Predicting bone formation in MSC seeded hydrogels using experiment based mathematical modelling

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Abstract

In vitro bone formation by mesenchymal stromal cells encapsulated in type-1 collagen hydrogels is demonstrated after a 28-day *in vitro* culture period. Analysis of the hydrogels is carried out by X-ray micro computed tomography, histology and immunohistochemistry, which collectively demonstrates that bone formation in the hydrogels was quantifiably proportional to the initial collagen concentration, and subsequently the population density of seeded cells. This was established by varying the initial collagen concentration at a constant cell seeding density ($3x10^5$cells/0.3ml hydrogel), and separately varying cell seeding density at a constant collagen concentration (1mg/ml). Using this data, a mathematical model is presented for the total hydrogel volume and mineralisation volume based on the observed linear contraction dynamics of cell-seeded collagen gels. The model parameters are fitted by comparing the predictions of the mathematical model for the hydrogel and mineralized volumes at day 28 with the experimental data. The model is then used to predict the hydrogel and mineralisation volumes for a range of hydrogel collagen concentrations and cell seeding densities, providing comprehensive input/output descriptors for generating mineralised hydrogels for bone tissue engineering. It is proposed that this quantitative approach will be a useful tool for gerenating *in vitro* manufactured bone tissue, defining input parameters that yield predictable output measures of tissue maturation.

Abbreviations: MSC –Mesenchymal Stromal Cell, CN – (initial) cell seeding number, CC – (initial) collagen concentration, μCT – micro computed tomography,

Statement of impact

This article describes a simple yet powerful quantitative description of in vitro tissue engineered bone by combining experimental data with mathematical modelling. The overall aim of the paper is to examine what is currently known about cell mediated collagen contraction, and demonstrate that this phenomenon can be exploited to tailor bone formation by choosing a specific set of input parameters in the form of cell seeding density and collagen hydrogel concentration. Our study utilises a clinically relevant cell source (human mesenchymal stem cells) with a biomaterial that has received regulatory approval for use in humans (collagen type 1), and hence could be useful for clinical applications, as well as furthering our understanding of cell/extracellular matrix interactions in determining in vitro bone tissue formation.

Introduction

The field of orthopaedic tissue engineering aims to replace and regenerate bone tissues that have become damaged through age, trauma and disease. *In vitro* engineered bone could lead to a significant step forward in addressing the currently limited gold standard treatments in orthopaedic medicine[1]. Treatment limitations include a lack of permanent bone implant procedures that result in a complete reintegration of implants with native tissue, without the risk of implant failure or donor site morbidity from autologous bone grafts[2]. One promising treatment strategy to overcome these limitations involves the *in vitro* expansion of human mesenchymal stromal cells (MSCs) followed by osteogenic preconditioning in biomaterial scaffolds prior to implantation. MSCs are multipotent progenitor cells that proliferate rapidly[11] and are easily isolated from different sources such as bone marrow or lipo-aspirates[3]. Collagen type 1 is the most abundant organic component of mammalian tissue, accounting for 20-30% of the body's total protein content[4] , and up to 90% of the organic phase

of *in vivo* bone tissue[5]. Additionally, collagen has demonstrated safety and efficacy as a bone substitute in humans when used in combination with osteo-inductive growth factors[6]. This ongoing proof of efficacy has established cell seeded collagen hydrogels as a promising candidate approach for bone regeneration, which has been shown in a number of animal studies to be effective in regenerating bone defects[7, 8, 9].

