Formation and characterisation of a modifiable soft macro-porous
hyaluronic acid cryogel platform

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A facile method for the synthesis of cell supportive, highly macro-porous hyaluronic
acid (HA) hydrogels via cryogelation is presented. Unmodified HA was chemically
cross-linked via EDC/NHS zero-length cross-linking at sub-zero temperatures to
yield cryogels with high porosity and high pore interconnectivity. The physical
properties of the HA cryogels including porosity, average pore size, elasticity and
swelling properties were characterised as a function of cryogelation conditions and
composition of the precursor solution. The HA cryogels swell extensively in water,
with the average porosities observed being \~90\% under all conditions explored.
The morphology of the cryogels can be controlled, allowing scaffolds with an
average pore size ranging from 18 ± 2 to 87 ± 5 μm to be formed. By varying the
cross-linking degree and HA concentration, a wide range of bulk elastic properties
can be achieved, ranging from \~1 kPa to above 10 kPa. Preliminary cell culture
experiments, with NIH 3T3 and HEK 293 cell lines, performed on biochemically
modified and unmodified gels show the cryogels support cell proliferation and cell
interactions, illustrating the biomedical potential of the platform.

**Keywords**: hydrogels; three-dimensional cell culture; cryogels; scaffolds; tissue
engineering; hyaluronic acid

1. Introduction

Synthetic and naturally derived hydrogels have been extensively explored as extracellular
matrix (ECM) mimics, suitable for numerous biomedical purposes, including \textit{in vitro} cell
expansion and as scaffolds for tissue engineering and regenerative medicine.\cite{1–3} The
incorporation of macro-pores into these materials adds tangible benefits over their non-macro-
porous counterparts for both \textit{in vivo} and \textit{in vitro} applications. The benefit of macro-porous
scaffolds in tissue engineering and regenerative medicine is exemplified by the promotion of
cell infiltration, vascular formation and tissue ingrowth.\cite{4,5} For \textit{in vitro} applications, macro-
pores provide significantly greater surface area per unit volume for cellular expansion and
allow the diffusion of metabolites and movement of cells throughout the structure.\cite{6,7}

Macro-porous hydrogels can be formed via a number of methods, which include salt and
other physical porogen templating,\cite{8,9} gas foaming,\cite{10,11} emulsion templating

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[12] and increasingly through 3D fabrication techniques.[13] In addition, cryogelation, the process of setting hydrogels below the freezing point of the solvent employed, is an attractive method. Under cryogelation conditions, i.e. temperatures below the solvent melting point, the majority of the hydrogel precursor solution solvent forms a crystallised solid phase, while a liquid micro-phase is maintained.[14,15] In the liquid micro-phase, the gel forming molecules are concentrated (cryo-concentration) and chemical cross-linking is able to occur.[16] Cryogels are inherently interconnected, due to the continued growth of solvent crystals until their collision. Since the ice crystals act as the porogens, there is no need to use potentially harmful or difficult to remove solvents and particles to create the desired open porous structure, which is often the case with other pore forming techniques.[15,17] Cryogels have been investigated for use in biomedical applications such as the replacement of bone,[18,19] cartilage,[20,21] neural[22] and skin tissue.[23] Despite the interest in cryogel scaffolds for use in these applications, there is still significant room for further understanding, particularly concerning the physical and chemical properties of these gels and how they can be optimised for specific applications. A platform to methodically investigate these parameters is therefore important. Additionally, the ability to tailor the biophysical properties of macro-porous hydrogels is fundamentally important to best mimic the natural cellular environment and ultimately optimise scaffolds for their application, whether for cell expansion in vitro or in vivo tissue engineering or regeneration.

As current tissue engineering methods are heavily focused on the use of biodegradable scaffolds combined with appropriate cell sources and biochemical signals to induce tissue regeneration, hyaluronic acid (HA) cryogels with modifiable physical and chemical properties are seen as highly advantageous.[24] HA plays an important role in angiogenesis,[25] is readily available and constitutes a substantial proportion of the native ECM, and therefore appears highly suitable for use in cell supportive scaffolds. HA has previously been cryogelled with other polymers, such as gelatin, silk, fibroin, chondroitin sulphate and collagen, to form co-gels.[26–28] However, the independent use of unmodified HA in this system removes the variability inherent in these biological polymers and enables more straightforward decoupling of the cellular responses from the scaffold properties. The cryogelation of HA differs from freeze drying and solvent evaporation techniques, in that cross-linking occurs while the precursor solution is being frozen. Non-macro-porous HA hydrogels have been formed using 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC)/N-hydroxysuccinimide (NHS) previously; however, stable gels were only formed after three days of solvent evaporation, prior to cross-linking, which resulted in stiff non-macro-porous HA discs.[29] Macro-porous HA scaffolds have been formed by cross-linking and freeze drying; however, pores were limited to a maximum of 20 μm in diameter.[30] Larger pores have been achieved by the freeze-drying method with HA and collagen; however, these scaffolds had porosities of less than 70%, significantly lower than is achievable by the cryogelation process.

