The use of antifreeze proteins to modify pore structure in directionally frozen alginate sponges for cartilage tissue engineering

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Abstract
It is thought that osteoarthritis is one of the world’s leading causes of disability, with over 8.75 million people in the UK alone seeking medical treatment in 2013. Although a number of treatments are currently in use, a new wave of tissue engineered structures are being investigated as potential solutions for early intervention. One of the key challenges seen in cartilage tissue engineering is producing constructs that can support the formation of articular cartilage, rather than mechanically inferior fibrocartilage. Some research has suggested that mimicking structural properties of the inferior cartilage can be used to enhance this response. Herein directional freezing was used to fabricate scaffolds with directionally aligned pores mimicking the mid-region of cartilage, anti-freeze proteins were used to modify the porous structure, which in turn effected the mechanical properties. Pore areas at the tops of the scaffolds were 180.46 ± 44.17 μm² and 65.66 ± 36.20 μm² for the AFP free and the AFP scaffolds respectively, and for the bases of the scaffolds were 91.22 ± 19.05 μm² and 69.41 ± 21.94 μm² respectively. Scaffolds were seeded with primary bovine chondrocytes, with viability maintained over the course of the study, and regulation of key genes was observed.

Introduction
Osteoarthritis is currently the leading cause of disability worldwide. In 2013 8.75 million people, aged over 45 in the UK alone required medical assistance as a result of osteoarthritis [1]. A number of interventions are available, however they are generally not suitable for long term use. Current treatments suffer from a number of risks. For example long term consumption of pain killers has been attributed as a risk factor to renal failure [2], and artificial joints have limited working lifetimes, with many patients requiring second surgeries for revision [3, 4]. This coupled with a rapidly ageing population means there is an increased chance of patients requiring multiple surgeries, and in addition means joint replacement is not always a suitable treatment for younger patients.

Tissue engineering seeks to combine biomaterials, cell delivery and bioactive factors to restore natural tissues [5]. A number of scaffold materials and fabrication techniques have already been approached for use in cartilage tissue engineering, for example electrospinning of PCL [6–8], or 3D printing of cellulose-alginate hydrogels [9]. There are also a number clinically available allografts on the market such as Chondrofix [10] and ProChondrix CR [11]. These are designed to be combined with microfracture techniques, whereby MSCs are bled into the scaffold from subchondral bone, with the aim of the MSCs differentiating into chondrocytes and producing cartilage [12]. Such techniques are employed to treat articular lesions that have been linked to the onset of osteoarthritis [13]. Although current research is promising current technologies and research are not without their own ethical and functional limitations [13, 14]. In many cases current results show the formation of mechanically inferior fibrocartilage in the wound space, or have been seen to fail mechanically resulting in the need for further intervention.

Previous work has demonstrated a number of methods for replicating the microstructure seen in various tissues [15, 16], with a number of efforts focused on cartilage, using a combination of multiple fabrication techniques [17–19] or directional freezing [20, 21]. Although such techniques have shown promise, there is often limited control over the porous
structure in such materials, particularly in methods like directional freezing. Directional freezing is a form of ice templating where pores are created by ice crystals forming along a single direction [20, 22, 23]. This is achieved by controlling crystal growth using either mould geometries or temperature gradients [20, 22–24]. This method has been used previously in a number of materials [20, 22, 23] and has successfully produced anisotropic structures capable of supporting chondrocytes [21]. The following work proposes a potential new method to provide additional control of pores in directionally frozen constructs, through the addition of Antifreeze Protein (AFP). Such proteins are known to have an influence over ice crystal formation [22, 23]. In this study a set of cross-linked alginate sponges were fabricated using directional freezing. One set incorporated AFPs while the other was AFP free. Structural characterisation, mechanical testing and biological assessment was performed, and comparisons drawn between the AFP and AFP free structures.

**Materials and methods**

Type III Antifreeze Proteins sourced from Arctic Pout were purchased from AF Protein. All other materials, unless otherwise stated were purchased from Sigma Aldrich.

