banerjee, R., 2014. Self-crosslinked oxidized alginate/gelatin hydrogel as injectable, adhesive biomimetic scaffolds for cartilage regeneration. *Acta Biomater.* 10, 3650–3663. doi:10.1016/j.actbio.2014.04.031
- Barnes, S.C., Lawless, B.M., Shepherd, D.E.T., Espino, D.M., Bicknell, G.R., Bryan, R.T., 2016. Viscoelastic properties of human bladder tumours. *J. Mech. Behav. Biomed. Mater.* 61, 250–257. doi:10.1016/j.jmbbm.2016.03.012
- Bartnikowski, M., Wellard, R.M., Woodruff, M., Klein, T., 2015. Tailoring hydrogel viscoelasticity with physical and chemical crosslinking. *Polymers (Basel)*. 7, 2650–2669. doi:10.3390/polym7121539
- Bouropoulos, N., Stampoulakis, A., Mouzakis, D.E., 2010. Dynamic Mechanical Properties of Calcium Alginate-Hydroxyapatite Nanocomposite Hydrogels. *Sci. Adv. Mater.* 2, 239–242. doi:10.1166/sam.2010.1092
- Burton, H.E., Freij, J.M., Espino, D.M., 2017. Dynamic viscoelasticity and surface properties of porcine left anterior descending coronary arteries. *Cardiovasc. Eng. Technol.* 8, 41–56. doi:10.1007/s13239-016-0288-4
- Cameron, A.R., Frith, J.E., Cooper-White, J.J., 2011. The influence of substrate creep on mesenchymal stem cell behaviour and phenotype. *Biomaterials*. doi:10.1016/j.biomaterials.2011.04.003
- Costa, A.M.S.S., Mano, J.F., 2015. Extremely strong and tough hydrogels as prospective candidates for tissue repair – A review. *Eur. Polym. J.* 72, 344–364. doi:10.1016/j.eurpolymj.2015.07.053
- Coutinho, D.F., Sant, S. V., Shin, H., Oliveira, J.T., Gomes, M.E., Neves, N.M., Khademhosseini, A., Reis, R.L., 2010. Modified Gellan Gum hydrogels with tunable physical and mechanical properties. *Biomaterials* 31, 7494–7502. doi:10.1016/j.biomaterials.2010.06.035
- D'Este, M., Eglin, D., 2013. Hydrogels in calcium phosphate moldable and injectable bone substitutes: Sticky excipients or advanced 3-D carriers? *Acta Biomater.* 9, 5421–30. doi:10.1016/j.actbio.2012.11.022
- Danoux, C., Pereira, D., Döbelin, N., Stähli, C., Barralet, J., van Blitterswijk, C., Habibovic, P., 2016. The Effects of Crystal Phase and Particle Morphology of Calcium Phosphates on Proliferation and Differentiation of Human Mesenchymal Stromal Cells. *Adv. Healthc. Mater.* 5, 1775–1785. doi:10.1002/adhm.201600184
- Espino, D.M., Shepherd, D.E.T., Hukins, D.W.L., 2014. Viscoelastic properties of bovine knee joint articular cartilage: dependency on thickness and loading frequency. *BMC Musculoskelet. Disord.* 15;205. doi:10.1186/1471-2474-15-205
- Fick, J.M., Espino, D.M., 2011. Articular cartilage surface rupture during compression: Investigating the effects of tissue hydration in relation to matrix health. *J. Mech. Behav. Biomed. Mater.* 4, 1311–1317. doi:10.1016/j.jmbbm.2011.04.018
- Fulcher, G.R., Hukins, D.W.L., Shepherd, D.E.T., 2009. Viscoelastic properties of bovine articular cartilage attached to subchondral bone at high frequencies. *BMC Musculoskelet. Disord.* 10;61. doi:10.1016/j.jmbbm.2011.04.018
- Gabrielsson, J., Lindberg, N.-O., Lundstedt, T., 2002. Multivariate methods in pharmaceutical applications. *J. Chemom.* 16, 141–160. doi:10.1002/cem.697
- Hu, J., Zhu, Y., Tong, H., Shen, X., Chen, L., Ran, J., 2016. A detailed study of homogeneous agarose/hydroxyapatite nanocomposites for load-bearing bone tissue. *Int. J. Biol. Macromol.* 82, 134–43. doi:10.1016/j.ijbiomac.2015.09.077
- Hukins, D.W.L., Leahy, J.C., Mathias, K.J., 1999. Biomaterials: defining the mechanical properties of natural tissues and selection of replacement materials. *J. Mater. Chem.* 9, 629–636. doi:10.1039/A807411I
- Jamshidi, P., Ma, P., Khosrowyar, K., Smith, A.M., Grover, L.M., 2012. Tailoring gel modulus using dispersed nanocrystalline hydroxyapatite. *J. Exp. Nanosci.* 7, 652–661. doi:10.1080/17458080.2012.724182
- Jethara, S.I., Patel, M.R., 2015. Optimizing Oral Controlled Release Drug Delivery Systems Using Experimental Designs. *Intellect. Prop. Rights Open Access* 3, 1–6. doi:10.4172/2375-4516.1000142
- Lawless, B.M., Barnes, S.C., Espino, D.M., Shepherd, D.E.T., 2016. Viscoelastic properties of a spinal posterior dynamic stabilisation device. *J. Mech. Behav. Biomed. Mater.* 59, 519–526. doi:10.1016/j.jmbbm.2016.03.011
- Lawless, B.M., Espino, D.M., Shepherd, D.E.T., 2017a. In vitro oxidative degradation of a spinal posterior dynamic stabilisation device. *J. Biomed. Mater. Res. Part B Appl. Biomater.* In Press, 1–8. doi:10.1002/jbm.b.33913
- Lawless, B.M., Sadeghi, H., Temple, D.K., Dhaliwal, H., Espino, D.M., Hukins, D.W.L., 2017b. Viscoelasticity of articular cartilage: Analysing the effect of induced stress and the restraint of bone in a dynamic

