An Injectable Alginate/Extra Cellular Matrix (ECM) Hydrogel Towards Acellular Treatment of Heart Failure

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Abstract (250 words)

As treatments for myocardial infarction (MI) continue to improve, the population of people suffering from heart failure (HF) is rising significantly. Novel treatment strategies aimed at achieving long-term functional stabilization and improvement in heart function post MI include the delivery of biomaterial hydrogels and myocardial matrix-based therapies to the left ventricle wall. Individually alginate hydrogels and myocardial matrix-based therapies are at the most advanced stages of commercial/clinical development for this potential treatment option. However, despite these individual successes, the potential synergistic effect gained by combining the two therapies remains unexplored. This study serves as a translational step in evaluating the minimally invasive delivery of dual acting alginate-based hydrogels to the heart. We have successfully developed new production methods for hybrid alginate/extracellular matrix (ECM) hydrogels. We have identified that the high G block alginate/ECM hybrid hydrogel has appropriate rheological and mechanical properties (1.6 KPa storage modulus, 29 KPa compressive modulus and 14 KPa dynamic modulus at day 1) and can be delivered using a minimally invasive delivery device. Furthermore, we have determined that these novel hydrogels are not cytotoxic and are capable of enhancing the metabolic activity of dermal fibroblasts *in vitro* ($p < 0.01$). Overall these results suggest that an effective minimally invasive HF treatment option could be achieved by combining alginate and ECM particles.

Keywords: Heart failure; Acellular hydrogel; Minimally invasive delivery catheter; Alginate; Decellularized ECM

1. Introduction

Since the mid 1990's enhanced coronary interventions have led to a decline in coronary heart disease mortality[1]. As a consequence of these treatments there has been an increase in chronic heart failure (HF) patient numbers[2]. As treatments for myocardial infarction (MI) continue to improve, it is predicted that the population of people suffering from HF will rise to 3% in Western societies by 2025[3]. At present approximately 50% of people diagnosed with HF, as a result of MI, will die within 5 years[4]. This is considered to be associated with the lack of treatment options that could eliminate (or decelerate) the negative remodelling that precedes congestive heart failure[5]. Currently, solutions are reactive and are initiated in the end stages of heart failure (New York Heart Association class IIIB/IV)[6,7]. In addition to this, they consist mainly of heart transplantation and left ventricular assist device implantation, both of which are highly invasive and have many limiting factors[7,8].

Novel strategies aimed at achieving long-term functional stabilization and improvement in heart function post MI include the injection of stem cells into the heart wall[9,10]. However, this approach has had limited success in a clinical setting to date. Embedding of stem cells in biomaterials prior to injection in the heart wall may be one way to increase benefit to the patient [11–14]. However, regardless of efficacy, clinical translation of cell based technologies remains burdensome due to regulatory pathway complexity, a lack of off the shelf availability, and high costs[15]. An alternative option for patients, that may achieve clinical translation in the short to medium term[16,17], is based on the myocardial injection of acellular biomaterials alone. Many materials, such as alginate, fibrin, collagen, chitosan and extracellular matrix (ECM)[18] to name a few, have been investigated for this application and some have demonstrated the preservation or increases of cardiac output or fractional shortening (FS). Some of these materials have even progressed to the late stage of clinical development. VentriGel (Ventrix, Inc., USA) (NCT02305602), Algisyl (LoneStar Heart, Inc., USA) (NCT01311791), and IK-5001 (Bellerophon Therapeutics, USA) (NCT00847964) are three such therapies that are in phase I and phase II/III clinical trials. VentriGel consists of the intramyocardial delivery of decellularized myocardial matrix following MI. This strategy has been disclosed in a number of publications and patent applications[19–22]. The injected material is capable of assembling into a nanofibrous network that allows cell migration and is structurally reminiscent of the native ECM[23]. The use of ECM is advantageous as it provides cells with the complex combination of proteins and polysaccharides seen *in vivo*[24]. This complex combination not only guides cellular attachment, but provides many survival, migration, proliferation and differentiation cues[24]. Injection of the matrix into porcine and rat MI models has demonstrated increased cardiac muscle presence, reduced fibrosis, prevention of negative left ventricular remodelling, and improved cardiac function[19,20]. In contrast to the injection of decellularized ECM, myocardial injection of alginate (Algisyl and IK-5001) is seen as a passive stabilising support in which the weakened ventricle wall is mechanically supported to reduce wall stress (based on LaPlace's Law) and prevent stress apoptosis and further LV remodeling. Additionally, the Algisyl treatment,

which consists of a circumferential pattern of intramyocardial injections across the left ventricle, aims to reshape the ventricle to a healthier and more efficient morphology. Injection of alginate can take place at either the acute (IK-5001 and Algisyl implant) or chronic (Algisyl implant only) phase post MI. Intracoronary injection of the IK-5001 implant has resulted in the reversal of LV enlargement, increased scar thickness and increased myofibroblast and collagen presence in swine[25]. Separately, patients injected with Algisyl have had significantly improved peak VO_2, six minute walking test and New York Heart Association functional class improvement at 12 months post treatment[17]. Previously, it has been shown that injection of stiffer hydrogels promotes better mechanical stabilisation of the heart[26,27]. ECM hydrogel (VentriGel) which has a storage modulus ≈5 Pa[28] is limited in this respect, as the modulus is orders of magnitude less than that of other injected materials such as alginate (3-5 kPa)[29]. Past studies have attempted to achieve this by crosslinking ECM hydrogel with glutaraldehyde[28] or by combining it with polyethylene glycol (PEG)[30]. Glutaraldehyde crosslinking resulted in a hydrogel with a storage modulus of ≈136 Pa, but further stiffening of the gel was restricted as glutaraldehyde has been shown to be cytotoxic at higher concentrations[31]. Combining ECM with PEG resulted in retention of the ECM hydrogels nanofibrous structure, but led to a maximum storage modulus of only 719 Pa, still much less than that of the previously mentioned hydrogels[29].

Minimally invasive delivery of hydrogel therapy offers many advantages over direct surgical injection, including lower procedure risks, reduced damage to target and surrounding tissue, shorter procedure times, quicker recovery times, improved suitability for repeat applications, higher potential for clinical integration, and potentially better cost effectiveness[32,33]. However, while some hydrogels, such as shear thinning gels[34,35], are naturally suited to this many are not. This has been indicated with materials that solidify too quickly which may result in clogging within the delivery device, or separately may lead to the destruction of the mechanical integrity of the hydrogel during its ejection[12,36]. Alternatively, materials that solidify too slowly may have limited material retention within the myocardium, or lead to prolonged procedure time[32]. In addition to gelation kinetics, a second significant complication of minimally invasive hydrogel delivery involves how a catheter's small lumen size and long length limits the maximum viscosity of the material that can be used[33]. Increased flow resistance may lead to very high forces during hydrogel ejection and consequently compromised operator usability or catheter component instability.

We hypothesise that a minimally invasively delivered hybrid biomaterial that has the potential to structurally stabilise the heart, while simultaneously providing the complex combination of proteins and polysaccharides will act synergistically as an acellular approach for the treatment of heart failure. Therefore, in this study we have combined decellularized ECM with two alginate-based hydrogels with differing gelation mechanisms and block make-ups. In the first material, high M block hydrogel, this system is analogous to that used by the IK-5001 implant. In the second material, high G block hydrogel, the same gelation mechanism that Algisyl used in its material was engaged[29]. Decellularized myocardial

matrix was then dispersed into each of these alginate bases. A minimally invasive catheter was developed to investigate injectability and resulting mechanical properties of the hydrogels. Cytotoxicity of the hybrid hydrogels was measured using an adapted International Organization for Standardization (ISO) cytotoxicity assessment protocol (EN ISO 10993-5).