Experimental studies continue to demonstrate the clinical relevance of cell seeded collagen hydrogels for bone tissue engineering. However, there is a lack of standardised protocols by which tissue engineers can repeatable generate *in vitro* bone constructs with known levels of tissue formation. Mathematical modelling in combination with experimental approaches has the potential to address this standardisation challenge by providing insights into the factors regulating tissue growth, and information that cannot be obtained from complex *in vitro* experiments alone. Additionally, mathematical models allow candidate experimental protocols to be tested more quickly and cheaply than by performing expensive and time-consuming *in vitro* experiments. There are many theoretical models of hydrogel formation[10] as well as mathematical models of culture, cell growth and fluid and solute transport in a variety of settings[11]. Multiphase modelling approaches, in which the tissue constituents are represented by a mixture of continua that occupy the same region of space, have been employed to investigate the impact of mechanical forces due to fluid flow in perfusion bioreactors on tissue growth and composition[12, 13, 14]. Theoretical models have also been developed to interrogate the relationship between tissue growth, mineralization, and hydrostatic pressure in a hydrostatic pressure bioreactor[15]. Recent reviews of mathematical models based on different *in vivo* physiological hypotheses (e.g. mechanical loading mediated stem cell differentiation) can be found in[16] and[17]. However, it is an open question whether these hypotheses are valid for *in vitro* tissue engineering studies[18]. Predictive mathematical models quantifying the relationship between experimental inputs and outputs

would be valuable tools to help determine suitable experimental protocols in studies of *in vitro* bone formation. To address this need, a combined quantitative modelling and experimental approach is presented to predict *in vitro* bone formation in self-assembling MSC/collagen hydrogels. Key determinants of mineralisation in an MSC cell seeded collagen hydrogel using X-ray micro computed tomography (μCT) are defined. The relationship between hydrogel volume and mineralisation volumes after the four-week culture period was determined as a function of the initial collagen concentration (CC) in hydrogels fabricated with an initial cell number (CN) of $3x10^5$ cells/hydrogel. In addition to this, the initial CN was varied at a constant CC of 1ml/ml. Simple assumptions were used to extrapolate beyond the experimental data to describe how the hydrogel and mineralisation volumes depend on experimental inputs CN and CC. The model was fitted using the experimental data, and then used to quantify bone formation in MSC seeded collagen hydrogels across a full range of CC and CN input parameters.

Results

Volume analysis

After an initial period of 24 hrs to allow for cell mediated contraction, the hydrogel volume for each group remained roughly the same for the duration of the experiment. The hydrogels were maintained in osteogenic culture media for 28 days before fixation and analysis using μCT. Assessment of hydrogel volume by μCT after 28 days (**Fig1)** shows a linear dependence of hydrogel volume on CC (**Fig1A, R^2=0.99)**. Lower initial CCs results in a much smaller final gel volume after the initial 24hrs contraction period. Hydrogel volume was less dependent on CN (**Fig1B, R^2=0.48)**, with a small linear decrease in volume as CN is increased (estimated as decreasing by $0.23mm^3/10^5$ cells). By contrast, varying the CC leads to a change in volume of $5.77\pm0.23mm^3$/mg collagen, which shows that changing CC

compared to the CN is more effective for generating hydrogels at a desired volume. Bright field images of the hydrogels (**Fig1C&D)** shows the change in volume for different experimental groups. The hydrogel at day 28 was approximately homogenous with a uniform cell distribution irrespective of the seeding parameters (**Supplementary Fig1**).

Mineralisation analysis

Evaluating the volume of the hydrogels at low vs high x-ray attenuation thresholds, the volume of mineralised phase relative to the total volume of the hydrogels was determined. Quantification of the mineralised volume phase shows a linear correlation between increasing mineralised volume and increasing CC (**Fig 2A, R^2=0.99**). Increasing CN result in a negligible change in the mineralised volume with increasing CN (**Fig 2B, R^2=0.66**). In contrast to **Fig 2A,** by considering the volume of mineralised tissue with respect to the total hydrogel volume, a decrease in the volume fraction of mineralised tissue is observed with increasing CC via an inverse power law (**Fig 2C, R^2=0.94)**. μCT reconstructions of the mineralising phase demonstrated an increase in the percentage mineralisation of the hydrogels (**Fig 2E,** indicated by solid grey regions) as CC decreased. Increasing the initial CN at a constant CC increased the volume fraction of mineralisation tissue linearly (**Fig 2D, R^2=0.77)**. The change in mineralisation with respect to CN groups is easily visualised in gels with different CNs, with prominent areas of mineralisation visible on the gel periphery at a higher CN (**Fig 2F**).