- environment. *J. Mech. Behav. Biomed. Mater.* 75, 293–301. doi:10.1016/j.jmbbm.2017.07.040
- Liu, Y., Lian, Q., He, J., Zhao, J., Jin, Z., Li, D., 2011. Study on the Microstructure of Human Articular Cartilage/Bone Interface. *J. Bionic Eng.* 8, 251–262. doi:10.1016/S1672-6529(11)60037-1
- Menard, K.P., 2008. *Dynamic Mechanical Analysis: A Practical Introduction*, 2nd ed. CRC press, Taylor & Francis Group, Boca Raton, Florida.
- Mennini, N., Furlanetto, S., Cirri, M., Mura, P., 2012. Quality by design approach for developing chitosan-Ca-alginate microspheres for colon delivery of celecoxib-hydroxypropyl- β -cyclodextrin-PVP complex. *Eur. J. Pharm. Biopharm.* 80, 67–75. doi:10.1016/j.ejpb.2011.08.002
- Mironi-Harpaz, I., Wang, D.Y., Venkatraman, S., Seliktar, D., 2012. Photopolymerization of cell-encapsulating hydrogels: Crosslinking efficiency versus cytotoxicity. *Acta Biomater.* 8, 1838–1848. doi:10.1016/j.actbio.2011.12.034
- Miyoshi, E., Takaya, T., Nishinari, K., 1996. Rheological and thermal studies of gel-sol transition in gellan gum aqueous solutions. *Carbohydr. Polym.* 30, 109–119. doi:10.1016/S0144-8617(96)00093-8
- Moriguchi, T., Yano, K., Nakagawa, S., Kaji, F., 2003. Elucidation of adsorption mechanism of bone-staining agent alizarin red S on hydroxyapatite by FT-IR microspectroscopy. *J. Colloid Interface Sci.* 260, 19–25. doi:10.1016/S0021-9797(02)00157-1
- Moxon, S.R., Cooke, M.E., Cox, S.C., Snow, M., Jeys, L., Jones, S.W., Smith, A.M., Grover, L.M., 2017. Suspended Manufacture of Biological Structures. *Adv. Mater.* 29. doi:10.1002/adma.201605594
- Nguyen, L.H., Kudva, A.K., Saxena, N.S., Roy, K., 2011. Engineering articular cartilage with spatially-varying matrix composition and mechanical properties from a single stem cell population using a multi-layered hydrogel. *Biomaterials* 32, 6946–52. doi:10.1016/j.biomaterials.2011.06.014
- Osmalek, T., Froelich, A., Tasarek, S., 2014. Application of gellan gum in pharmacy and medicine. *Int. J. Pharm.* 466, 328–40. doi:10.1016/j.ijpharm.2014.03.038
- Parmar, P.A., Chow, L.W., St-Pierre, J.-P., Horejs, C.-M., Peng, Y.Y., Werkmeister, J.A., Ramshaw, J.A.M., Stevens, M.M., 2015. Collagen-mimetic peptide-modifiable hydrogels for articular cartilage regeneration. *Biomaterials* 54, 213–225. doi:10.1016/j.biomaterials.2015.02.079
- Pearson, B., Espino, D.M., 2013. Effect of hydration on the frequency-dependent viscoelastic properties of articular cartilage. *Proc. Inst. Mech. Eng. H* 227, 1246–52. doi:10.1177/0954411913501294
- Radin, E., Yang, K., Riegger, C., Kish, V., O'Connor, J., 1991. Relationship between lower limb dynamics and knee joint pain. *J. Orthop. Res.* 9, 398–405.
- Sadeghi, H., Lawless, B.M., Espino, D.M., Shepherd, D.E.T., 2018. Effect of frequency on crack growth in articular cartilage. *J. Mech. Behav. Biomed. Mater.* 77, 40–46.
- Sadeghi, H., Shepherd, D.E.T., Espino, D.M., 2015. Effect of the variation of loading frequency on surface failure of bovine articular cartilage. *Osteoarthr. Cartil.* 23, 2252–2258. doi:10.1016/j.joca.2015.06.002
- Shepherd, D.E.T., Seedhom, B.B., 1997. A technique for measuring the compressive modulus of articular cartilage under physiological loading rates with preliminary results. *Proc. Inst. Mech. Eng. H* 211, 155–165. doi:10.1243/0954411971534278
- Suzawa, Y., Kubo, N., Iwai, S., Yura, Y., Ohgushi, H., Akashi, M., 2015. Biomaterial/agarose composite gels enhance proliferation of mesenchymal stem cells with osteogenic capability. *Int. J. Mol. Sci.* 16, 14245–14258. doi:10.3390/ijms160614245
- Tamaddon, M., Czernuska, J.T., 2013. Critical review The need for hierarchical scaffolds in bone tissue engineering 2, 1–8.
- Temple, D.K., Cederlund, A.A., Lawless, B.M., Aspden, R.M., Espino, D.M., 2016. Viscoelastic properties of human and bovine articular cartilage: a comparison of frequency-dependent trends. *BMC Musculoskelet. Disord.* 17;419. doi:10.1186/s12891-016-1279-1
- Vincent, J.F. V., 2012. Structural biomaterials, *Applied Biophysics*. doi:10.1002/9780470513156.ch15
- Wands, I., Shepherd, D.E.T., Hukins, D.W.L., 2008. Viscoelastic properties of composites of calcium alginate and hydroxyapatite. *J. Mater. Sci. Mater. Med.* 19, 2417–21. doi:10.1007/s10856-007-3364-3
- Zhang, L.-M., Wu, C.-X., Huang, J.-Y., Peng, X.-H., Chen, P., Tang, S.-Q., 2012. Synthesis and characterization of a degradable composite agarose/HA hydrogel. *Carbohydr. Polym.* 88, 1445–1452. doi:10.1016/j.carbpol.2012.02.050