2. Materials and methods

Alginate is known to be a whole family of linear copolymers containing blocks of (1,4)-linked βD-mannuronate(M) and α-L-guluronate (G) residues. The blocks are composed of consecutive G residues (GGGGGG), consecutive M residues (MMMMMM), and alternating M and G residues (GMGMGM) [37]. The composition (i.e., M/G ratio), sequence and molecular weight are critical factors affecting the physical properties of alginate and its resultant hydrogels[37,38]. Prior to hydrogel formation, sufficient decellularized cardiac matrix (ECM) for all experiments was manufactured using a whole porcine heart decellularization protocol as described in Section 2.1. Subsequently four separate hydrogels were constructed:

1. High M block alginate. These hydrogels were formed after the release of calcium from calcium carbonate upon addition of an acid.
    a. High M alginate alone
    b. High M alginate + ECM material
2. High G block alginate (similar to Algisyl). The hydrogels were formed by the exchange of gelling ions between soluble and insoluble alginate components.
    a. High G alginate alone
    b. High G alginate + ECM material

2.1 ECM preparation

Whole porcine hearts were sourced from a local abattoir (Lislin Meats Ltd, Mullagh, Co. Cavan) and decellularized according to a protocol adapted from Wainwright et al. and Remlinger et al.[39,40] and detailed in Supplementary Materials Section 1.1. Following decellularization the heart was drained for 1 hour prior to drying. Dissection of the right and left ventricles, and the ventricular septum then took place. At this point some matrix was removed for histological assessment. The remaining matrix was then placed in a -80 °C freezer for a minimum of 2 hours prior to lyophilisation. Following lyophilisation the decellularized matrix was cryomilled (6770 Freezer/Mill, Spex Sample Prep, USA) to form fine particles. Ground ECM particles generated from the ventricles and ventricular septum of all three hearts was then pooled. A small amount of this powder was sampled to conduct biochemical analysis of each decellularized heart. The resulting volume was then sterilised using a 12 hour ethylene oxide (EtO) cycle (Anprolene AN74i, Anderson Products, USA) before being stored in a -80 °C freezer until hydrogel preparation.

2.2 Histological and Biochemical analysis of ECM

Fresh and decellularized hearts were cut into sections and prepared in a tissue processor (Leica TP 1020), followed by embedding in paraffin wax. Samples were then sliced to 7 µm thickness and mounted on slides. This was followed by staining with H& E (Hematoxylin and Eosin, Sigma Aldrich, Ireland), and slides were observed under an Olympus BX41 microscope, as previously described[41,42].

10 mg per heart of lyophilized native and decellularized tissue was digested in papain (125 µg/mL) (Sigma # P3375), with 0.1 M sodium acetate, 5 mM L-cysteine HCl (Sigma # C7477), and 0.05 M EDTA (Sigma # E5134) at 60 °C under constant agitation for 18 hours. Assays were performed in accordance with the manufacturer's instructions with samples being evaluated in triplicate. The Quant-iT™ PicoGreen® dsDNA assay (Molecular Probes, USA) was used for DNA quantification. The total sulphated glycosaminoglycan (sGAG) content was quantified using the dimethylmethylene blue (DMMB) dye-binding assay (Blyscan; Biocolor Ltd., Carrickfergus, Northern Ireland). The total collagen content was determined by measuring the hydroxyproline content. Briefly, samples were hydrolysed at 110 °C for 18 hr in concentrated hydrochloric acid (Sigma # H1758) (38%) and assayed using a chloramine-T assay[43], at a hydroxyproline-to-collagen ratio of 1 : 7.69[44].

2.3 Hydrogel Preparation

2.3.1 High M block alginate hydrogel

The High M block hydrogel was prepared as follows: a sodium alginate powder with a 65-75% M-block content and a viscosity of 70-150 CPS at 1% (Protanal LF 120M, FMC Biopolymer, Norway) was dissolved in ultra-pure water and stirred overnight prior to neutral pH balancing using sodium hydroxide (Sigma # 55104) and hydrochloric acid (Sigma # H1758). Separately, calcium carbonate particles (Sigma # C5929) were suspended in ultrapure water and sonicated for 30 minutes at room temperature. The sonicated calcium carbonate and alginate components were then mixed to from a homogenous solution. The alginate/calcium carbonate solution and an acetic acid solution (Sigma # 695084) were then loaded into separate syringes. The syringes were connected using a luer-lock connector and material was transferred back and forth ten times. Final concentrations of 2% alginate, 0.4% calcium carbonate and 0.16% acetic acid were achieved. Sodium alginate and calcium carbonate were sterilised by EtO, as described previously, while the acetic acid was filter sterilized with a 0.2 µm syringe filter (# 431219, Corning, USA).

2.3.2 High G block alginate hydrogel

An internally gelling high G block alginate hydrogel was prepared based on the exchange of gelling ions between soluble and insoluble alginate as described previously by Melvik, Larsen and Lee et al.[29,45,46]. In the adapted protocol, both the soluble and insoluble components were made from sodium alginate powder with a 65-75% G-block content and a viscosity of 200-400 CPS at 1% (Protanal LF 200FTS, FMC Biopolymer, Norway). To prepare the insoluble component of the hydrogel, sodium

alginate and then sodium carbonate (# S7795) were dissolved in ultra-pure water to final concentrations of 2% and 0.52% respectively. The solution was rotated overnight prior to neutral pH balancing. Alginate beads were then formed by adding droplets of the solution into a continuously stirring pH neutral 1% calcium chloride bath (Sigma # C7902). The beads were then left to fully crosslink for 15 minutes. The precipitated calcium alginate was then washed 12 times by stirring in type one water. The washed beads were stored in 75 g batches in 5 inch petri dishes at 4°C. Prior to lyophilisation, dishes were stored at -20 °C for 35 minutes. Dishes were then removed and placed in a freeze-dryer that had been chilled to -20 °C. The vacuum was turned on immediately and lyophilisation then took place. The resulting dry beads were cryomilled for 30 seconds (6770 Freezer/Mill, Spex Sample Prep, USA), and sieved using meshes with a 75 and 125 µm pore size (Endecott, UK). Measured particles were then added to a 4.6% mannitol (Sigma # M4125) solution to give a concentration of 2.8% calcium alginate particles.

To prepare the soluble component of the hydrogel, sodium alginate was stirred into a mannitol solution overnight to give final concentrations of 2% and 4.6% respectively. Hydrogel gelation was initiated when equal volumes of the insoluble particle solution and the soluble solution were briskly transferred back and forth four times between two syringes that were connected with a luer-lock connector, as described previously[29,45]. Sodium alginate and calcium alginate were sterilised by EtO post sieving, while mannitol solutions were filter sterilized with a 0.2 µm syringe filter (# 431219, Corning, USA) prior to sodium alginate or calcium alginate addition.

2.3.3 Alginate/ECM hydrogels

Hybrid alginate/ECM hydrogel preparation took place by incorporating the ECM particles into the calcium carbonate alginate component of the high M block alginate or the soluble high G block sodium alginate component. The sodium alginate solution was chilled at 4 °C prior to use, and then placed on ice. ECM particles were removed from the -80 °C freezer prior to use and stirred into the sodium alginate/calcium carbonate solution using a magnetic bar stirrer while being kept on ice. Stirring took place for a minimum of 3 hours, or until a homogenous solution resulted. The ECM volume added to the respective sodium alginate was such that a final concentration of 6 mg/ml would be achieved post gelation. This concentration has previously been shown to result in improved cardiac function when injected alone into rats and pigs[19,20]. Gelation of the sodium alginate/ECM mixture was then initiated.

2.4 Scanning electron microscope imaging and microparticle analysis

Scanning Electron Microscopy (SEM) (Zeiss Ultra Plus, Zeiss, Germany) was used to characterise the structural morphology of lyophilised sections of decellularized hearts, the ECM particles alone, and all hydrogel types. In preparation for imaging 2-3 mm thick sections of decellularized matrix were cut and placed on carbon tape. ECM microparticles were suspended in 100% ethanol to aid dispersion onto carbon tape and then air dried at room temperature. Following SEM imaging, ImageJ software (U.S.

National Institutes of Health, USA) was used to quantify the major axis of calcium alginate and ECM particles. Major axis measurement was quantified by first thresholding images to highlight particles and then using the 'Analyse Particles' analysis tool.

2.5 Estimation of time to gelation and time to physiologic pH neutrality

Gelation temperature and gelation speed for all hydrogels was estimated using the "Visual Tube Inversion Method", previously described by Ur-Rehman[47].

The time to high M block hydrogels reaching normal physiologic blood levels (pH 7.35-7.45) was assessed by forming 200 µL samples and measuring their pH at intervals of 5 minutes. 200 µL samples were formed and left to solidify in moulds for two minutes. Samples were then removed to 5 ml of pH balanced Krebs-Henseleit buffer and maintained at room temperature. Two minutes before pH testing took place samples were removed from the buffer and gently shaken to expel excess fluid. Samples were then placed in a 500 µL micro-tube and a pH meter (Five Go, Mettler Toledo, Switzerland) was inserted into the gel so to record the pH. Hydrogel pH was measured at time intervals up to a total of 30 minutes.

2.6 Rheological and Mechanical Properties

Rheological assessment of each hydrogel took place by monitoring the storage (G') and loss (G") modulus using an AR1000ex Rheometer (TA Instruments, USA) as previously described[48,49]. Testing was performed at 20 °C using a shear stress sweep between 10 and 80 Pa, and a 40 mm diameter 4° cone. One millilitre of the alginate component of each hydrogel was tested, with each test being repeated five times. Samples were protected from evaporation with the use of a solvent trap.