Density analysis

Hydrogel density after 28 days is dependent on both CC and CN and follows the same trends as in **Fig2 (Fig 3A, R^2=0.74; Fig 3B, R^2=0.72)**. μCT image reconstructions visualises the local density through a representative cross section of the hydrogels (**Fig 3C**). The colour heat map depicts soft tissue in blue progressing through to green and finally red, representing

densely mineralised tissue (units expressed as $mgHA/mm^3$). An initial CC of 0.5mg/ml (**Fig 3C lhs)** shows densely mineralised (red) tissue throughout, whereas an initial CC of 5mg/ml is considerably less dense (blue-green). Density reconstructions of hydrogels in **Fig 3C, rhs** show changes in hydrogel density between high CN ($3x10^5$cells, right) and low CN ($1x10^4$cells, left), and further demonstrates that changes in the initial CC results yields a much higher level of control of hydrogel mineralisation compared with change changes in the initial CN.

Quantitative image analysis

Collectively, analysis by µCT indicates that the observed changes in mineralisation are a manifestation of the cell population density of individual hydrogels, i.e. if the same number of cells are present in a smaller volume the hydrogel has a higher final percentage mineralisation. Quantitative image analysis of haematoxylin and eosin stained paraffin embedded tissue sections (**Supplementary Fig1)** shows the total cell number within a given area to be directly proportional to the trends observed in µCT analysis (**Figure 4A R^2=0.99, 4B R^2=0.93)**. This indicates that the measured changes in hydrogel mineralisation by µCT is likely a manifestation of mineralisation in hydrogels with different cell densities. The total number of cells in each group is estimated using **Figure 4A&B,** on the assumption that each data point is representative of a 10µm thick histology section occupying a normalised area of $1mm^2$. Scaling up for each respective hydrogel volume, the total cell number in CC dependent groups is roughly equal **(Fig 4C)**. Analogously, increasing the CN produced an increase in total number of cells in the hydrogels **(Fig 4D).** Histology staining of bone mineral deposition using the Von Kossa method (**Supplementary Fig1A)**, together with immunohistochemistry (IHC) staining of organic protein composition in the hydrogels

(supplementary Fig2A), reveals that in densely populated hydrogels, the ECM is rich in osteocalcin. In addition to this, cells were interconnected with connexin 43 gap junctions **(supplementary Fig2B**, which is highly expressed in osteocytes in mature bone *in vivo*[19]. These findings provide two key assumption which can be used in formulating the mathematical model described in the next section; first, that the initial linear contraction dynamics of the collagen gels is a key determinate of subsequent bone tissue formation (as shown in **Figures 1-3**). Second, that cell number between the different groups is constant throughout the experiment (as shown in **Fig 4)**.

Predictive Mathematical Modelling

Based on the findings presented above, a data-driven mathematical model was developed to capture the mineralization process in the hydrogel as a function of initial CN and CC. We consider a homogeneous mixture of cells, collagen, and mineral in a volume V. It is assumed that that there is a short initial period of contraction, after which the total volume of the hydrogel is fixed, and so take this volume to be constant in time. We assume the volume is composed of a non-mineralised gel phase, V_g, and a mineral phase, V_m, where the volume of cells and other solutes is negligible, so $V=V_g+V_m$. Initially there is no mineralization, and hence at the start of the experiment we have $V=V_g$. As the total volume is fixed, this translates to a mineralization phase, which gradually replaces the gel phase over time.