Figure 1: The effect of changing hydrogel and HA composition on Young's modulus and rupture stress, representative curves of boundary compositions for both mineralised (A) agarose (AG) and (B) gellan (GG) constructs

Figure 2: The effect of changing hydrogel and HA composition on complex modulus (MPa) and loss factor ($\tan \delta$) with a) agarose and HA composition stiffness, b) gellan and HA composition complex modulus, c) agarose and HA loss factor and d) gellan and HA loss factor. Data represented as mean $\pm$ standard deviation ($n=5$).

Figure 3: A comparison of healthy human cartilage (HC) behaviour with synthetic analogues over a frequency range approximating normal human movement, with regards to A) complex modulus (E^*) and B) loss factor ($\tan \delta$). Data represented as mean $\pm$ standard deviation ($n=5$).

Figure 4: Micro-CT scans of mineralised hydrogels and human tissue, showing a) optimised agarose and gellan compositions b) the cartilage/bone interface and c) mineral particle sizing. Scale bars = 300 μm

Figure 5: Typical fluorescence micrographs illustrating the viability of MC3T3 osteoblast precursor cells after 1, 3, and 7 days of culture on optimised mineralised hydrogel compositions

Supplementary Information

Table 1. Summary of agarose/HA compositions tested

% w/v agarose	% w/v HA			
	0	0.5	12.75	25
2	x		x	
2.8		x	x	x
3.5	x		x	
4.3		x	x	x
5		x	x	x
5.8	x	x	x	x
6.5	x		x	

Table 2. Summary of gellan/HA compositions tested

% w/v gellan	% w/v HA				
	0	0.5	6	12.75	15*
1.5		x		x	
2.1		x	x	x	
2.8				x	
3			x		x
3.4		x	x	x	
4	x	x		x	
6	x				

* % w/v HA greater than 15% were not investigated as 15% was found to be the upper limit for successful homogeneous HA particle incorporation. Above 15%, composite homogeneity could not be maintained.