In addition to this, a frequency sweep (0.1 and 10 Hz) was performed at 37 °C under a 1% strain on formed hydrogels at day 1 with an 8 mm diameter crosshatch parallel plate geometry. Samples were prepared and maintained as discussed previously, with the exception that a 6 mm biopsy punch was used to form hydrogels in this case. Samples were protected from evaporation by placing a thin layer of low viscosity silicon oil along their edge.

The degradation behaviour of each hydrogel was investigated by placing samples in Krebs-Henseleit buffer maintained at 37°C, and subsequently measuring the compressive young's modulus at regular intervals. Briefly, 5 mL batches of hydrogels were mixed and allowed to gel for 5 minutes at room temperature in 4 mm deep moulds. 200 µL samples were then cut using an 8 mm circular biopsy punch. Each sample was then placed in 5 mL of Krebs-Henseleit buffer (Sigma # K3753). Well-plates were sealed with Parafilm-M (Parafilm, Pechiney, IL, USA) and stored at 37 °C. Samples (n=5 per group at each time-point) were removed at day 1, and at intervals of 7 days until 42 days. Unconfined uniaxial compression testing took place on the 200 µL samples using a Zwick with a 5 N load cell with an accuracy of ± 1% (Zwick Z005, Roell, Germany). All tests were performed in a PBS bath. A preload

of 0.01 N was applied to ensure that the surfaces of the gel constructs were in direct contact with the impermeable loading platens and to ascertain the height of the specimens. Samples were then preconditioned with 15 cycles of 0.005% of gauge length at 0.1% strain per second[27]. This was followed by a ramp to 20% compressive strain at 0.1% strain per second. The compressive modulus was determined as the slope between 10% and 20% strain of the resulting stress-strain curve. Dynamic tests were performed immediately after the compressive strain relaxation. The strain was reduced to zero and a cyclic strain of 1% was applied for 10 cycles at 1 Hz. Dynamic moduli at each frequency were calculated through the ratio of the determined stress amplitude to the applied strain amplitude.

2.7 Design and Development of a Minimally Invasive Delivery Catheter

A single lumen catheter, one meter in length, was designed and constructed as described in Figure 1A and B. The main body of the catheter consisted of polyimide tubing supplied by MicroLumen (MicroLumen, USA). The distal tip of the catheter terminated in a 26G needle that was adapted from a 26G needle (# 4665457, B. Bruan, Germany), and held in place using stainless steel tubing supplied by Unimed (Unimed S.A., Switzerland). 26G bevel tip needles were found to be the optimal for myocardial hydrogel injection previously[14]. A 20G needle (# 4710009040, Henke Sass Wolf GmbH, Germany), 22 mm in length, was inserted and secured at the proximal end of the polyimide tube to enable a leur connection to a syringe during hydrogel ejection.

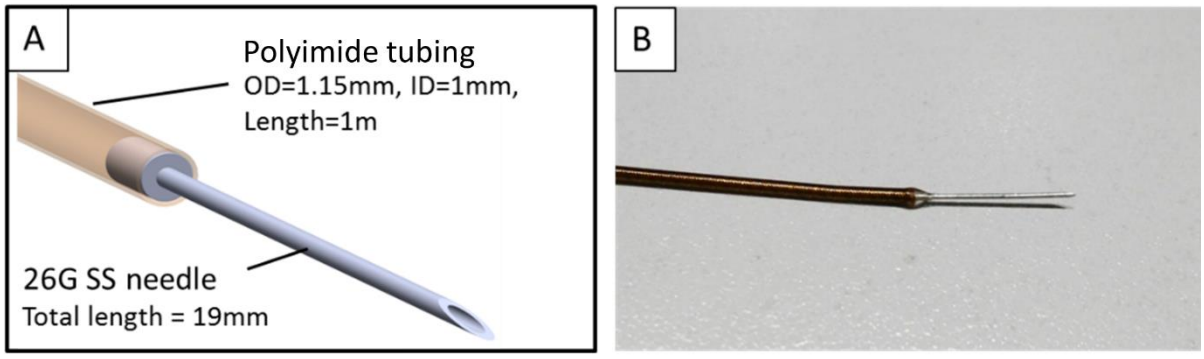

Figure 1: Catheter construction drawing and realised designs: (A) Catheter manufacture drawing and (B) final construction, where Outer diameter (OD), Inner diameter (ID).

2.8 Hydrogel ejection

A Zwick mechanical testing machine (Z050, Zwick/Roell, Germany) was used to determine the force required to inject the hydrogels through the needle and the catheter, as previously described[48,49]. A 200 N load cell an accuracy of ± 1% was fitted to the Zwick. A syringe holder that allowed the quick placement of 3 mL syringes (3 mL Soft-Ject, Henke Sass Wolf GmbH, Germany) was designed and 3D printed using PLA (Ultimaker 2+, Ultimaker, USA). The experimental set-up used during hydrogel

ejection is shown in Supplementary Figure 1. The hydrogel specimens ejected by the 26G needle and the catheter was premixed prior to loading into the 3 mL syringe. Each hydrogel was ejected 25 seconds post mixing. Hydrogel ejection was then repeated at 25 second intervals up to 200 seconds, and then every 200 seconds up to 600 seconds. Ejection of high M block hydrogels was stopped at 100 seconds. The same catheter was used throughout each experiment and the distal tip of the catheter was placed in a Krebs-Henseleit buffer (Sigma # K3753) bath during the timeframe between hydrogel ejections. 200 µL of hydrogel boluses were ejected through each needle and catheter at a rate of 1 mL/minute. Ejected hydrogels were collected in 200 µL 3D printed PLA moulds. Three minutes after hydrogel ejection, samples were removed from the moulds and prepared for mechanical testing as described in Section 2.6. A 70N maximum ejection force for the operator to apply was set, based on previously published data [48–51].

2.9 Effect of hydrogel degradation products on dermal fibroblasts

To investigate the potential effect of intramyocardial injection of the constructed hydrogels on surrounding cells, a conditioned media approach was carried out using primary dermal fibroblasts and an adapted International Organization for Standardization (ISO) cytotoxicity assessment protocol (EN ISO 10993-5) as previously described[52]. Hydrogels were prepared, as described above and added to DMEM (extraction medium) at a concentration of 0.1 g/ml. The hydrogel was then incubated for 24 hours. DMEM without any hydrogel sample was simultaneously incubated for 24 hours and served as a blind control. In parallel, primary isolated human dermal fibroblasts (passage 3-5) were seeded at a density of 20 000 or 10 000 cells per well in a 96 well plate and 200 µl of DMEM supplemented with 10% foetal bovine serum (FBS) was added. The cells were then incubated for 24 hours under normal conditions. After 24 hours, hydrogel samples were removed from the media and 10% FBS was added to all media samples, including the negative control. The media was removed from the fibroblasts and replaced with 200 µl of hydrogel conditioned media, blind control media or negative or positive control media. Negative control media consisted of DMEM with 10% FBS (100% proliferation), while the positive control consisted of DMEM, 10% FBS and 1% sodium dodecyl sulfate solution (0% proliferation). Each condition was performed in 6 wells, and this was repeated three times. Cells were then cultured in the hydrogel conditioned media, blind control media or negative or positive control media for a further 24 and 72 hours. Well-plates that were seeded at a density of 20 000 cells per well were observed at 24 hours, while those seeded with 10 000 cells per well were observed at 72 hours.

At each time-point a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assay was used to determine the metabolic activity of the cells. This was completed using the CellTiter 96 AQueous One Solution Cell Proliferation assay (# G3582, Promega, Mannheim, Germany) according to the manufacturer's instructions.

Following metabolic activity assessment, quantification and imaging of the cells took place. Each well was rinsed with PBS and fixed with 10% paraformaldehyde for ten minutes. Wells were rinsed with PBS and then stained with a 6-diamidino-2-phenylindole (DAPI) (# 10236276001, Roche, Germany) solution at a concentration of 5 mg/mL in PBS. Each well was imaged in the same three locations using fluorescent microscopy (Axiovert Zeiss, Carls Zeiss, Germany), and the cell number was quantified using image analysis (ImageJ, U. S. National Institutes of Health, USA).

2.10 Statistical Methods

All values were expressed as mean +/- standard deviation. A two-tailed student's t-test was used to test significance between separate groups and results with $p < 0.05$ were considered statistically significant. Bar charts are used to present multiple results, with error bars depicting one standard deviation.

3. Results

3.1 Characterisation of decellularized porcine heart

Qualitative confirmation of decellularization was achieved with H&E staining and SEM analysis of sections of decellularized sections, see Figure 2A-C. No cellular remnants could be observed post decellularization when compared to native tissue (Figure 2A and B). SEM analysis of the decellularized heart sections displayed no cell presence and an open collagen configuration (Figure 2C). Furthermore, quantification of the remaining DNA by a PicoGreen assay found that the right and left ventricles had 47 ± 5.3 ng/mg and 60 ± 4.2 ng/mg of DNA respectively, and when the decellularized heart components were pooled the remaining DNA was 53 ± 3.7 ng/mg, see Figure 2D.