The experimental data is used to inform the choice of constitute laws relating the dependence of each of V and V_m on CC and CN. Considering first the constitutive law for V, we see that there is a strong dependence of the hydrogel volume V on CC, whereas there is little dependence of the hydrogel volume on CN (**Fig1**). Thus it is assumed that V is linearly proportional to the CC, that is $V=\alpha g+\beta$, where g is the CC measured in mg/mm^3, and α and

β are constants having units of mm^6/mg and mm^3 respectively. Turning next to the constitutive relationship for V_m, it is seen from **Fig 5** that there is a strong relationship between V_m and CC (for fixed CN), and for fixed CC there is a weak dependence of $V_m$on CN. Additionally, it is assumed that collagen must be present for mineralisation to occur. This leads to a candidate constitutive law of the form

$V_m = \gamma g N + \delta g$, Where $\gamma$is in units m^6/ (10^5 cells mg), N is the CN measured in units of 10^5 cells and$\delta$is in units mm^6/mg. The δg term represents the impact of increasing collagen density in a hydrogel seeded with very few cells, though we do anticipate errors in this constitutive relation below some minimum threshold of cells necessary to initiate contraction and mineralisation.

We fit the steady state mineralised volume $V_m$and total hydrogel volume V to the experimental data in (**Fig5**) is used to estimate the parametersα , β , γ, and δ using the reported values of CC and CN, and we note a relatively small mean-squared error (MSEs). We fit the model for V andV_m to the experiemntal data for these quantities - in **Fig5A-D**. We note that the experimental data in **Fig5E&F** reflects the data shown in **Fig2C&D** showing V_m/V.

The data-driven model can be used to predict the volume of hydrogel and volume of mineralisation for values of CN and CC beyond those considered in the reported experiments (**Fig6)**. In Fig 6 it is shown how the volume, mineralised volume, and hence the percentage of mineralisation vary with N and g. For a given desired hydrogel volume and mineralisation volume (or equivalently, percent mineralization) these predictions can be exploited to select the appropriate the values of the CC and CN. For instance, if it is desirable to achieve approximately 30% mineralization of the construct, then the third contoured region (from the bottom-right) in (**Fig6C)** gives a region in which this mineralization is achieved. Then (**Fig6A)** can be used to pick a final hydrogel volume by selecting values of CN and CC that

lie within this contour and achieve the desired volume. Similar remarks can be made about selecting parameters to achieve particular mineralization densities.

Discussion

This paper details a quantitative description of *in vitro* bone formation in cell-seeded hydrogels measured by μCT analysis of hydrogel volume, mineralisation and density using CC and CN as input parameters. Histological assessment of mineral and protein production by encapsulated cells resembled the natural composition of bone, but also demonstrated that the measured changes in mineralisation were directly proportional to the population density of encapsulated MSCs. The experimental insights are used as the basis for the development of a simple mathematical model that describes hydrogel maturation as a function of the input parameters beyond the experimentally measured regimes. This combined experimental and theoretical approach could be useful for a number of different future applications. For example, from a tissue manufacturing perspective, the quantitative input x (e.g. CC or CN) → output y (hydrogel volume, degree of mineralisation and or density), could be used to define quality control parameters for large-scale manufacturing. Since these methods employ μCT as a primary assessment tool, it yields the potential to non-destructively screen batches for optimal properties based on mineralisation and density, thus minimising batch-to-batch variation without compromising construct integrity. In addition to this, from a research perspective, our results can be useful when considering future experiments to tailor the size and mineralisation of MSC seeded hydrogels. Previous work developing models of fracture repair using embryonic chick femurs have employed cell encapsulated alginate hydrogels[20, 21]. Whilst these studies provide promising preclinical data for potential cell therapies, predetermined characteristics of the scaffold design were not explored, increasing the likelihood of experimental error due to biological variability. The model presented in this

study has since been implemented for these scaffolds to generate an accurately scaled *ex vivo* femur defect model from which we are investigating scaffold pre-culture regimes and their effect on bone regeneration (data not published). Recently we have published new approaches to metrics for scaffold behaviour *in vitro* and *in vivo* using a standardised tissue turnover index[22].

