Bio-inspired design of a magnetically active trilayered scaffold for cartilage tissue engineering

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Abstract

An important topic in cartilage tissue engineering is the development of biomimetic scaffolds which mimic the depth-dependent material properties of the native tissue. We describe an advanced trilayered nanocomposite hydrogel (ferrogel) with a gradient in compressive modulus from the top to the bottom layers (p < 0.05) of the construct. Further, the scaffold was able to respond to remote external stimulation, exhibiting an elastic, depth-dependent strain gradient. When bovine chondrocytes were seeded into the ferrogels and cultured for up to 14 days, there was good cell viability and a biochemical gradient was measured with sulphated glycosaminoglycan increasing with depth from the surface. This novel construct provides tremendous scope for tailoring location-specific cartilage replacement tissue; by varying the density of magnetic nanoparticles, concentration of base hydrogel and number of cells, physiologically relevant depth-dependent gradients may be attained. © 2015 The Authors Journal of Tissue Engineering and Regenerative Medicine Published by John Wiley & Sons Ltd.

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Articular cartilage has an exquisite structural design, comprised of distinct zones (superficial, middle and deep), allowing it to absorb joint loads and provide very low-friction articulation over a lifetime (Brady et al., 2015). When articular cartilage is lost, due to trauma or disease, engineering design suggests that replacement cartilage should mimic the features found in native tissue (Zhang, 2012). Articular cartilage displays depth-dependent non-homogeneity: from the articular surface, there is an increasing biochemical gradient of, for example, increasing sulphated glycosaminoglycan (sGAG) (Nguyen et al., 2011) and a corresponding gradient of mechanical properties, with the compressive modulus increasing from the articular surface to the deep zone (Schinagl et al., 1997). Under loading, a strain gradient also exists; the superficial layer absorbs more strain than the middle and deep layers (Chen et al., 2001; Erne et al., 2005; Neu et al., 2005). To engineer new cartilage recapitulating these complex depth-dependent gradients while directing cell fate using remote signalling cues, advanced functional nanocomposite materials are required. Here, we propose the use of magnetic nanoparticles (MNPs), since magnetic forces offer a promising approach for directing cellular and matrix organization, conferring the major advantage of remote application (Torres-Lugo and Rinaldi, 2013; Fayol et al., 2013). We envision a novel construct for cartilage tissue engineering (TE) applications, in which MNPs and cells are embedded in layers of a trilaminar scaffold to produce an advanced smart nanocomposite biomaterial with stiffness and biochemical gradients, and which can respond to a remote external magnetic field (see supporting information Figure S1) to experience a strain gradient. Spatial variation of applied loading can be achieved by tailoring the concentration of MNPs, the strength of the magnetic field and the local stiffness of the material.

We used a hydrogel as the base material, due to its biocompatibility, ease of fabrication and tunable physical, chemical and biological properties (Annabi et al., 2014). We first engineered a trilayered agarose (type VII; Sigma-Aldrich, UK) construct with a stiffness gradient to provide...
a starting point for the nanocomposite hydrogel. The stiffness gradient was achieved by varying the concentrations of agarose in the top, middle and bottom layers (1, 2 and 3 wt%, respectively). Partial thermal setting (optimal partial setting time = 2 min) enabled integration of the layers; the method of partial thermal setting of agarose can be found in Supplementary materials and methods (see supporting information Figure S2). The measured compressive moduli of the individual layers (top to bottom) were 33 ± 2.8, 127 ± 14 and 278 ± 9 kPa. The overall compressive modulus of the trilayered scaffold was 64 ± 4 kPa, close to the theoretical value of 71.8 kPa computed from individual layer stiffnesses and thicknesses.

Our next objective was to integrate commercially available, dextran-coated MNPs (Micromod, Rostock, Germany; product code 84-00-102; average particle size 100 nm and starting density 25 mg/ml) into the distinct layers of the biomimetic agarose gel to create a trilayered ferrogel. Dextran-coated MNPs have been shown to be fully biocompatible (Wisselader et al., 1989), are extensively used in MRI applications and, in 1996, the United States Food and Drug Administration (FDA) approved the human use of these carbohydrate-coated, nanoparticle-based, iron oxide imaging agents for the treatment of liver lesions (Tassa et al., 2011). Further, after implantation, any MNPs released are treated as foreign substances and are readily removed from the body; smaller particles (ca. 10 nm) are taken up by the reticuloendothelial cells throughout the body and larger particles (ca. 30 nm) are rapidly collected by the liver and spleen (Pankhurst, 2006).