Here, we provide a detailed study of the mechanical and morphological properties of a purely HA-based cryogel system that is derived via zero-length cross-linking of unmodified HA. This system exhibits an easily reproduced material platform with a range of physical properties that covers the range of soft tissues. In addition, we exploit methods for the incorporation and presentation of bioactive molecules as well as rapid cell loading.
2. Materials and methods

2.1. Materials

High (1500 kDa) and low (60 kDa) molecular weight sodium hyaluronate (HA) was purchased from Lifecore Biomedical (Landec Corporation), Minnesota, USA. 1-Ethyl-3-(3-dimethylaminopropyl)carbodiimide (EDC), N-hydroxysuccinimide (NHS), N-hydroxysulfosuccinimide sodium salt (sNHS), 2-(N-morpholino)ethanesulfonic acid (MES), 5(6)-carboxyfluorescein diacetate N-succinimidyl ester (CFSE), streptavidin-FITC from *Streptomyces avidinii*, paraformaldehyde and phosphate buffered saline (PBS) tablets were purchased from Sigma Aldrich, New South Wales, Australia. Dulbecco’s modified Eagle’s medium (DMEM), Roswell Park Memorial Institute 1640 medium (RPMI), foetal bovine serum (FBS), GlutaMAX™ (Gibco®), Antibiotic–Antimycotic (Gibco®), Alexa Fluor® 594 Cadaverine, alamarBlue® and fibronectin (FN) (human) were purchased from Life Technologies Australia (Mulgrave, Victoria, Australia).

DMEM and RPMI were supplemented with 10 vol% FBS, 1 vol% Antibiotic–Antimycotic and 1 vol% GlutaMAX™. Ethanol (undenatured 100%) was obtained from Chem-Supply (Gillman, South Australia, Australia). Ethandiol (Ajax Finechem) was obtained from Thermo Fisher Scientific Australia (Scoresby, Victoria, Australia). C (RGDFK) peptide was purchased from Peptides International (Louisville, Kentucky, USA). Dapi Fluoromount™ was purchased from ProSciTech (Kirwan, Queensland, Australia). Biotinylated bovine serum albumin was prepared in-house at the Commonwealth Scientific and Industrial Research Organisation (CSIRO), Materials Science and Engineering, Clayton, Australia using Sulfo-NHS-LC-biotin (ThermoScientific) and following the manufacturer’s instructions (http://www.piercenet.com/instructions/2161776.pdf). In brief, this involved a two-step procedure where BSA was added to 10 mM Sulfo-NHS-LC-biotin and the excess biotin was removed by dialysis. All water used, unless specified, was purified by Milli-Q water purification system (Merk Millipore, Massachusetts, USA).

2.2. Cryogel fabrication

HA cryogel scaffolds were prepared by EDC- and NHS-mediated zero-length cross-linking of the HA polymer chains. Briefly, either high or low molecular weight HA was dissolved in water at 4 °C. EDC at a concentration of 0.4 M and NHS at 0.5 M were added in a 2:1 molar ratio, depending on the desired number of cross-links per disaccharide repeat unit. Precursor solutions were vortexed for 10 s then sandwiched between two glass slides, using a one millimetre thick acrylic polymer spacer to keep a consistent distance between the glass surfaces. The glass slide moulds were then placed in a circulating cooling bath at one of three different temperatures (−12, −16 or −18 °C) and maintained at the chosen temperature for the duration of the cross-linking period. Precursor solutions were left for 72 h to ensure comprehensive cross-linking. Gels were thawed in excess water, removed from the glass moulds and then stored in PBS at 4 °C.

2.3. Swelling properties and gel fraction yield

HA cryogels were dried under vacuum (Dynavac FD5 vacuum freeze drier, Boston, MA, USA) for 48 h and then weighed to determine their dry mass ($m_d$). Equilibrium-swelling ratios were calculated using Equation (1), where wet mass ($m_w$) was determined from cryogels swollen in PBS for 12 h at room temperature.
Equilibrium swelling ratio $= \frac{(m_w - m_d)}{m_w}$ \hspace{1cm} (1)

Gel fraction yield was also determined gravimetrically. Once cryogel samples had been returned to room temperature, the samples were weighed to determine an expected dry mass ($m_d$) based on the polymer content of the cryogels prepared. Samples were washed in water for 5 days at room temperature and then dried under vacuum, to determine a dry mass ($m_d$). Gel fraction yield was then calculated using Equation (2).