This study also provides a useful framework for future studies aiming to provide mechanistic insights into different stem cell phenotypes. It would be interesting for example to study different cell sources in parallel to generate comparative data on changes in the contractility of MSCs residing in different *in vivo* niches such as bone marrow, adipose tissue or dental pulp. MSC biology has been found to vary between different organ niches, which plausibly directly affects the therapeutic potential of future treatments[23]. In addition to this, the mechanical stimulation of cells in bioreactors for tissue engineering has been shown to be a key factor in subsequent tissue maturation[24, 25] with a number of dynamic protocols for generating tissue engineered bone established[26, 27]. Within these systems mathematical modelling has been employed to characterise the mechanical milieu within a cell seeded hydrogel exposed to externally applied hydrostatic pressure[28], and these models have been proposed to potentially predict *in vitro* bone formation[15]. Thus, for example, the volume predictors in **Fig 5** could be used to study how changes in the mechanical field reflect changes in bone formation as a result of hydrostatic stimulation, which is well known to have a positive influence on osteogenic differentiation of MSCs during *in vitro* culture[29, 30, 31].

This data driven model is able to predict steady state values for hydrogel volume and mineralised volume. The availability of more complex spatiotemporal experimental data would permit the development of more sophisticated mathematical models, able to capture how the spatial development of hydrogel mineralization during the experimental timeframe. Such a model could for example be utilised to determine the experimental values of CC and

CN ensuring that the mineralisation process occurs on a desired timescale. Although, the data-driven model presented here is unable to provide mechanistic insight into the complex interplay between collagen and MSC leading to mineralisation, experimental maps of the spatial distribution of mineralised content throughout the hydrogel would permit the development of multiphase mechanistic mathematical models. These models would allow candidate hypotheses for the nature of cell-collagen interactions leading to mineralisation to be tested.

Collagen itself has long been known to follow very conserved contractile kinetics, owing to a persistence length which leads to viscoelastic, semi-flexible polymer like behaviour[32, 33]. We have previously reported an extreme case of this, which predicts at sufficiently high concentrations, collagen undergoes spontaneous liquid crystal self-assembly[34, 35]. The present findings demonstrate that the association between the input and output parameters manifests from the conserved contraction kinetics of type 1 collagen. This idea in itself has been reported previously. A recent study investigated the contraction kinetics of different populations of fibroblasts in 3D collagen lattices to model different cross-sectional cellular distributions following gel contraction[36]. Their simulations have multiple comparative similarities to our data, first in terms of the relative change in size of the hydrogels between experimental groups, but also in terms of cell distribution, predicting nodal clustering of cells at high CN **(Supplementary Fig1)**. Whilst such investigations of cell mediated contraction are common in the literature[37, 38, 39], few studies have quantified how the initial contraction dynamics of these gels determines tissue formation after an extended *in vitro* culture. Furthermore, implementation of μCT provides a quantitative assessment of the size and physical characteristics of the matured hydrogels. A previous study of the effect of CN and CC on chondrogenesis in hMSC seeded collagen microspheres established a direct correlation between CN, CC and chondrogenic tissue maturation[40]. In agreement with these

findings, their results showed a positive correlation between increasing CN and chondrogenesis. However, in contrast to this work, a negative correlation between lower CCs and chondrogenesis is reported, suggesting that MSC fate can be significantly re-routed by a delicate balance between chemical signals and the mechanical environment of the surrounding ECM. It has previously been reported that spontaneous mineralisation of acellular collagen based hydrogels can occur via precipitation and nucleation of constituents in cell culture media[41]. However, it is unlikely in this experimental setup to have a significant impact on the observed mineralisation due to the nature of fabrication in the hydrogels. The fabrication of the hydrogels relies on cell-mediated attachment contraction of collagen fibrils under mildly acidic conditions, followed by transferral to neutral buffered culture media after 24hrs. Hence in acellular conditions, fibrillogenesis would not occur and the collagen fibrils remain in solution. In addition to this, the observed mineralisation after 4 weeks measured by μCT is found to be directly proportional to the number of cells/mm^2 in both CC and CN groups (**Supplementary Fig2B)**, strongly inferring cell mediated as opposed to spontaneous hydrogel mineralisation.