To determine the relative number of MNPs required in each layer, we first performed a theoretical analysis of the mechanics of a trilayered hydrogel to determine local strains. The total force/unit volume, \( F_z \), acting on a gel in a magnetic field, is (Zhang et al., 2009):

\[
F_z(z) = \left( \frac{Z}{\mu_0} \right) \phi_{MNP} V_{MNP} B(z) \frac{dB}{dz} (z) \tag{1}
\]

where \( \mu_0 \) is the permeability of free space, \( B(z) \) is magnetic field strength, \( \chi \) is magnetic susceptibility, \( \phi_{MNP} \) is the nanoparticle volume fraction and \( V_{MNP} \) is the volume of a single MNP. Considering a trilaminar construct with three distinct layers, basic mechanics yields the following equations for the stress (\( \sigma \)) and strain (\( \varepsilon \)) in each layer \( j = \{1,2,3\} \) (surface, middle and bottom, respectively):

\[
\sigma_j(z) = F_z(z)(z_{j,upper} - z) + \sigma_{j-1}(z_{j,upper}); \sigma_j(z) = \sigma_j(z) \frac{E_j}{E_j} \tag{2}
\]

where \( z_{j,upper} \) refers to the location of the upper surface of layer \( j \) and \( \sigma_0 = 0 \). Given local material stiffnesses (\( E_j \)), external magnetic field strength and gradient, the ratio of MNP volume fractions between layers was calculated. Physiologically relevant target strains were obtained from the literature; under physiological loading of explants (1.29–2.57 kPa), strain varies non-linearly with depth, with maximum amplitude of 11% at the joint surface (Neu et al., 2005). Further, embedded chondrocytes in the superficial zone experience 19.1% deformation, whereas middle and deep zone chondrocytes experience 14.8% and 15.7%, respectively (Erne et al., 2005). With these values, suitable volume percentages of MNPs in the surface, middle and deep layers were determined to be 20, 7 and 10 wt%, respectively.

The blending method was used to fabricate each layer, since this method can be easily adapted to incorporate live cells. This involved sequential preparation of MNPs and the liquid hydrogel precursor solution; the MNPs and liquid hydrogel were then mixed and crosslinked, resulting in encapsulation of the MNPs in the hydrogel (Li et al., 2013). Briefly, for acellular constructs, the MNP solution was filtered using 0.45 and 0.2 \( \mu \)m sterile filters, and then centrifuged for 90 min at 4000 rpm to obtain a pellet of known concentration. The supernatant was removed and the pellets resuspended in working volumes of growth medium [GM; Dulbecco’s modified Eagle’s medium (DMEM)-high glucose; 10% fetal bovine serum and 1% penicillin–streptomycin (P/S; all from Sigma)] to obtain the required MNP wt%, which was subsequently mixed with the required concentration of liquid agarose.

Using 3D light microscopy, we assessed the distribution of MNP aggregates throughout the acellular matrices. Briefly, samples of different MNP weight percentages were prepared, fixed and imaged; 3D reconstruction enabled qualitative analysis of the distribution of MNP aggregates throughout the depth and breadth of the scaffold. Single sections (x-y plane) of blank agarose gel and 1 and 5 wt% MNP-agarose gels (Figure 1A–C) showed a relatively uniform distribution of MNP aggregates. Further, a z-stack of all sections showed fairly uniform distribution of MNP aggregates throughout the hydrogel (Figure 1D, showing representative 3D renditions of 1 and 2 wt% MNP aggregate distribution, in 1 wt% agarose, after image post-processing with Matlab and Amira software). Increasing the weight percentage of MNPs produced smaller aggregates, which appeared more uniformly distributed (movie files of serial sections through gels with higher MNP concentration are available in the supporting information, Movies S1–S4). This analysis established that the blending method enabled the successful integration and distribution of dextran-coated MNPs in agarose throughout the breadth and depth of the newly formed ferrogels.

We next quantified the magnetocompressive response of acellular ferrogels to an external magnetic field, which were expected to depend primarily on three parameters: magnetic field strength, MNP density and agarose wt% (the latter determining the stiffness of the matrix). Rare earth NdFeB magnets (Grade N42; E-Magnets, UK) were used to apply the magnetic field (0.4, 0.5 or 0.75 T). Engineering strain, defined as change in thickness over original thickness (\( AL/L \)), was used as a measure of magnetocompression. First, magnetic field strength (0.5 T) was held constant with MNP density varied (0–10 wt%), showing an approximately linear correlation between strain and MNP density for both 1 and 2 wt% agarose (Figure 1E). Next, with MNP density (10 wt%) and agarose concentration (1 wt%) constant, strain was...
Figure 1. (A–C) Distribution of MNP aggregates (0, 1 and 5 wt% MNPs) in single histological sections of acellular 1 wt% agarose ferrogels fabricated using the blending method. Note that individual MNPs are not visible by light microscopy; thus, we observed aggregates. (D) 3D visualization of MNP aggregate distributions (left, 1 wt%; right, 2 wt%) in agarose after image post-processing to remove blank agarose. The magnetocompressive behaviour of acellular ferrogels was investigated as follows. (E) Increasing MNP density in acellular ferrogels of different agarose wt% increases strain (mean ± SD: n = 3 experiments); the slopes of the regression lines were 2.147/wt% and 0.186/wt% for the 1 and 2 wt% agarose gels, respectively (both different from 0; p < 0.0001). (F) Increasing magnetic field strength (agarose 1 and 10 wt% MNP) increased strain. (G) After removal of the magnetic field, shape recovery was evaluated; acellular ferrogels (1 wt% agarose) of different MNP wt% all returned to within ± 0.02 strain. Error bars = SD