\[
\text{Gel fraction yield} = \frac{m_d}{m_e}
\] \hspace{1cm} (2)

2.4. Morphology analysis

The morphologies of the prepared cryogels were assessed by environmental scanning electron microscopy (ESEM, FEI Qanta 200, Oregon, USA) and confocal microscopy (CLSM, Leica TCS SP2, Wetzlar, Germany). Samples for ESEM required no additional preparation and were imaged at 2 °C under low vacuum at 12.5 kV with a spot size of 3 mm. CLSM samples were prepared by the incorporation of fluorescently labelled HA into the precursor solution prior to cryo-cross-linking. HA was labelled with Alexa Fluor 594 cadaverine, through EDC/sNHS cross-linking. Briefly, 10 mg of HA was dissolved in excess MES buffer, to which 100 μl of EDC (50 mM stock solution), 50 μl of sNHS (50 mM solution) and 270 μl of Alexa Fluor 594 Cadaverine (2 mM stock solution) were added. The solution was then vortexed and left at room temperature for 16 h. After cross-linking overnight, the HA solution was transferred into dialysis tubing and placed in excess water. After 4 days of purification in dialysis tubing at room temperature, the HA solution was transferred to a 15 ml polypropylene conical base centrifuge tube and submerged in liquid nitrogen. The frozen HA sample was then vacuum dried to obtain the fluorescently tagged HA. Fluorescently labelled HA was incorporated into precursor solutions at 1%w/w of the total amount of HA used in a given cryogel preparation. Image analysis was performed on labelled cryogel scaffolds, vertically cross sectioned, as described by Dainiak et al., using ImageJ (1.48i) software and Granulometry (D.Prodanov at lumc.nl) and Voxel Counter plugins (W.Rasband, wsr at nih.gov).[23] The Granulometry plugin was used to estimate the pore size and wall thickness across samples for three areas throughout the height of the gel. The granulometric filtering plugin is used to identify structures of a certain size range by applying sieves of increasing size to the image, removing structures that match or are smaller than the sieve at a given sieve size.[23]

Cryogel porosity (method 1) was estimated using the Voxel Counter plugin, whereby image stacks of 50 μm were made binary and a ratio of gel pixels to non-gel pixels was determined.[23] In addition to image analysis, cryogel porosity was calculated gravimetrically (method 2) using Equation (3), where the vapour-hydrated mass ($m_v$) was determined from cryogels hydrated with water vapour (method 2). Hydration was achieved by storing gels at 37 °C in a sealed humidifying chamber (which only hydrates the gel portion of the cryogels without filling the pores).[14] Upon reaching equilibrium, determined by cryogels re-obtaining their shape and external dimensions, vapour-hydrated cryogels were weighed and assigned a vapour-hydrated mass ($m_v$) for porosity determination.

\[
\text{Porosity} = \frac{(m_w - m_v)}{m_w}
\] \hspace{1cm} (3)
2.5. **Rheological measurements**

Mechanical properties of HA cryogels were tested with an ARES controlled strain rheometer (Rheometric Scientific). Plate–plate (50 mm) geometry was selected with gap in the range of 1.1–1.3 mm. Frequency sweeps ($10^2$–$10^{-1}$ rad/s) were performed at 2% strain at 25 °C. The storage modulus of the cryogels was recorded as the average of the storage modulus ($G'$) across the linear region between 1–10 rad/s.[31] A minimum of four replicates for each sample type was used.

2.6. **Scaffold bio-functionalisation**

Prior to coupling reactions and/or cell culture use, cryogels were cut with a circular 6 mm diameter biopsy punch and washed extensively in 80% ethanol. Scaffold bio-functionalisation was qualitatively investigated with biotinylated bovine serum albumin (biotinylated BSA). Cryogels were first washed in 0.1 M 2-(N-morpholino)ethanesulfonic acid (MES) buffer at pH 5.5. Gels were then activated with 50 μl of EDC and NHS (80 mM EDC and 40 mM NHS) in MES buffer. Cryogels were incubated in the EDC reaction solution for 25 min at room temperature. Following activation, cryogels were washed in MES buffer and transferred to 100 μl biotinylated BSA solution (54 μg/ml) and incubated at 4 °C for 16 h. Control gels were also swollen in biotinylated BSA. Biotinylated BSA-modified HA cryogels and control HA cryogels were incubated in streptavidin–FITC for 6 h. The cryogels were then washed in 50 ml of PBS overnight prior to imaging. CLSM was used to assess the qualitative extent of cryogel bio-functionalisation.

2.7. **In vitro cell culture**

HeLa cells were fluorescently labelled with CFSE cell tracking reagent by incubating washed cells in a 20 μM CFSE solution in PBS at 37 °C for 15 min. Labelled cells were washed in PBS and re-suspended in DMEM containing 10% serum at a concentration of 2 million cells/ml. Before cell loading, HA cryogels were partially dehydrated with sterile gauze. A 10 μl cell suspension of labelled cells was then pipetted onto the gels.