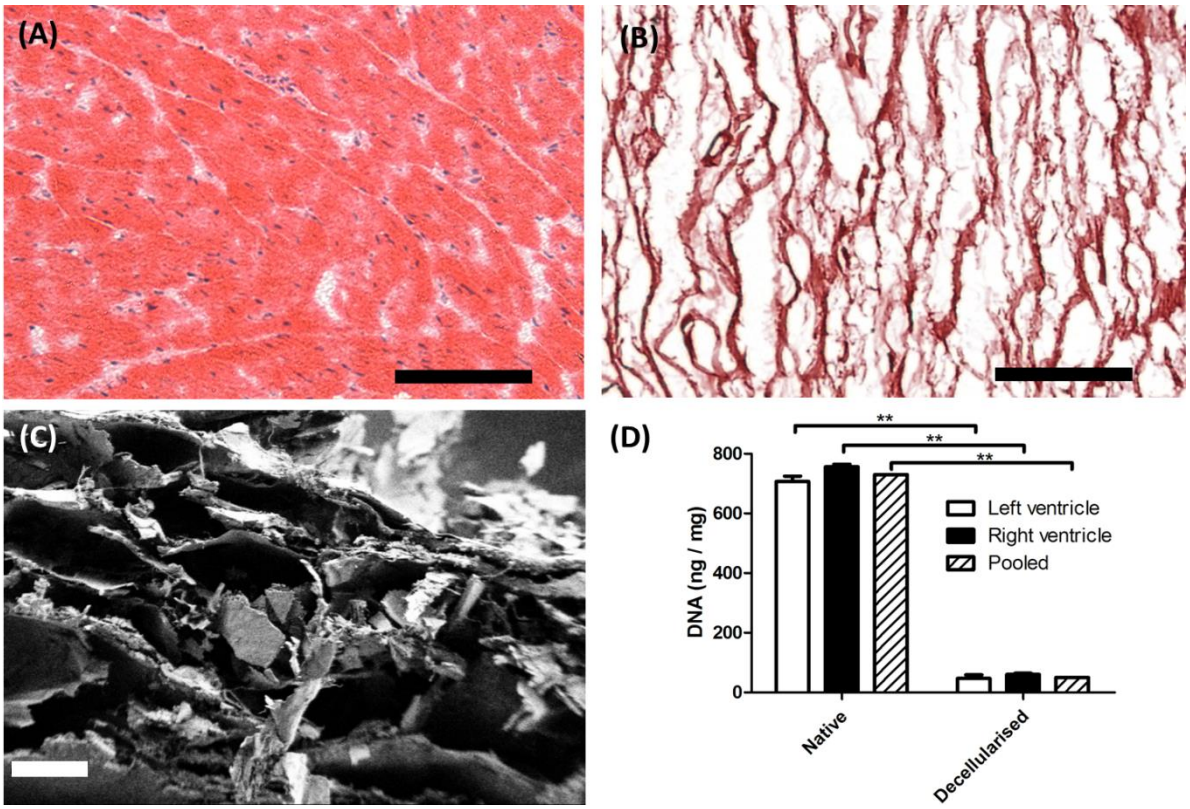

Figure 2: Characterisation of native and decellularized porcine cardiac matrix. Representative H&E stained sections of (A) native and (B) decellularized porcine cardiac tissue. Staining revealed the presence of nuclei in native ventricular sections (see zoomed in image highlighted in A), and their absence in decellularized samples. (C) Representative SEM cross section of freeze dried decellularized matrix sections. SEM images displayed no cell presence and an open collagen configuration. (D) Quantitative analysis of DNA content using a Pico Green assay. Decellularized matrix had a significantly decreased DNA content in the ventricles of the heart and in pooled samples. Significance: ** denotes statistical significant difference with $p < 0.01$. n=3 for D. Scale bar: black = 100 µm, white = 0.1 µm.

SEM analysis of cryomilled ECM particles revealed that 77.73 ± 8.65 % of particles had a long axis between 10 and 25 μm, Figure 3A. Characterisation of the GAG content demonstrated that the decellularized matrix had statistically significantly less GAG than that measured in the native ventricle tissue ($p < 0.01$), see Figure 3B. Characterisation of the collagen content demonstrated that the decellularized matrix had a statistically significantly higher proportion of collagen than that measured in the native ventricle tissue ($p < 0.01$), see Figure 3C.

3.2 Hydrogel characterisation

SEM of calcium alginate particles for the high M and high G block base hydrogels revealed that the majority of the particles had a long axis between 10 and 25 μm, Figure 3D. No statistical difference in time to gelation was found between the high G block hydrogel base and the high M block equivalent, see Figure 3E. It was also noted that while the high M block base hydrogel appeared to have a consistent rate of gelation, the high G block base hydrogel remained at a low viscosity for approximately 35 seconds before experiencing an increase in gelation speed. Addition of ECM to each of the hydrogel bases resulted in statistically significant slower gelation times for the high G block alginate and statistically significant faster gelation times for the high M block alginate, see Figure 3E. The pH of the high M block alginate alone hydrogel was found to stabilise at pH 7.35-7.45 after 20 minutes, Figure 3F.

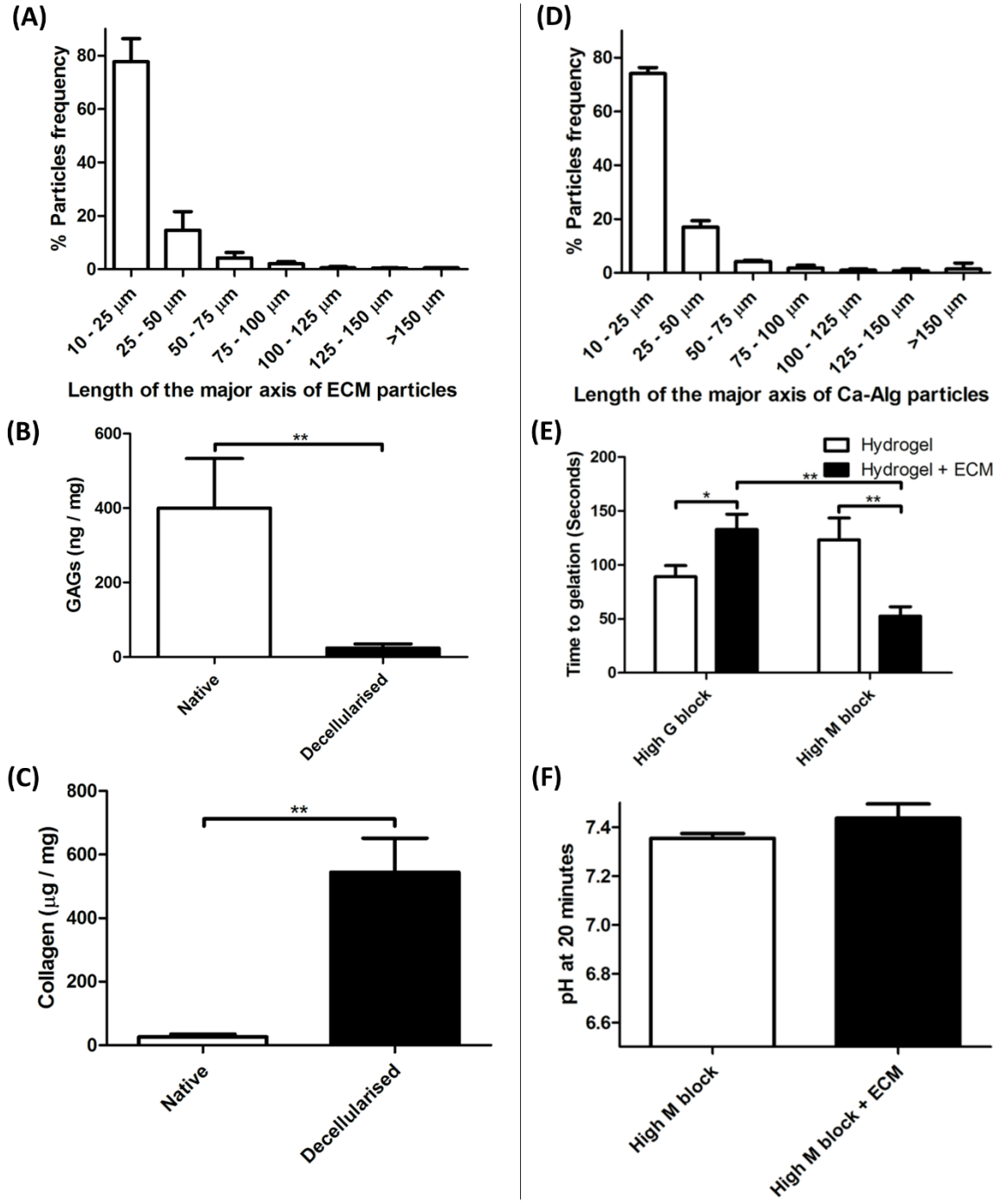

Figure 3: Characterisation of native and decellularized porcine cardiac matrix particles and calcium alginate particles, time to gelation and time to neutrality for hydrogels. (A) Distribution of major-axis length of decellularized ECM particles. The vast majority of ECM particles were found to have a major axis length of between 10 and 25 microns. (B-C) Quantification of GAGs and collagen in native and decellularized matrix particles. GAGs measured were significantly lower (B), and collagen content was significantly higher (C), in the decellularized matrix than in the native ventricular tissue. Parts B and C are based on lyophilised samples. (D) The majority of particles were found to have a major axis length of between 10 and 25 microns. (E) No significant difference in time to gelation existed