shown to depend on magnetic field strength (Figure 1F). We then asked whether ferrogels would return to their original thickness upon removal of the external magnetic field. Shape recovery was observed (Figure 1G) for ferrogels (1 wt% agarose) of different MNP densities (1, 5, 10 and 20 wt%), with all ferrogels returning to their original thickness ± 2% strain. Regression analysis and testing the slope of the regression line were used for statistical significance; the results are expressed as mean ± SD of three separate experiments.

The final objective of this work was to seed cells into the ferrogels and characterize cell viability, biosynthesis of the cartilage ECM component sGAG, magnetocompression (strain) and compressive modulus (stiffness) for each layer. Bovine chondrocytes (BCs) were isolated and provided by Dr Seth McCullen (Department of Materials, Imperial College London, UK). Briefly, cartilage was harvested from the lower legs of young calves. The ankle joint was cut open along the joint line, and cartilage was cut with a scalpel into thin sections parallel to the subchondral bone, and removed. The cartilage was then digested [0.2% w/v pronase (Invitrogen, UK) for 1 h and then 0.04% w/v collagenase type I (Sigma, UK) overnight]. After digestion, isolated chondrocytes were filtered through a 70 μm pore size filter, centrifuged at 250 × g for 3 min and plated in standard GM. In this study, BCs were used at passage 1 or 2 and cultured in standard GM, equipped with a 50 N load cell was used to measure the compressive modulus of all nanocomposite hydrogels. Samples. Strain, mechanical properties and biochemical analysis was carried out at days 1, 7 and 14. Strain was calculated via thickness measurements, using a digital Vernier caliper. An Instron (Model 5866) testing machine equipped with a 50 N load cell was used to measure the compressive modulus of all nanocomposite hydrogels. Samples were preloaded to 0.05 N, allowed to equilibrate for 1 min and then compressed at a crosshead speed of 100 μm/s. The tangent modulus was calculated from stress–strain curves at 10% strain. sGAG content was quantified using the Blyscan sGAG assay (Biocolor), according to
the manufacturer’s instructions. All sGAG amounts were normalized to the DNA content, quantified using the PicoGreen® double-stranded DNA assay (Invitrogen).

After 7 days of magnetic stimulation, a strain gradient was present, with the surface layer experiencing more strain (9 ± 4%) than the middle and bottom layers (7 ± 2% and 1 ± 0.5%, respectively) (Figure 2D). This same trend was present after 14 days. Similarly, after 7 days of magnetic stimulation, a biochemical gradient was present (Figure 2E), with low levels of sGAG in the surface layer (2 ± 1.6 μg/μg) and significantly increased levels in the middle (7 ± 5 μg/μg; *p < 0.05) and deep (19 ± 6 μg/μg; **p < 0.001) layers. Further, the stiffness of the matrix increased with time in culture in the surface, middle and deep zones (Figure 2F), with an apparent stiffness gradient at day 7 from the bottom to the surface layer. However, after 14 days in culture, there was no significant difference in stiffness between any of the layers.

In conclusion, we describe the development of an advanced cell-embedded nanocomposite hydrogel that, upon external magnetic stimulation, exhibits depth-dependent strain and biochemical gradients after 14 days in culture. Further work could consider varying the number of MNPs, the stiffness of the matrix and external magnetic field, and functionalizing the embedded MNPs with growth factors (e.g. TGFβ3 for chondrogenic differentiation) or drugs. Further, zonal differentiation of mesenchymal stem cells is envisaged, as well as matrix organization by aligning MNPs. Finally, excitation protocols can be developed for the application of dynamic loading (which has been shown to enhance in vitro cartilage ECM formation), by applying time-varying magnetic fields.

**Conflict of interest**

The authors have declared that there is no conflict of interest.
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Supporting information on the internet

The following supporting information may be found in the online version of this article:

Figure S1. Measured magnetic field strength vs distance from magnet face, for magnets used in this study

Figure S2. Protocol of partial setting agarose of different wt% to obtain a trilayered agarose construct, with a gradient of mechanical properties, and custom-built mould for trilayered ferrogels