Human embryonic kidney cells (HEK 293 cells) were used to assess cell viability and cell-surface interactions in HA cryogels. For this purpose, HA cryogels were primed using the same bio-functionalisation protocol (subsection 2.6. Scaffold bio-functionalisation); however, 25 μl of fibronectin (FN, 20 μg/ml) or c(RGDfK) (c-RGD, 20 μg/ml) was used in place of b-BSA. For the assessment of cell viability, a number of control cryogel types were prepared in addition to FN and cRGD-coupled gels. These included cryogels prepared using the same bio-functionalisation method using FBS, cryogels swollen in FN (20 μg/ml) and finally unmodified control cryogels swollen in 5% RPMI. All cryogels were washed extensively in PBS before finally being washed and stored in 5% FBS in RPMI and left for 16 h at 37 °C prior to use. Cryogels were partially dehydrated with sterile gauze and placed into the wells of a Corning® Costar® 96-well cell culture plate. The semi-hydrated cryogels were then loaded with 25,000 cells in 20 μl of 5% RPMI and incubated at 37 °C for four hours. Following this four-hour incubation period, 80 μl of 5% RPMI was added to each well. On day 5, 10 μl of AlamarBlue® cell viability reagent (Invitrogen, Life Technologies) was added to each cryogel containing well. After four hours of incubation at 37 °C, 50 μl of liquid was drawn from each well and placed into a
new 96-well plate. The new 96 well plate was then analysed at 570 and 600 nm in a UV–vis micro-plate reader (Cary® 50 UV–vis Spectrophotometer, Varian). Tukey’s HSD (honesty significant difference) test in conjunction with an ANOVA was used to test the difference between the means of each group analysed. A corrected \( p \)-value <0.05 was predetermined to indicate significance. For the assessment of cell-surface interactions, HEK-293 cells were fluorescently labelled with CFSE cell tracking reagent by incubating washed cells in a 20 \( \mu \)M CFSE solution in PBS at 37 °C for 15 min. Fluorescently labelled cells were loaded following the same protocol described above. On cell culture day 2, cell loaded cryogels were washed gently with PBS and incubated in 4% paraformaldehyde overnight. The following day, gels were washed in PBS and swollen in DAPI Fluoromount™. Cryogels were imaged using CLSM (Leica TCS SP2).

3. Results and discussion

3.1. Preparation

HA cryogels were prepared by cross-linking unmodified HA with the zero-length cross-linker, EDC. HA, modified by the incorporation of hydrazide or aldehyde groups,[32] is commonly used for the formation of HA hydrogels, as unmodified HA hydrogels cross-linked at room temperature possess unsuitable mechanical properties.[33,34] Due to the cryo-concentration of HA in the liquid micro-phase, which occurs during solvent crystallisation, and subsequent cross-linking of the densified polymer, HA does not need to be modified to form mechanically robust and stable cryogels. The cross-linking of unmodified HA in this system is a significant advantage, as it both removes the need for additional synthetic steps and does not require the use of potentially harmful compounds to introduce crosslinkable groups.

Three temperatures −12, −16 and −18 °C were used to assess the impact of temperature upon cryogel fabrication. In addition to variations in temperature, HA content (mass/volume%, wt%) and cross-link degree (% cross-linking) were also explored. The cross-link density was reported as a percentage of the number of theoretical hydroxyl groups available and the number of EDC molecules provided.

Precursor solutions containing 5 wt% and 8 wt% HA were prepared with 60 kDa HA, and 2 wt% gels were prepared with 1500 kDa HA. The lower molecular weight HA was shown to produce unstable gels at 3 wt%, which dissolved in culture media at 37 °C over 5 days. In contrast, high molecular weight HA could not be used to generate gels with comparatively high HA content (>3 wt%) due to the high viscosity of the solutions and the difficulty in efficiently preparing and handling the solutions. The lower boundary of the HA content (wt%) (i.e. the wt% below which no stable gels are obtained) for the 60 kDa gels suggests that polymer chain entanglements in addition to polymer cross-linking play a role in gel stability. This assumption is supported by the stability of the high molecular weight HA gels at low HA percentages.

3.2. Morphology

The morphology of the prepared cryogels was investigated using ESEM and CLSM. Light microscopy proved to be inappropriate for pore size analysis due to the transparent nature of the hydrogel walls and the difficulty in discriminating between unrelated and related pores (Supplementary material SI.1). Images obtained via CLSM were used
to estimate the degree of porosity, pore size and wall thickness (Table 1), while the greater depth of field presented in ESEM micrographs shows the mesh-like morphology of the cryogels and confirms the substantial pore interconnectivity in the samples analysed (Figure 1).