between the high G block and high M block hydrogel alone groups. Addition of ECM to each of the hydrogel bases lead to statistically significant longer gelation times for the high G block alginate but to statistically significant faster gelation times for the high M block alginate. (F) pH of high M hydrogel samples with and without ECM particles at 20 minutes. Significance: * denotes statistical significant difference with $p < 0.05$. ** denotes statistical significance with $p < 0.01$. n=3 for all groups.

3.3 Rheological and Mechanical properties at initial and degraded time points

The viscosity of the high G block alginate solutions were statistically significantly higher than those of the high M block alginate solutions at all shear stresses tested, $p < 0.01$, see Figure 4A. All viscosities decreased with increasing shear, showing shear-shinning, with greater decreases being revealed for the high G block based solutions. At all shear stresses tested, the addition of ECM to the high M block alginate led to statistically significantly higher viscosities than those of the high M block alone solution, $p > 0.01$, while no difference was seen in the high G block alternatives.

Rheometry performed at day 1 confirmed gelation as the storage modulus was larger than the loss modulus for all hydrogels (loss modulus figures not reported). The storage modulus for the high G block alone hydrogel was significantly higher, $p< 0.01$, than that of the high M block alone hydrogel, 1.642 ± 0.112 KPa and 0.72 ± 0.047 KPa, respectively, see Figure 4B. Addition of ECM to the high G block alginate did not significantly change the storage modulus, however, ECM addition did significantly lower the storage modulus of the high M block hydrogel ($p<0.01$), 1.554 ± 0.075 KPa to 0.611 ± 0.028 KPa, see Figure 4B.

Over the 42 day degradation study, a general trend of reduced mechanical properties was found for all hydrogels. Comparison of the high G block alginate with and without ECM found no differences existed between the two groups for the compressive modulus at day 1, see Figure 4C. However, from day 7 to 28 the high G block hydrogel with ECM had a significantly higher compressive modulus ($p<0.01$), see Figure 4C. Comparison of the compressive moduli of the two high M block hydrogels found that the hydrogel containing ECM was not statistically different at day one, but was significantly higher from day 7 until day 28 ($p<0.01$), see Figure 4C. From day 28 until the end of testing no significant differences were found between these two hydrogels. Both high G and high M hydrogels containing ECM were found to have improved compressive modulus preservation over time than their alginate alone equivalents. A significant reduction in compressive modulus for the high G alone hydrogel occurred at day 21 (when compared to day 1), while the respective alginate with ECM hydrogel did not have a significant reduction (when compared to day 1) until day 28, see Figure 4C. For the high M alone hydrogel, it was found that a significant reduction in compressive modulus occurred at day 7 (compared to day 1), while for the respective alginate with ECM hydrogel this did not occur until day 14.

Comparison of dynamic moduli between hydrogel groups with and without ECM found no significant differences at any time-point between the high G hydrogels, with a significant decrease in dynamic modulus seen at day 21 between the high M hydrogels with and without ECM, see Figure 4D. The reduction in the dynamic modulus over the 42 day degradation period was minimal. The dynamic modulus of the high M alginate with ECM was found to be significantly higher than its alginate alone equivalent at day 21, but this was the only significant difference found in any of the groups.

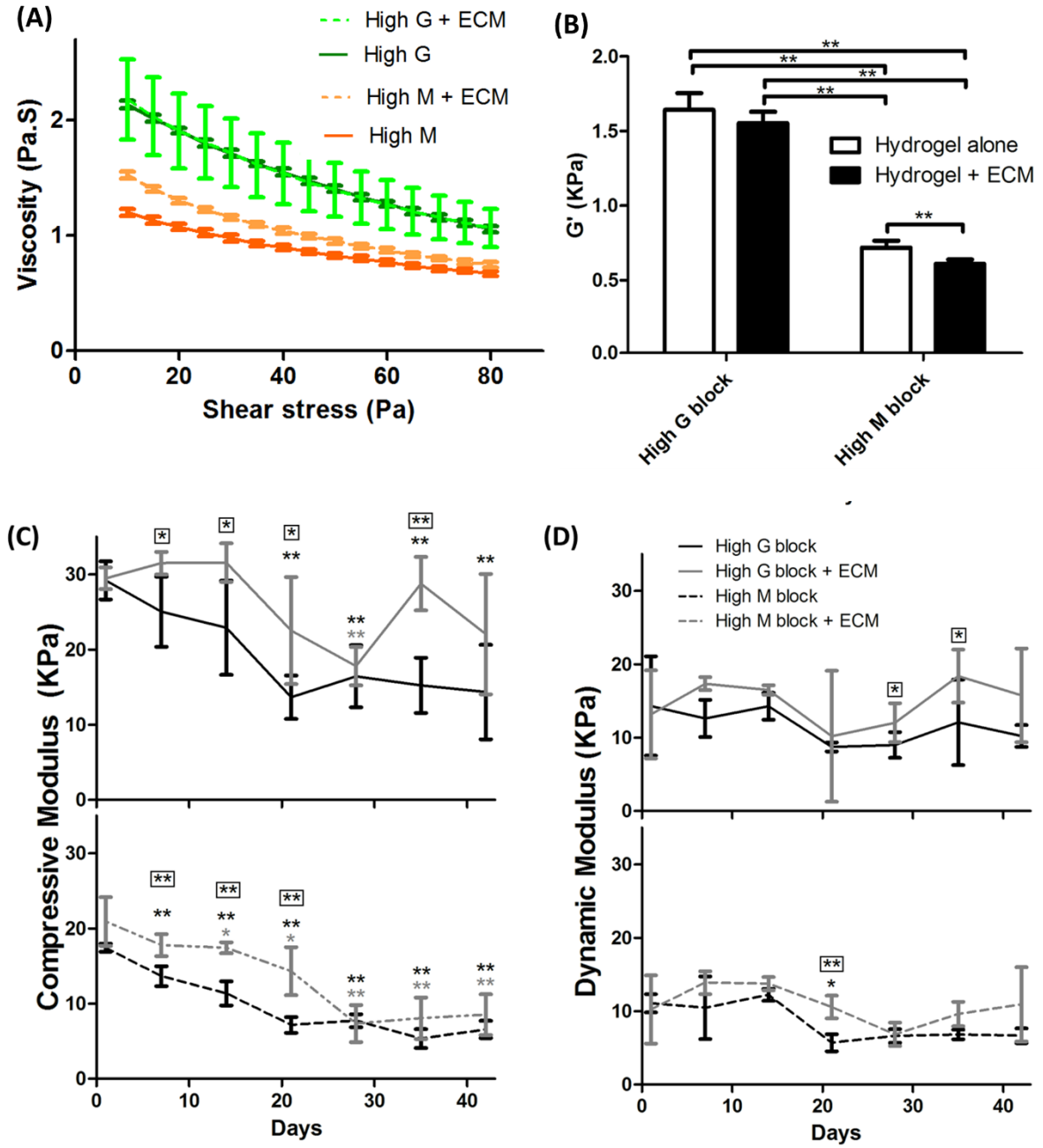

Figure 4: Viscosity and Mechanical properties of hydrogels. A) The viscosity of the high G block alginate solutions were statistically significantly higher than those of the high M block alginate solutions at all shear stresses tested, p >0.01. All solution viscosities decreased with increasing shear when

compared to baseline. Greater decreases were revealed for the high G block based solutions. (B) Storage moduli as measured at day one for all hydrogels. (C) Compressive modulus of hydrogels up to 42 days (D) Dynamic modulus of hydrogels up to 42 days. All samples were stored in Krebs-Henseleit buffer at 37 °C. Significance: * denotes statistical significance with $p < 0.05$** denotes statistical significant difference with $p < 0.01$. Unboxed asterisks denote significance between relevant time point and day one. Boxed asterisks denote significance between hydrogels alone and with ECM at that time point. n=5 for all groups.

