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Novel Freeze-Drying Methods to Produce a Range of Collagen–Glycosaminoglycan Scaffolds with Tailored Mean Pore Sizes

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The pore structure of three-dimensional scaffolds used in tissue engineering has been shown to significantly influence cellular activity. As the optimal pore size is dependant on the specifics of the tissue engineering application, the ability to alter the pore size over a wide range is essential for a particular scaffold to be suitable for multiple applications. With this in mind, the aim of this study was to develop methodologies to produce a range of collagen–glycosaminoglycan (CG) scaffolds with tailored mean pore sizes. The pore size of CG scaffolds is established during the freeze-drying fabrication process. In this study, freezing temperature was varied (−10°C to −70°C) and an annealing step was introduced to the process to determine their effects on pore size. Annealing is an additional step in the freeze-drying cycle that involves raising the temperature of the frozen suspension to increase the rate of ice crystal growth. The results show that the pore size of the scaffolds decreased as the freezing temperature was reduced. Additionally, the introduction of an annealing step during freeze-drying was found to result in a significant increase (40%) in pore size. Taken together, these results demonstrate that the methodologies developed in this study can be used to produce a range of CG scaffolds with mean pore sizes from 85 to 325 μm. This is a substantial improvement on the range of pore sizes that were possible to produce previously (96–150 μm). The methods developed in this study provide a basis for the investigation of the effects of pore size on both *in vitro* and *in vivo* performance and for the determination of the optimal pore structure for specific tissue engineering applications.

Introduction

Porous scaffolds are used extensively in tissue engineering to provide a three-dimensional structure on which tissue synthesis can occur. The pore architecture of these scaffolds has been shown to have a significant effect on both physical properties and cellular activity.[1–5] It has been hypothesized that the pore diameter must be large enough to allow infiltration of the cells toward the center of the scaffold, while being small enough to present sufficient ligand density for cellular attachment.[4,6] In addition, the pore size also determines permeability, which in turn influences the diffusion of nutrients and waste products within the scaffolds as well as the stimulus applied by flow perfusion bioreactors.[5,7] Consequently, the optimal pore size is dependant on both the cell type and specifics of the tissue engineering application. Endothelial cells, for example, show favorable attachment to pores in the range of 20–80 μm, whereas osteoblasts require pores larger than 100 μm for bone formation.[2,3,8,9] Therefore, for a particular scaffold to be suitable for multiple applications, the ability to alter the pore size over a wide range is essential.

In our laboratory, we use a freeze-drying process to fabricate porous collagen–glycosaminoglycan (CG) scaffolds, which have been used in a variety of tissue engineering studies.[8,10,11] Using this technique, a suspension of collagen and chondroitin sulfate is frozen, trapping the CG fibers between growing ice crystals. This produces a continuous network of ice crystals surrounded by CG fibers. Subsequent sublimation of the ice crystals during the drying phase leads to the formation of a highly porous CG scaffold.[12,13] As the pore structure of the scaffold mirrors the ice crystal structure formed during freezing, pore structure can be effectively controlled by altering the freezing process used during freeze-drying.[14] Accordingly, modifications to this process are the key to creating a range of scaffolds with different pore structures.

Previous work has investigated the effects of cooling rate and freezing temperature (T_f) used in the freezing process.[4,5,13] Cooling rate was found to determine the homogeneity of the structure, whereas T_f influenced the pore size. The results showed that pore size decreased with T_f down to −40°C. However, because of technical limitations, the study was restricted to a T_f of −40°C; the freeze-dryer used in the current study has the ability to reach a T_f of −70°C, allowing

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the work of O'Brien *et al.*[4] to be expanded upon. We hypothesize that reductions in T_f below $-40°C$ will result in scaffolds with a smaller mean pore size. Another method to vary the scaffold pore size might be through the introduction of annealing during lyophilization. Annealing is an additional step in the freeze-drying cycle that involves raising the temperature of the frozen suspension after freezing to increase the rate of ice crystal growth by reducing viscosity.[15] Although annealing has been used to create larger ice crystals to facilitate shorter drying times for freeze-dried pharmaceuticals, the effects of annealing on the pore structure of freeze-dried scaffolds have yet to be investigated.[16–18] We hypothesize that annealing can be used to increase the pore size of CG scaffolds. Therefore, the specific objectives of this study were to determine (1) the effects of reducing T_f to below $-40°C$ on scaffold pore size and (2) to test our hypothesis that the introduction of an annealing step during freeze-drying would lead to an increase in the pore size of CG scaffolds.