As the temperature of cryo-cross-linking is dropped, ice crystals form more rapidly, resulting in the formation of a greater number of smaller pores.[15,35] Pore sizes calculated from ESEM and CLSM images confirm the impact temperature had on morphology. Across the relatively small temperature range investigated (−12 to −18 °C), both imaging techniques show pore size range was significantly influenced by the temperature of cryo-cross-linking (Figure 1). The average pore size for cryogels of 5 wt% HA with 30% cross-linking set at −12, −16 and −18 °C was 79 ± 4, 40 ± 4 and 18 ± 2 μm, respectively, based on semi-automated image analysis. The pore size distributions and images (Figure 1) underline the inherent and normal heterogeneity in scaffold morphology observed within a single cryogel type. Such heterogeneity is normal for macroporous hydrogel-based scaffolds, irrespective of the technique employed to induce large pores and the method of image analysis.[22,23,36,37] Wall thickness was affected by cryogelation temperature. Cryogels with 5 wt% HA and 30% cross-link degree, gelled at −12, −16 and −18 °C, show wall thicknesses of 13 ± 1, 10 ± 1 and 6 ± 1 μm, respectively (Figure 1).

The ability to manipulate average pore size, and to a lesser extent wall thickness, is of particular interest in the context of cell culture and tissue engineering applications. As previously discussed, pore size and more broadly scaffold morphology have been shown to play an important role in cell infiltration, vascular formation and tissue ingrowth.[4,5] Purportedly, the significance of pore size and its role in angiogenesis varies based on the target tissue.[38,39] Additionally, the ability to control these properties opens up the potential for optimisation of pore size depending on the intended application and experimental outcomes.

CLSM image analysis indicated the degree of cross-linking did not show any significant impact on pore size and wall thickness; however, HA weight percentage was shown to have a noticeable effect on pore size. This is most evident when comparing HA cryogels set at −16 °C with 3 wt%, 5 wt% and 8 wt% HA (low molecular weight), which had an average pore size of 87 ± 5, 40 ± 4 and 21 ± 1 μm. Similarly, when HA molecular weight was increased, but total wt% kept constant, the average pore size was significantly decreased. Low molecular weight HA gels of 3 wt% had an average pore size of 87 ± 5 μm, while high molecular weight gels of the same weight percentage had an average pore size of 16 ± 1 μm. As detailed by Ivanov et al., increasing polymer content decreases the volume of free solvent (in this case water) that can be

Table 1. Pore properties of HA cryogels prepared at different temperatures.

| Characteristics | Pore diameter (μm)\(^a\) | Porosity (%) |
|-----------------|--------------------------|--------------|
| Cryogelation temperature (°C) | HA wt% | Cross-link degree % | Method 1\(^a\) | Method 2\(^b\) |
| −12 | 5 | 30 | 79.8 ± 4.4 | 94.8 ± 0.4 | 91.3 ± 1.0 |
| −16 | 5 | 30 | 40.2 ± 4.4 | 95.0 ± 0.4 | 90.9 ± 2.1 |
| −18 | 5 | 30 | 17.7 ± 2.2 | 92.2 ± 0.9 | 91.7 ± 0.7 |

\(^a\) Pore diameter and porosity as obtained from CSLM images using ImageJ (n = 3, mean and standard deviation are reported).

\(^b\) Porosity as measured by swelling response in water.
crystallised and so form pores at a given temperature.[40] This is supported by the noticeable increase in HA solution viscosity, resulting from both increased weight percentage and molecular weight of the HA.

Figure 1. (A–C), CLSM images of fluorescently labelled 5 wt% HA and 30% cross-link degree cryogels set at −12, −16 and −18 °C, respectively, and ESEM micrographs (D–F) of the same gels. Scale bar 100 μm. (G) Pore size and (H) wall thickness distributions of 5% HA (30% cross-link density) cryogels set at three different temperatures. The average pore size and standard deviation across three samples for each data set are also indicated on the graphs.
In addition to pore size and wall thickness, CLSM image z-stacks were used for the quantification of percentage porosity. All HA cryogels were shown to have high percentage porosity (~90%) irrespective of cryogelation temperature, HA content and degree of cross-linking. Additionally, the porosity of cryogel samples was determined by swelling using Equation (2). Conventionally, squeezed mass is used as part of this calculation as it seeks to represent the mass of the hydrated gel component of a cryogel with empty macro-pores.\cite{41} The vapour-loaded mass of cryogels (which was used in place of squeezed mass) was deemed a more suitable measurement since it removes user bias from the squeezing method. The results complement the data acquired through CLSM image analysis, with all samples determined to have porosity of 90% and above, reiterating their highly porous nature. Variations in HA percentage, cross-linking density and temperature did not significantly impact porosity (Table 1).