3.4 Hydrogel Ejection through a Minimally Invasive Catheter

Hydrogel ejection through the catheter led to significantly higher ejection forces for all hydrogels tested when compared to ejection through the needle, $p < 0.01$, as a result of the long length profile of the catheter, see Figure 5A and B. This occurred for all hydrogels except for the high M block alginate alone hydrogel, see Figure 5A, where force remained below the 70N ejection force limit which we have identified. ECM addition to the high M block hydrogel led to statistically significantly higher ejection forces when compared to ejection forces for its respective hydrogel alone variant, $p < 0.01$, see Figure 5A.

Hydrogel ejection forces reached maximums of 58.79 ± 1.17 N and 57.85 ± 1.13 N for high G block hydrogels at 600 seconds, with and without ECM respectively, when ejected through the needle only. Ejection forces reached maximums of 144.46 ± 8.97 N and 163.28 ± 10.53 N for high G block hydrogels at 600 seconds, with and without ECM respectively, when ejected through the catheter. ECM addition to the high G block hydrogel had minimal effect on ejection force compared to its alginate alone variant; see Figure 5B. Ejection forces for hydrogels ejected through the needle and the catheter also increased statistically significantly with increased time after hydrogel mixing, $p < 0.01$, see Figure 5B. Ejection of the high G block hydrogels remained below the 70N limit until ~125 seconds, which corresponds with the time to gelation as shown in Figure 3E. Based on these results the author's recommend that the hydrogel solutions should be ejected through the catheter ≤ 2 minutes after preparation.

3.5 Compression moduli of Hydrogels after Ejection Through Catheter

Representative images of hydrogel samples 24 hours after preparation can be seen in Figure 5B. High M block hydrogels ejected through the catheter disintegrated during the 24 hour maintenance period between hydrogel ejection and mechanical testing. For this reason, the compression moduli are not reported as for these samples, see Figure 5D. High M block alginate alone hydrogel had a significant reduction in compressive modulus when injected through the needle only, see Figure 5D. Other high M groups had largely reduced properties at all time points, see Figure 5D.

High G block alginate alone hydrogel had a significant reduction in compressive modulus at 400 seconds when ejected through the needle, and at 600 seconds when ejected through the catheter, Figure 5D. Addition of ECM to the high G block alginate was found to have no statistically significant effect on the compression moduli of this hydrogel over extended time-points, Figure 5D.

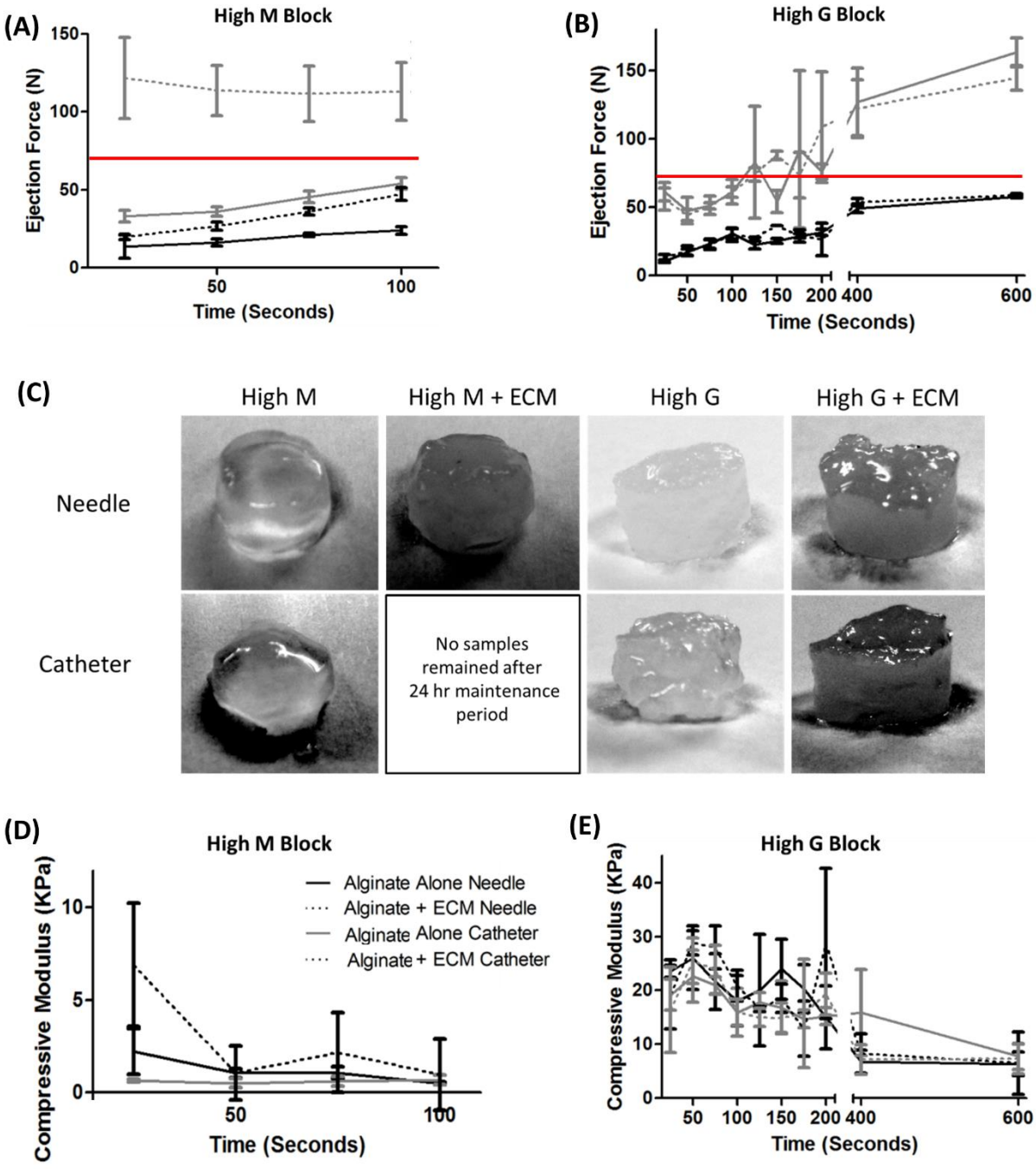

Figure 5: Representative images of hydrogels, ejection forces and compressive moduli of ejected hydrogels. Ejection forces for the high M block (A) and high G block (B) hydrogels ejected through the needle and catheter. Red line represents max allowable ejection force of 70N set by the authors. C) Representative images of hydrogel samples 1 days after ejection. High M block alone hydrogels had a

greater transparency than their high G block equivalent. The hybrid hydrogels were more opaque (off-white in colour) than their alginate alone counterparts. Compression moduli for the high M block (D) and high G block (E) hydrogels ejected through the needle and catheter. *Note High M Block Alginate + ECM Catheter group not included in (D) as samples disintegrated during the 24 hour maintenance period.

3.6 The effect of hydrogel degradation on dermal fibroblasts

The hydrogels did not dissolve or break down within this 24-hour culture period and no macroscopic differences were observed between the high M and G block hybrid hydrogels (data not shown). All fibroblasts groups exposed to hydrogel conditioned media were found to have a significantly increased metabolic activity 72 hours post exposure, compared to negative controls, see Figure 6A. Additionally, fibroblasts cultured with conditioned media from hydrogels containing ECM, and the high G block alone hydrogel, were found to have significantly increased metabolic activity at 24 hours, compared to negative control, see Figure 6A. When the metabolic activity of high G block hydrogels with and without ECM were compared to the negative control at both time-points, the alginate alone variant was significant ($p < 0.05$), while the ECM variant had a larger significance ($p < 0.01$), Figure 6A. Similarly, at 24 hours, the high M block hydrogel containing ECM had a significant increase in metabolic activity ($p < 0.05$) while the variant without ECM had no significant difference, Figure 6A. Both high M hydrogels had equal increases in metabolic activity, $p < 0.05$, at 72 hours.

In contrast to the increased metabolic activity demonstrated for all hydrogels, no increase in proliferation was found for any treatment groups compared to the blind controls, Figure 6B. Interestingly there was a significant decrease in proliferation in the high M + ECM group at 72 hours, $p < 0.05$.