Materials and Methods

Slurry fabrication

To prepare the CG slurry, a suspension of type I bovine collagen (Integra Life Sciences, Plainsboro, NJ), chondroitin-6-sulphate (Sigma-Aldrich, Seelze, Germany), and 0.05 M glacial acetic acid were blended together at 15,000 rpm using an overhead blender (Ultra Turrax T18; IKA Works, Wilmington, NC). Blending was carried out in a reaction vessel that was maintained at 4°C using a circulation cooling system (WKL 230; Lauda, Lauda-Konigshofen, Germany). The resulting CG suspension contained 0.5% (w/v) collagen and 0.044% (w/v) chondroitin-6-sulfate. The suspension was degassed in a vacuum desiccator for 60 min to remove trapped air bubbles after blending.

Freeze-drying

The CG slurry was lyophilized following the protocol developed by O'Brien *et al.*[13] to produce CG scaffolds with a homogeneous pore structure. Briefly, 67.25 mL of the CG suspension was pipetted into a stainless-steel tray (5×5 in., grade 304 SS). The tray was placed onto the freeze-dryer shelf (Advantage EL; VirTis, Gardiner, NY) and the freezing cycle was started. Several variations of the freezing cycle were used in this study. To determine the effect of T_f on pore size, scaffolds were produced with a T_f of $-10°C$, $-40°C$, $-50°C$, $-60°C$, and $-70°C$ (Fig. 1).

Additionally, an annealing step was introduced into the freezing process (Fig. 2). The samples were cooled to a temperature of $-20°C$ at a constant cooling rate of 0.9°C/min and held at this temperature for 20 min to allow the suspension to freeze. The temperature was then increased to $-10°C$ to start annealing and held there for the specific annealing time. Annealing times of 0.25, 6, 12, 18, and 48 h were used to determine the effects of annealing on pore size (Fig. 2, E). Once freezing was complete, the ice crystals were removed via sublimation for 17 h at 0°C and 200 mTorr. Samples were then crosslinked in preparation for histological analysis.

Crosslinking

After freeze-drying, dehydrothermal (DHT) treatment was used to crosslink the CG scaffolds. DHT treatment was carried out by placing the scaffolds in an aluminium foil packet inside a vacuum oven (Vacucell 22; MMM, Munich, Germany) under a vacuum of 0.05 bar at 105°C for 24 h. This is the standard protocol used in several studies.[4,13,19–21] Following DHT treatment, samples were prepared for pore size analysis.

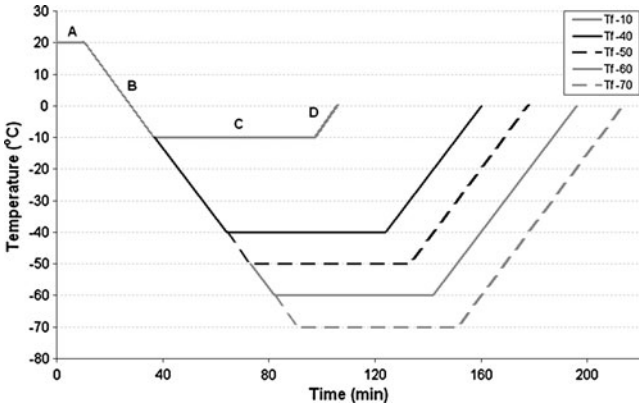

FIG. 1. Time–temperature plot illustrating the standard freeze-drying cycle and alterations to the final freezing temperature (T_f). (A) Temperature is held at 20°C for 20 min to allow the suspension to reach a uniform temperature. (B) The shelf is cooled to the final T_f at a rate of 0.9°C/min and (C) held at this temperature for 60 min to allow the suspension to freeze. (D) The sample is then heated to 0°C at a rate of 0.9°C/min before the onset of the drying cycle. The cycle outlined in blue ($T_f - 40$) is the standard freeze-drying cycle developed by O'Brien *et al.*[4]