**Scaffold fabrication**

Alginate scaffolds were fabricated by dissolving 6% Alginic Acid Sodium Salt, in ultrapure water over night. Solutions were sonicated to ensure alginic acid had fully dissolved. Following this solutions were either used or the addition of 0.1 mg ml−1 AFP was made, these solutions were then left on a roller for 1 h to allow for dispersion of the AFP. Solutions were poured into an aluminium mould, pre-cooled to −30 °C on a cold plate (Cambridge Reactor Design Polar Bear Plus). After 1 h the frozen solutions in moulds were covered in foil and freeze dried (LABCONCO FreezeZone -105 4.5 L) overnight. A 6 mm bioposy punch was then used to punch scaffolds out, and scaffolds were placed into a 6% calcium chloride solution overnight to induce ionic crosslinking.

**SEM**

Cross-linked scaffolds were flash frozen in liquid nitrogen and freeze dried overnight (LABCONCO FreezeZone −105 4.5 L). Scaffolds were gold sputter coated using an Elmscope FLM-007. Images were acquired using a Hitachi TM4000 SEM at 15 kV with mixed detection mode.

**Porosity**

Average pore area and pore distribution of top and base section of the scaffolds was determined from SEM images using MATLAB 2019. Overall porosity was estimated per equation (1) [16], with an alginate density of 1.6 g cm−3 [24].

\[
Porosity = \left(1 - \frac{\text{Density of Scaffold}}{\text{Density of Polymer}}\right) \times 100
\]

**Mechanical characterisation**

Compressive properties of the scaffold were assessed in unconfined compression using an Instron 3367 (Instron, UK) test rig. Scaffolds of 6 mm diameter and 3 mm height had a pre-strain of 0.01% applied, followed by compression up to 30% strain at a rate of, at 1%/min, stress relaxation was measured for 5 min Incremental moduli were calculated between 0–5, 5–10, 10–15 and 15%–20% strain.

**Cell isolation and culture**

Bovine Chondrocytes were isolated from Bovine Phalangeal joints from cows aged approximately 30 months (sourced from a local abattoir). Before dissection started all specimens were washed and sterilised in 70% ethanol. Tissue was then removed up to the synovial capsule. Samples were then transferred to a sterile hood and the joint was opened. Sections of cartilage were removed from the joint with a scalpel and placed into 15 ml of high glucose culture media, with 1% anti/anti overnight. 5 ml of 0.5% w/v pronase was then added and samples were incubated for 1 h at 37 °C with 5% CO2. The pronase solution was then aspirated out, and the cartilage samples were washed three times with 10 ml of non-supplemented media. 5 ml of 0.2% Collagenase II was added to the cartilage dish with 5 ml of non-supplemented media and incubated for 24 h. Digested solutions were then filtered using a 100 μm cell strainer, in to a petri dish, which was washed four times with non-supplemented cell media, with the aspirated media also being passed through a cell strainer. Solutions were centrifuged, cells were counted and re-suspended into culture media containing 50 μg/ml L-Proline, 0.1 mM sodium pyruvate, 10 ng ml−1 TGF-β3, 1% ITS and 1% anti/anti, media changes were performed every 2–3 days until use.

**Cell seeding**

At P1 cells were trypsinzed, and counted. Scaffolds were washed 3 times in 70% ethanol, and 3 times in PBS. Scaffolds were soaked for 2 h in medium. Medium was removed and 150,000 cells were pipetted directly on to the surface of each scaffold. Cells were allowed 2 h to attach and then 1.5 ml of chondrocyte culture media (containing 50 μg ml−1 L-Proline, 0.1 mM sodium pyruvate, 10 ng ml−1 TGF-β3, 1% ITS and 1% anti/anti) was added to each well. Media was changed every 2–3 days.
Cell viability
Cell viability was assessed at 24 h, 7 days, 14 days and 21 days using CellTitre-Blue viability assay (Promega), performed per the manufacturer’s protocol. Scaffolds were placed in new wells with 400 µl of fresh media and 100 µl of CellTitre-Blue. Plates were incubated for 3.5 h and samples were read with the Modulus™ II microplate reader at an excitation of 525 nm and emission range of 580–640 nm.