### 3.3. Swelling properties

As a result of their hydrophilic open pore structure, HA cryogels show rapid swelling and de-swelling properties which are comparable to those of a sponge. Typical of cryogels, HA cryogels reach their equilibrium-swelling ratio in less than 5 min.\cite{42,43} Storage of these samples overnight in PBS had no significant impact upon their equilibrium-swelling ratio. As summarised in Table 2, swelling is primarily impacted by polymer content and cross-link density. The impact of cross-link density is most obvious and statistically significant when comparing gels of 10% and 50% cross-link degree ($p < 0.02$). The more densely cross-linked gels show less swelling, an observation shown to occur at all investigated temperatures. Likewise there is a significant difference between gels of varying HA wt% (Table 2), where an increase in HA content from 5 to 8% is shown to reduce the swelling degree. ($p < 0.05$) Due to the greater HA content, we expect the cryogel of 8% HA to have a tighter hydrogel network structure and hence expect the observed lower swelling degree. Cryogels of 8% HA have a smaller pore microstructure than cryogels formed with 5% HA, which may contribute to the lower swelling degree. The impact of pore size, however, does not appear to have a significant impact on swelling degree, as there is no significant difference in the swelling nature of cryogels formed at $-12$, $-16$ and $-18$ °C. Indeed, cryogelation temperature was shown to have no statistically significant impact upon swelling degree, when HA wt% and cross-link degree were kept constant.

In addition to swelling degree, the gel fraction ratio of HA cryogels was also investigated. As is typically observed in cryogels, HA cryogels mostly showed very

### Table 2. Effect of HA content and cross-linking on swelling of HA cryogels.

| Cryogelation temperature (°C) | HA percentage (wt%) | Cross-link degree | Swelling degree | Standard deviation ($n = 3$) |
|-------------------------------|---------------------|------------------|------------------|-----------------------------|
| $-16$                         | 5                   | 10               | 37.3            | 3.0                         |
| $-16$                         | 5                   | 50               | 28.1            | 1.2                         |
| $-16$                         | 3                   | 30               | 56.9            | 6.8                         |
| $-16$                         | 5                   | 30               | 35.0            | 2.2                         |
| $-16$                         | 8                   | 30               | 26.1            | 4.8                         |
| $-12$                         | 5                   | 30               | 30.6            | 5.0                         |
| $-18$                         | 5                   | 30               | 26.3            | 7.0                         |
high gel fractions, greater than 90%.[44,45] The impact of cross-linking degree indicated that at 10% cross-linking, the gel fraction yield is deleteriously impacted. Cryogels of 10% cross-linking (set at −16 °C) showed an average gel fraction yield of 78.5% compared to gels of 20% and 30% cross-linking degree that had a gel yield greater than 95%. The lower gel fraction yield is likely due to insufficient cross-linking. The cryogelation temperature, HA% and HA molecular weight showed no significant impact upon gel fraction yield.

3.4. Mechanical properties

The mechanical properties of bioengineered materials have been shown to play an important role in cellular functions such as differentiation, proliferation and migration.[46,47] Consequently, there is an interest in the ability to manipulate these properties based on the intended use of the scaffold. HA cryogels were tested with a controlled strain ARES rheometer to describe the range of mechanical properties achievable and the impact cryogelling temperature, cross-linking density, and HA wt%. Hydrogel and cryogel samples under dynamic mechanical analysis typically show a $G''$ (loss modulus), a magnitude lower than $G'$ (elastic modulus), which was observed across all samples analysed here.[48] In the case of cryogels and other types of macroporous hydrogels, mechanical properties are dependent on both the composition of the precursor solution (Figure 2) and the porous morphology of the gel. As discussed previously, lower cryogelation temperatures result in smaller and more numerous pores. The relationship between cryogels set at −12 °C and their counterparts set at −16 °C suggests the smaller pores result in a greater storage modulus (Figure 2). However, assessment of the mechanical properties of gels prepared at −18 °C does not support this assumption, as mechanical properties were not enhanced by diminishing pore size. The observed drop-off in storage modulus at −18 °C may be explained by insufficient cross-linking at the lower temperature or due to the thinner pore walls (Figure 1).

![Figure 2. Storage modulus of 5% HA cryogels versus cross-linking degree gelled at −12 °C (solid), −16 °C (striped), −18 °C (no fill) (n = 4). Cryogelation time 72 h.](image)
−18 °C, the efficiency of the EDC/NHS cross-linking could be sufficiently impeded, compared to cryogels set at −16 °C, to result in weaker gels following the designated cross-link period. Additionally, it is possible at −18 °C the pore walls are thinner to such an extent that mechanical stability suffers. To assess this, a precursor solution of 5 wt% HA and 30% cross-linking was gelled at −18 and −16 °C and investigated after 24, 72 and 168 h of cross-linking (Figure 3). After 24 h, cryogels set at −18 °C produced weak and clear gels, which were unable to maintain their structure and were unsuitable for mechanical analysis. In contrast, solutions set at −16 °C produced typical spongy opaque and mechanically stable cryogels after 24 h. The weak and transparent nature of the cryogels set at −18 °C over 24 h was indicative of inefficient cross-linking indicating cross-linking efficiency is limited at −18 °C over 24 h.