Pore size analysis

Pore size was determined using a histological technique previously described by O'Brien *et al.*[13] From each scaffold variant, four sets of cylindrical samples (8 mm diameter, 4 mm height) were cut using a punch. The samples were then embedded in JB4 glycomethacrylate (Polysciences, Eppelheim,

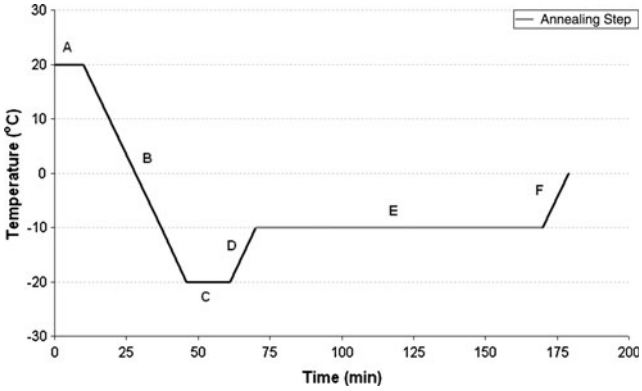

FIG. 2. Time–temperature plot for a standard annealing cycle. (A) Temperature is held at 20°C for 20 min to allow the suspension to reach a uniform temperature. (B) The shelf is cooled to $-20°C$ at a constant cooling rate of 0.9°C/min and (C) held at this temperature for 20 min to allow the suspension to freeze. (D) The frozen solution is then heated to $-10°C$ at a rate of 0.9°C/min and (E) held there for a set annealing time, which was varied from 0.25 to 48 h in this study. (F) The sample is then heated to 0°C at a rate of 0.9°C/min before the onset of the drying cycle.

Germany): two samples in the longitudinal plane and two in the transverse plane. The embedded samples were sectioned at 5 μm thickness using a microtome (Leica RM 2255; Leica, Vertrieb, Germany). Every 20th section was mounted on slides and stained using 2% aniline blue, giving a gap of 100 μm between sections for subsequent analysis. Digital images were recorded using a microscope at 125× magnification (Optimphot2; Nikon, Tokyo, Japan). A total of 10 digital images were taken from each of the samples to give a total of 40 images from each scaffold variant.

Analysis of the digital images was carried out using a Matlab program developed in conjunction with the Sigmedia Research Group from the Electrical Engineering Department at Trinity College Dublin. Analysis was carried out in two stages. The program first thresholds the image, converting it into a binary image consisting of black and white pixels, and an algorithm is run to remove blotches from the image. The second stage consists of pore analysis; briefly, the program picks a random pixel in the image and then detects the pore walls surrounding it. The major and minor axes are then calculated and the pixels within the pore are marked as selected. The program repeats this process until at least 70% of the pixels within the image have been marked as selected. In this fashion the average pore size can be calculated for each section. The pore size referred to throughout this article is the diameter of a circle with a cross-sectional area equivalent to that of the best fit ellipse that is generated by the software.

Differential thermal analysis

Differential thermal analysis (DTA) was used to gather further information and explain the structural changes that take place as the T_f of the CG suspension is varied. DTA measures the difference in temperature between the sample and a reference, thus monitoring exothermic or endothermic changes observed as a solution melts. These exothermic and endothermic changes indicate subtle changes in the viscosity of a frozen sample as it is melted. For example, if the viscosity of a sample reduces suddenly as it melts, the rate of increase in temperature will rise because of improved mobility within the frozen suspension. Using DTA, this will be observed as a reduction in the temperature difference (delta T) measured between the sample and reference. DTA analysis was carried out using Lyotherm 2 (Biopharama, Winchester, United Kingdom). The temperature of the CG sample together and the reference material (AnalaR water) were measured at 5-s intervals as they were heated from −150°C. Raw data were exported, and interpretation of the cooling profile was carried out to determine the temperatures of the significant events that could be attributed to structural changes occurring in the CG suspension.

Statistical analysis

Results are expressed as mean ± standard deviation. One-way analysis of variance followed by pairwise multiple comparison procedures (Student Newman Kuels [SNK] test) was used to evaluate the effects of T_f and annealing on the pore size of CG scaffolds. Statistical significance was declared at $p \leq 0.05$.