DNA quantification
DNA quantification was performed using Quant-iTTM-PicoGreen® assay. In short cell seeded scaffolds at 7, 14 and 21 days were digested in 1 ml papain overnight at 60 °C. A Modulus™ II microplate reader was used to quantify DNA content with fluorescence, using an excitation wavelength of 490 nm and an emission of 510–570.

sGAG quantification
A Sulphated GAG assay (Blyscan Biocolor) was used per the manufacturer’s protocol to quantify Glycosaminoglycan production at 24 h, 7 days, 14 days and 21 days. Samples were digested in 1 ml of Papain overnight at 60 °C. A Modulus™ II microplate reader was used to assess absorbance at 656 nm, and GAG content was calculated from a standard curve.

Live/Dead imaging
Live/Dead® staining was used at 24 h to qualitatively assess the presence of live and dead cells on the scaffold after seeding. In short scaffolds were washed in PBS before a solution of 0.1% v/v calcein and 0.05% v/v ethidium homodimer-1 in PBS was added to each scaffold. Scaffolds were then incubated for 30 min before 3× PBS washes to remove excess agents. When ready for imaging scaffolds were placed on to a glass microscope slides, and images were acquired using the Zeiss Axioimager Fluorescent microscope at ×25 magnification.

Gene expression
Gene expression was assessed using real time quantitative polymer chain reaction (RT-qPCR). Scaffolds were placed into 1 ml of Trizol tri-reagent at each time point to lyse cells, to homogenize the solution 200 µl of chloroform was added, and 400 µl of ethanol was used to precipitate the RNA. RNA was then purified using Qiagen’s RNeasy spin columns. Promega’s ImProm-II Reverse Transcription System was used to synthesize cDNA. RT-qPCR was performed using a LightCycler® 480 II (Roche Life Science) with the QuantiT nova SYBR Green kit (Qiagen). Collagen I, Collagen II, Aggrecan and GAPDH primers were used. Gene expression was quantified using the $2^{-\Delta\Delta CT}$ method, with gene expression relative to scaffolds at 24 h, and results normalized to GAPDH.

Statistical analysis
All Statistical analysis was performed in origin 2020. A one way ANOVA, with Tukey’s post Hoc test were carried out at a confidence interval of 95%. Adjusted p values were used to assess significant difference for “P < 0.05, ”<P0.01 and ***<P0.001.

Results
Structural characterization
SEM images were used to assess differences in pore structure qualitatively. Figure 1 shows representative images of the sponge tops, bases and cross section views. It is possible to see a difference in the pore formations at the top of the sponges, with the AFP sponges showing smaller rounder pores, while AFP free sponges showed a larger elongated morphology.

Figure 1. Representative SEM images of alginate sponges with and without AFP. Images show the tops, bases and a cross section of the sponges.
Additionally the cross sectional views show slight differences with the pores appearing less aligned in the AFP scaffolds.

SEM images were also used to assess pore size and porosity on the top and base surfaces. Table 1 shows the porosity and pore area which were assessed using MATLAB. A significant difference to \( p < 0.05 \) was present for surface porosity between the AFP free top and the bases of both scaffolds. For pore size a significant difference of \( p < 0.05 \) was observed between the AFP free scaffold tops when compared to the AFP scaffold top and the bases of both scaffold groups.

Calculated porosities are detailed in table 2. Porosity was significantly higher in AFP Free scaffolds to a significance of \( p < 0.05 \).