After 72 and 168 h of cross-linking, the desirable opaque sponge like cryogels were formed at both −16 and −18 °C. Mechanical measurements of gels set at −18 °C showed markedly improved properties for gels set for 168 h compared to gels set for 72 h, although they were still weaker than the gels formed at −16 °C. In contrast, no significant difference was calculated between gels set at −16 °C cross-linked for 72 or 168 h (Figure 3). Cryogels set at −12 °C over 72 and 168 h, like gels set at −16 °C, showed no significant difference in storage modulus \(p < 0.05\) T-Test). This demonstrates the cross-linking rate is inhibited at lower temperatures (Figure 3), and to achieve sufficient cryo-cross-linking with EDC/NHS at low temperatures, longer incubation times are required.

Figure 4 shows the impact of HA percentage on the bulk storage modulus of HA cryogels. As HA % is increased, bulk storage modulus also increases. However, as previously discussed, an increase in HA % decreases the average pore size. It would be expected that gels with a tighter, smaller micro-structure would have higher bulk mechanical properties. It is therefore not possible to attribute the increase in storage modulus entirely to HA wt%.

Figure 3. Storage modulus of 5% HA cryogels (30% cross-linked) set at −16 °C (striped) and −18 °C (no fill) after 72 h and 168 h of cross-linking \(n = 4\).
3.5. Bio-modification and cell loading

A method for coupling of proteins and growth factors to the internal cryogel surface was explored to expand the potential of the unmodified HA cryogels as scaffolds for use in cell culture and tissue engineering applications. To maintain the simplicity of the described platform, protein coupling was applied post-fabrication using a similar chemistry used in the formation of the gels. To qualitatively confirm the bioactivation of HA cryogel surfaces, a biotinylated bovine serum albumin (b-BSA) solution was used in the coupling reactions. As shown in Figure 5(A), probing with streptavidin-FITC and subsequent CLSM analysis indicated the coupling reaction utilised was successful in coating HA cryogel surfaces with b-BSA, which demonstrates the potential to decorate the porous structures of the cryogel scaffolds with bioactive molecules. A control experiment in which a cryogel was swollen in b-BSA showed that without the coupling reaction, the gel surface was not stably coated with b-BSA (Figure 5(B)). This protein coupling method is broadly suitable for surface modification of the hydrogels, as shown in the two examples (i.e. FN and cyclic-RGD (c-RGD) surface modification) discussed below.

One limitation often encountered in the practical application of porous cellular scaffolds is finding an efficient cell loading method so as to best exploit the vast internal surface area of the gels. To efficiently load cells throughout the cryogel scaffolds obtained in this study, their rapid swelling properties were exploited. Briefly, fluorescently labelled cells were spun down and re-suspended to a concentration of 2,000,000 cells/mL. Cryogel samples were partially dehydrated and then loaded with the concentrated cell suspension (10 μL suspension per cryogel with the following dimensions: Ø 6 mm × 1 mm), resulting in almost instantaneous hydration of the gel and highly efficient distribution of cells throughout the gel. As presented in Figure 6(C), the labelled cells can be seen evenly distributed throughout the scaffold, thanks to the
large and highly interconnected macro-pores. As the scaffold is dehydrated and then rehydrated with the concentrated cell suspension, the cells are efficiently distributed throughout the gel. This method of cell loading is much more efficient than loading cells on to the upper surface of scaffolds, which relies on gravity to distribute cells evenly throughout the scaffold. Loading cells directly onto the surface of scaffolds can lead to poor cell distribution, especially if the scaffold has small pores or low pore interconnectivity.

Human embryonic kidney 293 (HEK 293) cells cultured on FN-coupled cryogels proliferated to a greater extent in comparison with other gel types assessed, with similar physical properties (storage modulus: 5.3 ± 0.60 kPa, average pore size: 40 ± 4.4 μm) (Figure 6). Media coupled gels, which had been treated with the same coupling method as the FN coupled gels using serum-containing medium in place of FN, indicate the coupling method utilised does not impose any noticeable cytotoxic effects on the scaffolds, as expected based on the past use of EDC.[49,50]

The increased number of viable cells present within the FN coupled gels over the control gel types, in particular the FN swollen gels, supports the conclusions made from the CLSM data on successful surface modification and additionally indicates the coupled FN is bioactive. ANOVA of the experimental means highlights the observed difference between the FN-modified gels and the control gels are significant, with a p-value <0.05.