Results

Effect of T_f *on scaffold pore size*

Pore size was found to decrease with T_f until −50°C ($p < 0.05$), but subsequent reductions in T_f to −70°C did not result in a further significant change in pore size (Fig. 3). The results show that alterations of the T_f can be used to produce scaffolds with a mean pore size ranging from 84.6 to 324.9 μm. A T_f of −10°C produced scaffolds with the largest pores at 324.9 μm, whereas scaffolds produced using a T_f of −60°C had the smallest pore size at 84.7 μm (Fig. 4).

Effect of annealing on scaffold pore size

Results show that as annealing time increased an initial decrease in pore size can be seen from 0.25 to 6 h, after which the pore size increases to a peak at 18 h (Fig. 5). Annealing times beyond 18 h revealed no additional increases in pore size. Annealing for 18 h produced a 40% increase in scaffold pore size when compared with scaffolds annealed for 0.25 h (0.25 h, $p < 0.001$), resulting in a mean pore size of 191.4 μm (Fig. 6).

Differential thermal analysis

DTA was used to determine the temperatures at which structural changes occur within the frozen CG suspension (Fig. 7). Delta T represents the difference in temperature between the CG sample and pure water as they are heated from −100°C. An increase in delta T indicates an increase in the viscosity of the sample, whereas a reduction in delta T indicates the opposite. DTA shows three distinct phenomena as the frozen sample was heated from −100°C to 0°C. An increase in viscosity (or hardening) can be seen up to −66°C (Fig. 7, A), followed by a reduction in viscosity (softening) at −42°C (Fig. 7, B). Finally, the onset of melting occurs at −6°C (Fig. 7, C).

Although these data relate to changes in the structure as the sample melts, it should be noted that the behavior during freezing will be the opposite. For example, a reduction in the viscosity of a frozen suspension during melting points to an increase in viscosity at the same temperature during freezing. Therefore, DTA indicates ice crystal nucleation at −6°C, followed by an increase in viscosity of the frozen suspension at −42°C, and finally, a reduction in viscosity at −66°C. The increase in viscosity at −42°C may indicate the glass transition temperature (T_g) of the suspension and explain why reductions in T_f have a limited effect below −40°C (Fig. 3).

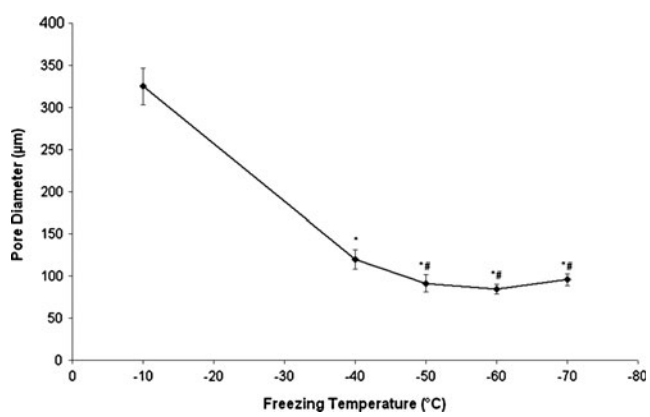

FIG. 3. The effect of T_f on pore size ($n = 4$ per group; $^{*}p < 0.001$ compared with −10°C group; $^{\#}p < 0.05$ compared with −40°C group; no differences were found between −50°C, −60°C, and −70°C groups).