### Table 1. Porosity and pore areas based off of image analysis in MATLAB of SEM images of the sponges.

|                     | Free scaffold top | AFP scaffold top |
|---------------------|-------------------|------------------|
| Surface Porosity (%)| 13.90 ± 2.71      | 10.82 ± 1.49     |
| Average Pore Area (\( \mu m^2 \)) ± STD | 180.46 ± 44.17 | 65.66 ± 36.20 |

### Table 2. Average calculated porosity for AFP and AFP free scaffolds.

| Porosity ± SD       |
|---------------------|
| Free Scaffold       | 95.56 ± 0.36     |
| AFP Scaffold        | 94.46 ± 0.64     |

### Mechanical characterisation

Mechanical properties of the scaffolds were assessed using compression testing. Figure 2 shows the average compressive moduli calculated at strain regions 0–5, 5–10, 10–15 and 15%–20%. It is possible to see from these results that AFP free sponges were significantly stiffer, with peak compressive moduli being roughly 60 kPa, while AFP scaffolds had a peak around 15 kPa.

### Biological characterisation

#### Cell viability

Cell viability at 24 h, 7 days, 14 days and 21 days were assessed and are shown in figure 3, where it can be seen that cells were viable at each time point. Viability in AFP free scaffolds remained consistent over 3 weeks. Viability was shown to increase significantly between 24 h and 14 days in AFP scaffolds, with viability at 14 days also shown to be significantly higher in AFP scaffolds when compared to AFP free scaffolds.

### Discussion

Alginate has been explored as a material for a number of biomedical applications, it is considered biocompatible and has been shown it can be modified in a number of ways, allowing it’s use as a drug delivery vehicle [28] or a scaffold for tissue engineering [29]. Likewise AFPs have been investigated for use in food preservation [25], cryopreservation of biological samples [26] and manufacturing of porous ceramics [30]. AFPs are thought to control ice crystal formation through binding to the basal planes of ice crystals and repelling surrounding water molecules with a hydrophobic end, which helps to prevent further ice crystal growth [31]. Here Alginate and AFPs were combined for the first time with directional freezing, a method used previously to fabricate scaffolds capable of mimicking the mid-region of articular cartilage, typically these methods rely on varying temperature gradients, or varying mould geometries [20–24]. The addition of AFPs to Alginate was performed to

### DNA quantification

DNA production was also assessed at days 7, 14 and 21. Results can be seen in figure 4. No significant differences were observed between time points or groups across the course of the study.

### sGAG quantification

sGAG content for each scaffold at 24 h, 7 days, 14 days and 21 days was assessed biochemically. Results shown in figure 5 show significant differences in sGAG content overtime in the AFP Free scaffolds, with differences seen between 24 h, 7 days and 21 days. In addition sGAG content was shown to be significantly higher in AFP free scaffolds at 14 days compared to AFP scaffolds.

### Live/Dead imaging

Live/Dead images were acquired at 24 h to assess the presence of cells following seeding. Figure 6 is a representative image of those acquired through live dead staining, with the images showing regions of green demonstrating live cells were present 24 h after seeding.

### Gene expression

Gene expression was used to assess the phenotypic function of the chondrocytes while being cultured on the scaffolds. Figure 7 shows gene expression results for Collagen I, Collagen II and Aggrecan, all genes were normalised to 24 h AFP free scaffolds and GAPDH housekeeping gene. No significant differences were observed during the study for either scaffold type. Both showed reduced expression of collagen II and Aggrecan over time, while Collagen I expression was shown to increase in AFP free scaffolds over the course of the study.
investigate the potential of AFPs as an alternative method for the control of the directional freezing.