In conclusion, this study demonstrates a quantitative *in vitro* culture platform for generating tissue-engineered bone constructs using a clinically relevant cell source and clinically approved ECM scaffold. Developing such methodologies could provide a valuable resource for researchers and industry, allowing standardized, reproducible tissue constructs that can be used to test different biochemical or mechanical stimuli. In addition to this, the development of mathematical models that accurately describe *in vitro* bone formation will help realise the implementation of tissue engineering strategies in the clinic. By generalising insights from simple experiments to larger parameter regimes, costly and exhaustive therapies can be streamlined, reaching the clinic more efficiently with more impactful patient outcomes.

Methods

MSC/Collagen hydrogel fabrication

Human bone marrow derived mesenchymal stromal cells were isolated from a bone marrow aspirate (Lonza) by plastic adherence. Adherent MSC colonies were expanded in proliferation media (DMEM, 10%FBS (Bio sera), 2% Pen-Streptomycin (Lonza), 1% L-glutamine (Lonza), 1% Non-essential amino acids(Sigma)) until passage 5 before encapsulation in collagen. Collagen hydrogels of concentration (0.5-5mg/ml) were fabricated by diluting cells suspended in proliferation media (PM) in a stock solution (9.22mg/ml) of acidic type-I rat tail collagen (BD Biosciences, UK). 0.3ml MSC/collagen solutions were pipetted into non-adherent round bottom 96 well plates. After 24hrs, contracted hydrogels were transferred to 6 well cell culture inserts (Corning). Hydrogels were maintained in osteogenic induction media (OM) (PM above + 150μg/ml ascorbic acid, 10^{-8}M dexamethasone and 2mM sodium β-glycerophosphate) under static culture conditions (37°C with 5%CO_2) for 28 days. After 28 days, hydrogels were washed twice in PBS and fixed in neutrally buffered formalin for 1 hr at room temperature followed by two 15 minute washes in PBS.

X-ray micro tomography

Hydrogel volume and density was measured by X-ray micro tomography (μCT) using a Scanco μCT40 (beam energy: 55 kVp, beam intensity: 145 μA, 200 ms integration time, spatial resolution: 10 μm). Each sample was analysed at two different x-ray attenuation thresholds (low and high), firstly to determine the total (volume) of the construct, then

separately at a higher attenuation threshold to determine the volume of the denser mineralising phase. The selection high and low thresholds are based on previously published data investigating the mineralisation of developing *ex vivo* chick femurs[28, 27]. Total hydrogel volume is given in units of mm^3 and hydrogel density in units of mg-Hydroxyapatite/mm^3, which is a pre calibrated according to the manufacturer.

Quantitative Image analysis

ImageJ was used to quantify the average number of cells per unit area as a measure of cellular distribution between experimental groups. The number of cells within a given area was counted on three separate gel sections using a cell counter plugin in ImageJ, with the corresponding area calibrated to measure in mm^2. The final values were normalised to cells/mm^2, allowing for direct comparison of cellular distribution between different gel concentrations and seeding densities. We then calculated the total cell number in each hydrogel using the formula $\left(\frac{cells}{0.01\,mm^3}\right) * total\ hydrogel\ volume$.

Data & statistical analysis

Experimental results were plotted in OriginPro 9.0 with statistical analysis performed using linear and non-linear iterative fitting algorithms where appropriate. Standard error of the mean is shown in all figures. Fitting of the mathematical models was done using linear regression and plotted in MATLAB.

Additional information:

Supplementary methods and results are available in the supporting information, provided with this manuscript. Raw data for all experiments performed are available on request.

Competing financial interests

The authors declare no competing financial interests.

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Author contributions

J.C.P (A[1,4]), A.K (B[2]), S.W(C[2]) and A.J.E.H (D[1,3]) conceived and initiated the work. J.C.P (A[1,4]) performed experimental work and prepared main text and supplementary figures. A.K B[2] performed the modelling work editing and revisions performed by A.K B[2], S.W(C[2]) and A.J.E.H (D[1,4]).

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