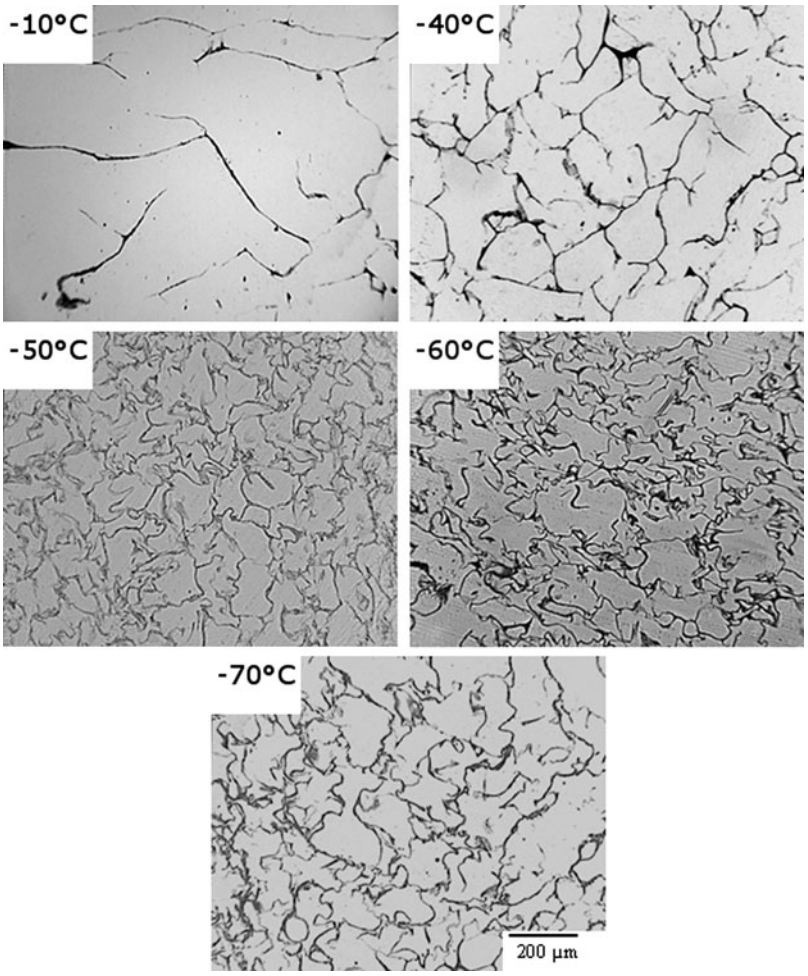

FIG. 4. Digital images of the stained scaffold sections captured at 125× magnification. The mean pore size decreases with T_f until −50°C, with no further significant change in pore size resulting at lower temperatures of −60°C and −70°C.

Discussion

The pore structure of three-dimensional scaffolds used in tissue engineering has been previously shown to influence cellular activity.[2–4] With this in mind, the aim of this study was to develop methodologies to produce a range of CG scaffolds with tailored mean pore sizes, which could be used in further studies. Scaffolds were fabricated using several alterations to the freeze-drying protocol developed by O'Brien *et al.*[13] The T_f was varied from −10°C to −70°C and an annealing step was introduced to the cycle. We found that the pore size of the scaffolds decreased with decreasing T_f until −50°C, after which no significant changes in pore size were observed. Additionally, the introduction of an annealing step during freeze-drying was found to result in a significant increase (40%) in pore size after 18 h. Taken together, these results demonstrate that methodologies developed in this study can be used to produce a range of CG scaffolds with mean pore sizes ranging from 85 to 325 µm (Table 1). This is a substantial improvement on the range of pore sizes that were possible to produce previously (96–150 µm).[4] This facilitates the investigation of the effects of pore size on both *in vitro* and *in vivo* performance, and the determination of the optimal pore structure for specific tissue engineering applications.

In this study, we have investigated the effects of T_f and annealing on the pore structure of CG scaffolds. The effects of T_f on pore structure in scaffolds produced from a variety of biomaterials have been investigated in previous studies.[22–25] However, in these studies, the cooling rate was also varied in conjunction with T_f, making it difficult to determine the specific mechanisms behind changes in pore structure. Several studies have investigated the effects of cooling rate on the pore structure of freeze-dried scaffolds. It has been shown that slow cooling rates (<1°C/min) produce scaffolds with

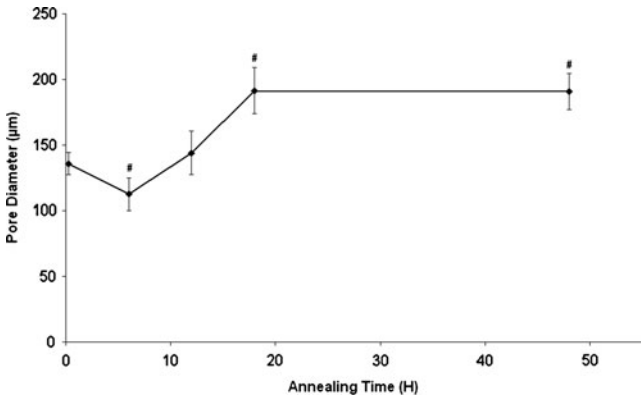

FIG. 5. The effect of heat annealing on pore size ($n = 4$ per group; $^{\#}p < 0.05$ compared with 0.25 h group; no differences were found between 0.25 and 12 h groups).