Results of the study demonstrated clear structural differences between AFP and AFP free scaffolds, with the AFP scaffolds showing smaller pores and a lower porosity on the top surface compared to the AFP free structures. Furthermore in cross sections of the scaffolds (figure 2) it was possible to see AFP scaffolds had less order in their pores when compared to the linearity of the AFP free scaffolds. These structural differences suggest the AFPs may have reduced, or slowed the ice crystal growth during directional freezing, a similar effect on crystal growth rate was previously seen by Fukushima et al [30] when looking at porous ceramics. It should be noted that pore order was qualitatively assessed due to the destructive techniques used to prepare the scaffolds for imaging, and further characterisation using micro-CT scanning is needed to further quantify the order of the pores in these scaffolds. Compression testing also demonstrated that the inclusion of AFPs significantly reduced the compressive properties of the scaffolds, this may be linked to the order seen in the AFP pores where the discontinuities in the pore direction meant that there was less resistance to compressive forces applied perpendicular to the mean pore direction, when compared to AFP free scaffolds. It should also be noted that neither scaffold had compressive moduli comparable to that of healthy articular cartilage, (ordinarily in the range of 1–18 MPa depending on activity [32]), although it may
not be beneficial to match the mechanical properties of healthy cartilage, as it has been observed that lower scaffold stiffness is beneficial for cell viability [33]. Additional biochemical assessments were performed, to assess the effect the sponges had on cell viability and function. Cell-Titre Blue demonstrated that both AFP and AFP free scaffolds were able to support cell viability over 3 weeks, DNA content showed no significant changes over time, but increases are visible in the groups over time. sGAG quantification showed that sGAG remained constant across all time points within the AFP variable, but differences were observed in the AFP free scaffold groups, showing a slight increase in sGAG production over time. These results are supported by other studies by where the effect of scaffold stiffness on cell viability and function has been investigated. These studies have shown softer scaffolds support higher viability, while stiffer scaffolds lead to better chondrogenic function [33, 34]. In this case the AFP scaffolds that were softer had greater cell viability, but the AFP free scaffolds showed greater sGAG production. Further investigation showed gene expression in each scaffold type using RT-qPCR was downregulated for Collagen II and Aggrecan in both scaffold types, when normalised to 24 h AFP free scaffolds, with a slight increase in collagen I over time in the AFP free scaffolds. Similar results have been seen previously, showing that when stiffer scaffolds are used to culture...
chondrocytes, increased phenotype expression is observed, while lower stiffness is correlated to increased cell viability \[33\]. While these results show a relationship between scaffold stiffness, viability and phenotypic expression, it is understood that morphology also plays a role in cell response \[35, 36\], it would therefore be beneficial for future work to investigate a wider range of groups with AFPs to identify any morphological effects on cell behaviour achieved through this fabrication technique.

Current strategies for the treatment of articular cartilage damage range from total joint replacement \[37\], autologous chondrocyte implantation \[14\] and microfracture \[38\]. At present no strategy has demonstrated the ability to fully regenerate damaged cartilage, with the closest successes being limited to the filling of damaged regions with mechanically inferior fibrocartilage \[14\]. Although this may in part be linked to the poor regenerative capacity of cartilage itself \[39\], some studies have shown that mimicking the natural structure of cartilage can be beneficial \[17, 18\]. In this work we sought to further explore the use of directional freezing for mimicking the perpendicular structure seen in the mid-region of articular cartilage, AFPs were used to further modify the freezing process to investigate if it was possible to use these scaffolds for cartilage tissue engineering. In addition to investigating the effects of AFP on the scaffold structure, this study also investigated the influence of AFP modified, and AFP free scaffolds on chondrocyte survival and repair functions. Although the scaffolds were able to support cell viability clear evidence of dedifferentiation was present. Further work is therefore needed to refine these scaffolds to better support cartilage regeneration. This may be achieved by additional functionalisation that could improve cell attachment, as it is well known that alginate does not provide natural binding points for cells \[40\]. Furthermore refinement of the fabrication process is needed to understand the full capability of AFPs to help modify structures produced through cold fabrication techniques.

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Figure 6. Live/Dead fluorescence imaging of AFP (A) and AFP free (B) scaffolds after 24 h. Green represents live cells, and red represents dead cells.

Figure 7. Gene expression for cells cultured on AFP and AFP free constructs over 3 weeks, with values normalised to GAPDH and relative to 24 hour AFP free scaffolds. \*p < 0.05, one-way ANOVA with Tukey’s post hoc test. Errors bars represent standard error.
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