Lastly, we investigated the morphology of HEK 293 cells grown on peptide-modified and unmodified HA cryogels. The HA cryogels used had a storage modulus of 5.3 ± 0.60 kPa and pores of an average of 40 ± 4.4 μm. As such, these gels had mechanical properties suitable for soft tissue engineering applications and large pores to enable efficient cell infiltration. As expected, cells grown on unmodified HA cryogel scaffolds exhibited clumping and a rounded morphology (Figure 6(B)), an indication of cells not interacting well with the gel surface, preferring instead to adhere to one another. The presence of the rounded cells is typical of cells cultured on soft planar HA-based hydrogels,[51] and macro-porous HA hydrogels.[42] Cells grown on modified HA cryogels (Figure 6(A)), however, exhibited significantly smaller cell clumps in comparison with unmodified HA cryogels (Figure 6(B)), a trend which was observed over several days in culture, and seen in repeat experiments across both FN (Supplementary material SI.2) and c-RGD-modified gels (Figure 6(A)). Additionally, the spread morphology of HEK cells cultured on c-RGD-modified gels (Figure 6(A) and SI.3) indicates the cells are adherent. The extent of spreading is naturally less than that seen

Figure 5. CLSM micrographs showing immunodetection of biotinylated BSA in (A) modified and (B) control HA cryogels by streptavidin – FITC. Scale bars: 200 μm.
on hard culture plastic surfaces due to the soft mechanics of the cryogels (5.3 ± 0.60 kPa) relative to the cellular traction forces. This observation indicates the coupled c-RGD influences the interaction of HEK 293 cells with the cryogel surface and provides a plausible explanation for the increased viability of cells cultured in modified HA cryogels, relative to the controls discussed earlier (Figure 6). These results follow similar observations made in cross-linked acrylated HA hydrogels, where encapsulated mesenchymal stem cells were shown only to spread when an adhesive peptide was incorporated.[52]

In summary, we show surface modification of the hydrogel platform is readily achieved via facile EDC/NHS chemistry, whereby proteins and peptides such as BSA, FN and c-RGD are stably bound to the hydrogels’ surfaces yet maintain their bioactivity. Further, such peptide modification alters the interaction of cells with the hydrogel surfaces such that it influences cell attachment and proliferation. These findings indicate the gels studied here could be suitable candidates for tissue engineering applications, and the described system is suitable for a range of cell supportive applications.

Figure 6. Micrographs showing HEK 293 cells cultured in (A) a c-RGD-modified cryogel and (B) an unmodified HA cryogel at culture day 2 (scale bars: 50 μm) (blue: DAPI staining of nucleus; green: cytoplasmic CFSE stain). (C) CLSM image of fluorescently labelled HeLa cells distributed through a vertical cross section of a fluorescently labelled HA gel, 2 h after cell loading (scale bar: 200 μm) (green: cytoplasmic CFSE stain, red: Alexa Fluor 594 labelled HA). (D) The number of HEK 293 cells present after 5-days culture was compared across modified and unmodified HA cryogels, as compared to the control standard, media swollen gels. * Indicates statistically significant deviation (p < 0.05, ANOVA) from FN coupled gels (n = 4). (Please see the online article for the colour version of this figure: doi: 10.1088/09205063.2015.1065597.)
4. Conclusion

In this manuscript, we present a facile cryogelation process for the formation of macro-porous and highly interconnected HA hydrogel scaffolds, without the need to first chemically modify the precursor HA polymer. The use of unmodified HA not only reduces the complexity of the platform, but also additionally avoids potential adverse biological responses linked to modified HAs. Additionally, we show an important connection between cryogelation time, temperature and the concentration of the extensively used zero-length cross-linking agent, EDC.

We have shown that the bulk elastic stiffness of these gels can be altered via manipulations in cross-linking density, which has been shown to have an insignificant impact on morphology, or via the HA weight percentage of the gelling solution. Further, a simple process for the coupling of bioactive molecules to the internal surfaces of the scaffolds is shown, with in vitro cellular responses to FN-modified and c-RGD-modified scaffolds showing improved proliferation.

The ability to manipulate the chemical and physical properties of a HA macro-porous gel to mimic those observed in natural tissues make the described cryogelation platform an attractive method to explore the syntheses of a wide range of biomaterials suitable for a variety of applications. The ability to tailor these properties is especially important for optimisation in a number of soft tissue engineering and in vitro cell culture applications, which require delicate fine-tuning of the physical and/or chemical properties of the host material to achieve the desired tissue and cellular responses.

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Disclosure statement

No potential conflict of interest was reported by the authors.

Supplemental data

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