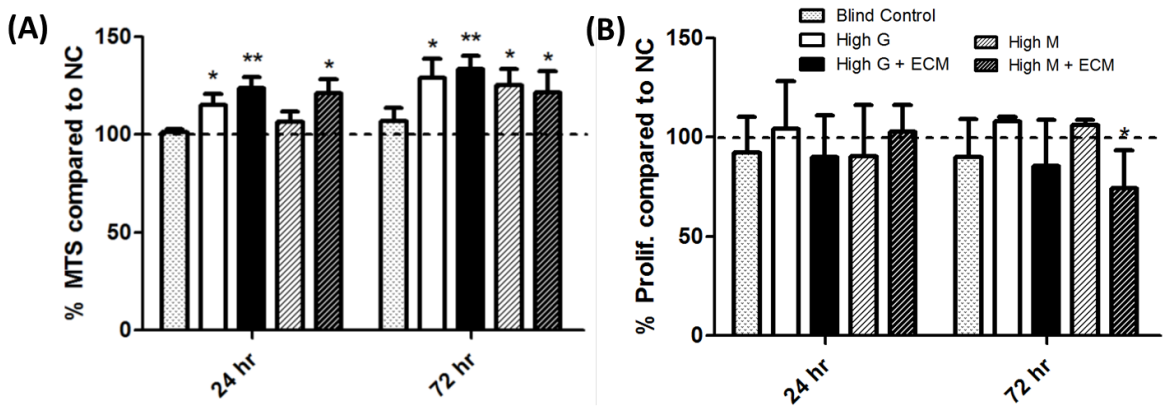

Figure 6: Metabolic activity and proliferation data indicating the impact of hydrogel degradation products on human dermal fibroblasts. (A) MTS and (B) proliferation analysis of dermal fibroblasts, at 24 and 72 hours, cultured with hydrogel conditioned media for 24 hours at 37 °C. Significance: * denotes statistical significant difference with $p < 0.05$. ** denotes statistical significant difference with $p < 0.01$. Results are given compared to negative controls, which are taken as 100% (represented by the dotted line). Positive controls (0%) are not shown. n=3 per group.

4. Discussion

Individually alginate and myocardial matrix based therapies are at the most advanced stages of commercial/clinical development for this potential treatment option[17,19,20]. However, despite these individual successes, the potential synergistic effect gained by combining the two therapies remains unexplored. In this study we have taken a translation step towards developing a hybrid alginate/ECM hydrogel suitable for cardiac stabilization via intramyocardial injection. We have successfully developed hybrid alginate/ECM hydrogels and have shown that the hydrogels have rheological properties appropriate for minimally invasive delivery and initial and degraded mechanical properties within a similar range of epicardially delivered alternatives such as Algysil (3-5 KPa)[17,29] which is used for cardiac stabilization. Furthermore, we have determined that they are not cytotoxic and are capable of enhancing the metabolic activity of dermal fibroblasts *in vitro*.

We have identified the high G block base alginate hydrogel as the optimal hydrogel, for this application, due to its superior storage and compressive modulii, as well as its ability to be injected through a minimally invasive catheter with reasonable force (<70N[48] for up to 2 minutes). The composition of G and M blocks in the alginate base is a critical factor affecting the physical properties of alginate and its resultant hydrogels[37,38], important factors when designing a hydrogel for cardiac stabilization. Interestingly, the high G block base alginate hydrogel has the most similar make-up to the alginate hydrogel used by Algysil. The hybrid high G block alginate/ECM hydrogels had an over two fold increase in storage modulus over previously modulated PEG/ECM hybrid hydrogels[30], and an over three hundred fold increase in storage modulus over unmodified cardiac ECM hydrogels[28]. This result suggests that a HF treatment option can be achieved by combining alginate and ECM particles. Although the high G block hydrogel formed in this study had a substantially higher storage modulus (1.5 kPa) than previously modified or unmodified myocardial matrix based hydrogels, they remained below the storage modulus (3-5 kPa) reported for Algisyl[29]. However, a higher compressive modulus than that reported by Larsen et al.[45] for a similar hydrogel was achieved. There currently exists a lack of specific details in the data published by Algisyl concerning parameters such as the molecular weight of the alginate used[16]. This makes it difficult to replicate the exact methodology used in the manufacturing process of commercial Algisyl, as such could potentially be the reason why differences

between the storage modulus or compressive modulus of the constructed high G hydrogel and Algisyl were found in this study. Furthermore, the compressive moduli for the G block materials is maintained when the material is ejected through catheter tested in this study. Differently, the modulus of the M block materials is significantly reduced when the material is ejected through the same catheter technology. Overall, the results demonstrate that materials produced were stable as they maintained a significant portion of their initial mechanical properties. An example can be seen with the hybrid high G material that maintained 74.8% of its initial compressive strength after 42 days. The results of this study clearly demonstrate that it is possible to produce a mechanically robust high G block alginate gel loaded with cardiac ECM particles.

Examination of the ejection force of high G block hydrogels revealed significantly increased ejection forces with increased in-catheter or in-syringe dwelling time. Nilson et al. report a maximum male pinch force of 70N[51] whereas Peebles and Norris found that the maximum female thumb pad press force of 133 ± 33.4N[50]. Ejection of the high G block/ECM hybrid hydrogels remained below the self-imposed 70N limit until ~125 seconds, which corresponds with the time to gelation. Based on these results the author's recommend that the high G block/ECM hybrid hydrogel solutions should be ejected through catheter ≤ 2 minutes after preparation. While minimally invasive delivery of ECM material has been demonstrated[20], this has yet to be exhibited for alginate therapies. Injectability of the material through the catheter is directly related to the gelation time and is an important feature to consider when designing a delivery device. The solution must gel rapidly at the site of injection in order to retain the therapy at the desired site, while premature gelation may block the catheter, rendering the therapeutic and catheter unusable, thus preventing many hydrogels from being delivered in a minimally invasive manner.

The results of the conditioned media study revealed that no cytotoxic effect was seen when dermal fibroblasts were exposed to media conditioned with hybrid hydrogels, and instead had significantly higher metabolic activity than those of the negative control. Furthermore, though fibroblasts treated with media conditioned with hybrid hydrogels led to higher increases of significance compared to negative control, it is interesting to note that conditioned media from the alginate alone hydrogels also led to significantly increased metabolic activity. This result shows that the hydrogels are inducing cellular metabolic activity which is not related to cell death or proliferation proving that they are not simply inert, in this case there would be no difference in metabolic activity to the controls. As dermal fibroblasts were used in this study (based on the ISO cytotoxicity assessment protocol (EN ISO 10993-5)), it is hard to ascertain what the impact of such increased metabolic activity might be *in vivo*. One possibility is that it may lead to increased connective tissue presence, and in turn increased scar thickness, as seen after the myocardial injection of alginate[25,53]. However, increased scar thickness does not necessarily correspond to attenuation of negative remodelling or increased cardiac function[54], so this does not specifically account for success of the Algisyl treatment. The cell experiments presented in this study were designed to determine if any negative cellular aspects would occur in response to the

developed hybrid hydrogels, not to explore potential positive effects. A future experimental programme related to these hydrogels would include *in vitro* experiments focused on the potential benefits that media conditioned by the hybrid high G hydrogel would have on cardiac fibroblasts and HL-1's. These include culturing hypoxia treated cells and quantifying cell growth factor and protein production.

Despite the promising results presented there were some limitations associated with our experiments including the limited insight into the effectiveness of whole heart decellularization and pooling of ECM particles. Use of tools such as Liquid Chromatography Mass Spectrometry (LC-MS/MS) would provide a more comprehensive depiction of the biochemical complexity and the potential of the resulting decellularized particles. LC-MS/MS would be of particular benefit here as it allows for the identification of important proteins and proteoglycans that have been shown to positively modulate cell function[55,56]. In addition to this assays that quantify elastin, growth factor, fibronectin and laminin content would be of use as suggested by Gilbert et al.[57].

This study serves as a translational step in evaluating the minimally invasive delivery of dual acting alginate-based hydrogels to the heart. In this study we have successfully developed new production methods for alginate/ECM hybrid hydrogels. We have identified that the high G block/ECM hybrid hydrogel has appropriate rheological and mechanical properties and can be delivered using a minimally invasive delivery device. Furthermore, the conditioned media study reinforced this research direction by revealing that this hybrid hydrogel was not cytotoxic, and instead had significantly higher metabolic activity than that of the control. Overall these results suggest that an effective minimally invasive HF treatment option could be achieved by combining alginate and ECM particles.

5. Conflict of Interest

All authors declare that they have no conflict of interest.

6. Acknowledgements

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Supplementary Material

Supplementary Materials and Methods

1.1 ECM preparation – Whole heart decellularization

Briefly, freshly harvested porcine hearts were retrieved and excess fat and tissue was trimmed, taking care to keep the atria and aortic root intact. Hearts were subsequently weighed before storing in a -80°C freezer for a minimum of 24hr. Prior to perfusion each heart was completely thawed in 3L of deionised water at 4 °C. A ½″ to ¼″ barbed reducer (# 27215, Qosina, USA) was inserted into the aorta, above the aortic valve, and secured with two cable ties. The heart was then submerged in deionised water to remove air. A ¼″ internal diameter silicone outflow tube (Masterflex L/S 17, Cole-Palmer, USA) attached to the roller pump (FH100 M, Thermo Scientific, USA) was then attached to the barbed reducer. The tubing had previously been filled with deionised water and was then secured using cable ties. Excess air was removed using an air release valve. 3L of hypotonic type one water (Consisting of 0.16% trizma hydrochloride (Sigma # T3253) and 0.589% ethylenediaminetetraacetic (EDTA) acid disodium salt dihydrate (Sigma # E5134) balanced at pH 8 with 1 mM sodium hydroxide (Sigma # 655104)) was then circulated at 0.4 L/min for 25 min. The solution was changed at 0, 5 and 15 min. 3L of 2X phosphate buffered saline (PBS) was then circulated at 0.7 L/min for 15 min, with this solution also being changed every 5 min. 3 L of type one water was then perfused through the heart at 0.75 L/min for 10 min. 3 L of a 0.02% trypsin (Sigma # T4049), 0.05% ETDA acid (Sigma # E9884) and 0.05% sodium azide (Sigma # S2002) solution was then warmed to 37 °C using a water bath. This solution was then perfused through the heart at 1.2 L/min for 1hr, 1.5 L/min for a second hour, and 1.8 L/min for a third hour, while remaining in the 37 °C water bath. 3 L of a 3% Triton X-100 (Sigma # X100), 0.05% ETDA acid (Sigma # E9884) and 0.05% sodium azide (Sigma # S2002) solution was then perfused at 2 L/min for 1hr at room temperature. This solution was then replaced before perfusion at 2.1 L/min for a further 1.5 hr. 3 L of a 4% deoxycholic acid (Sigma # 30970) solution was then perfused at 2.2 L/min for 3 hr.

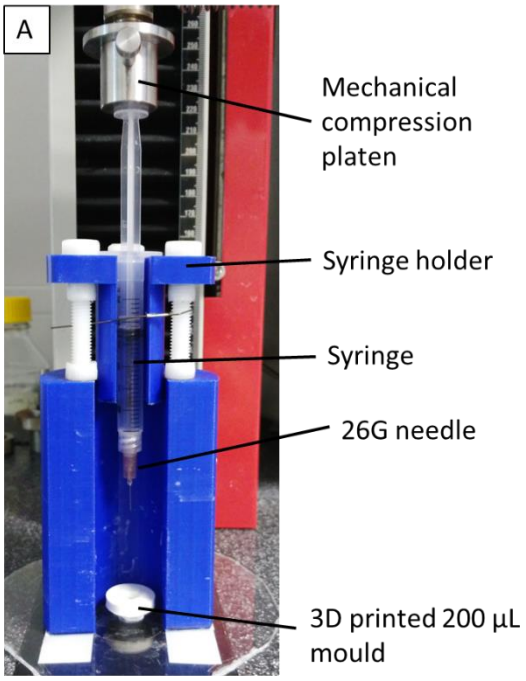
After each chemical perfusion, a two part rinse cycle took place to aid cell lysis and enable efficient removal of cellular debris and chemical residues. The rinse cycle consisted of perfusing 3 L of type one water for 10 min, followed by 3 L of 2 X PBS for 15 min. The perfusate was changed every 5 min for both solutions after every chemical wash at this stage. Disinfection of the decellularized matrix then took place. This was accomplished by perfusion with 3 L of a 0.1% peracetic acid (Sigma # 77240) / 4% ethanol (Sigma # 4935446) solution at 2.2 L/min for 1.5 hr. This was followed by a final rinse cycle consisting of perfusion with 5 L of 1X PBS for 15 min, followed by two 5 min washes with 3 L of type one water. This two part cycle was completed twice at flow rate of 2.2 L/min. Supplementary

Supplementary Table 1: Decellularization agents and their methods of actionSupplementary Table outlines the method of action of the decellularizaiton agents used in this protocol.

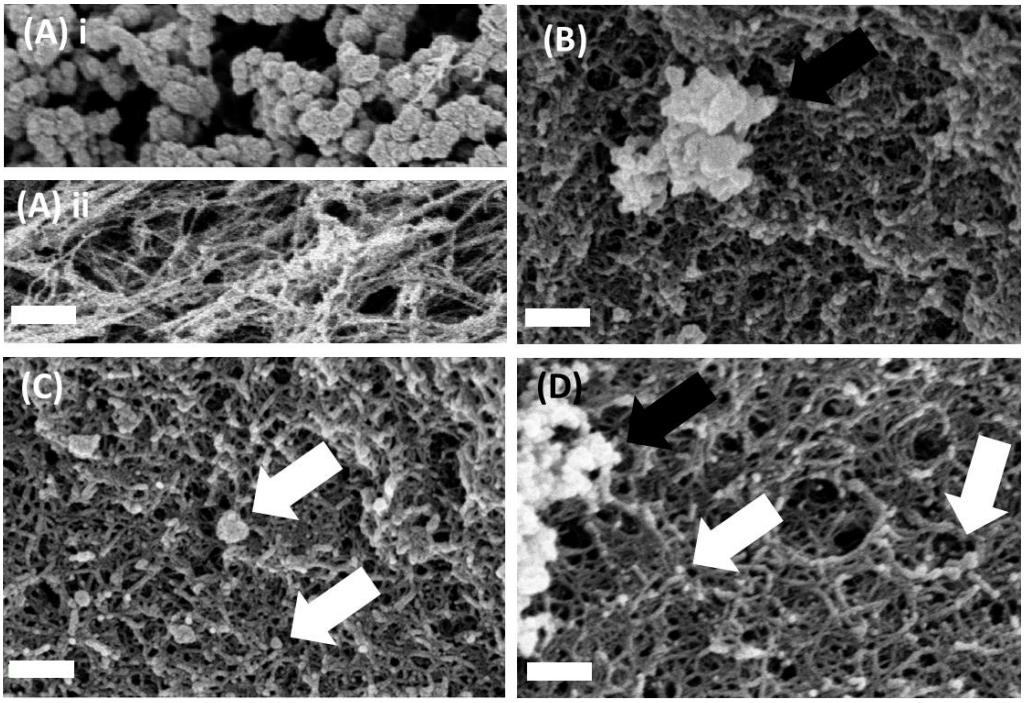
Supplementary Table 1: Decellularization agents and their methods of action (adapted from Crapo et al 2011[58]).

Agent	Mode of action
Hypotonic and hypertonic solutions	Induces cell lysis by osmotic shock and disrupt DNA-protein interactions. For maximum effect tissues are cycled through several hyper- and hypotonic cycles. Such solutions also help rinse cell residue from within the tissue following lysis
Trypsin	A type of protease, trypsin removes ECM constituents such as collagen, laminin, fibronectin, elastin, and GAG. Its disruptive effect on tissue ultrastructure can improve the penetration of subsequent decellularization agents. Therefore, exposure to trypsin in the early stages of a tissue decellularization protocol can aid the complete removal of cell nuclei from dense tissues.
Triton X-100	Disrupts DNA-protein interactions and lipid-lipid and lipid-protein interactions. To a lesser degree it also disrupts protein-protein interactions. Use of this detergent can result in some breakup of the ultrastructure and removal of GAG.
Sodium deoxycholate	Solubilizes cell and nucleic membranes. This makes it effective at removing nuclear remnants and cytoplasmic proteins from dense tissues. These actions can also disrupt the ultrastructure, remove GAG and growth factors, and damage collagen
Peracetic acid	Disinfection agent that doubles as a decellulariztion agent by removing residual nucleic acids with minimal effect on the ECM composition and structure.

Supplementary Figures



Supplementary Figure 1: Experimental set up for the ejection of hydrogel through needles and catheter.



Supplementary Figure 2: SEM characterisation of hydrogels with and without ECM. SEM images of high G type hydrogel, alone (A), and with ECM (C). The high G hydrogel without ECM hydrogel, (A), was found to have two distinct structural morphologies, type i and ii. These morphologies were found in approximately equal measure. Type i appeared to be crystalline nature and have a larger pore size, while type ii was more fibrous and had a smaller pore size. The high G with ECM hydrogel, (C), had a uniform and fibrous nature and ECM particles were visible throughout, see white arrows. (B) and (D) are SEM images of high M type hydrogels, alone, and with ECM particles, respectively. The high M hydrogel without ECM, (B), was fiberous in nature and undissolved calcium carbonate particles were seen to be present, see black arrow. Similarly, the high M gel with ECM, (D), was also found to host unreacted calcium carbonate crystals, indicated by the black arrow, as well as ECM particles, indicated by the white arrows. Scale bar 0.2µm.