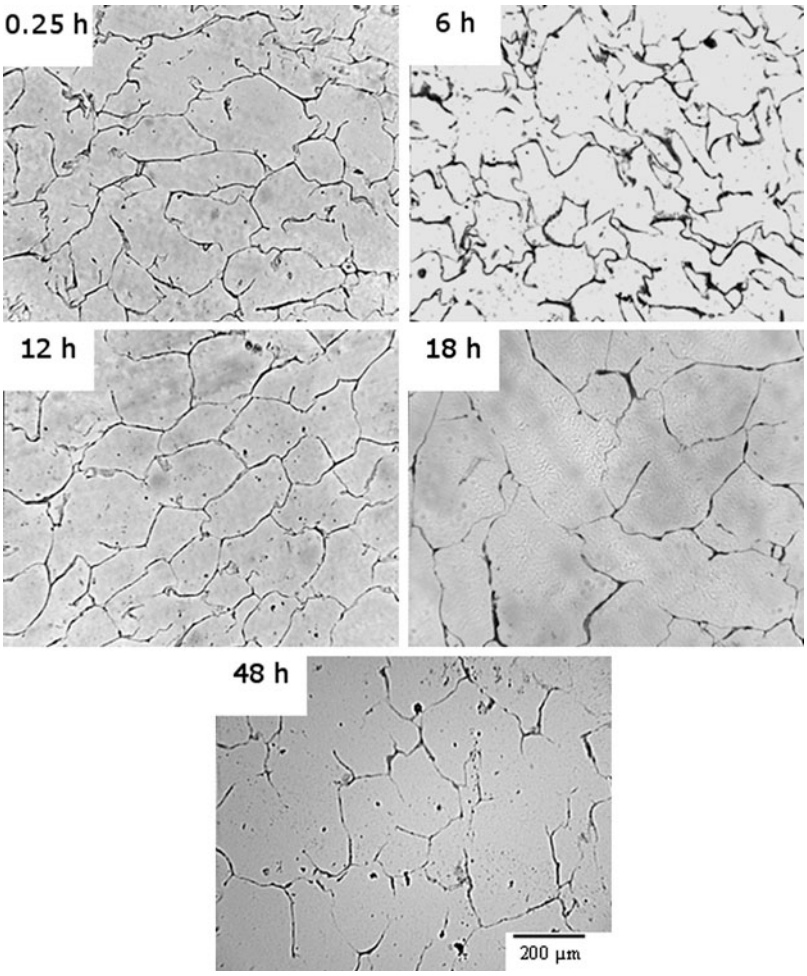

FIG. 6. Digital images of the stained scaffold sections captured at 125× magnification. The mean pore size increases up to an annealing time of 18 h, after which no further changes in mean pore size are observed.

large pores, whereas fast cooling rates (>1°C/min) produce scaffolds with smaller pores.[22,25] However, a study by O'Brien *et al*.[13] has shown that cooling rate also affects the uniformity of the pore structure. It was found that a cooling rate of 0.9°C/min produced scaffolds with homogeneous pore structure, whereas quicker or slower cooling rates reduced the uniformity of the structure formed.[13] This has also been shown to be the case with freeze-dried alginate scaffolds.[23] On this basis, it was decided to keep a constant cooling rate of 0.9°C/min and modify alternative aspects of the freezing cycle in this study. In addition, freeze-drying in this study was carried out using a freeze-dryer equipped with a temperature-controlled shelf. This allowed control of the cooling rate and eliminated the possibility of sample thawing during transport between the freezing and drying apparatus, which were separate devices in many studies.[22,23,25] The use of annealing to alter the pore size of a freeze-dried scaffold is a novel aspect of this study. Annealing has been used previously to encourage crystallization and reduce the drying cycle times of pharmaceuticals,[17,26,27] but has yet to be investigated for its effects on the structure of freeze-dried scaffolds.

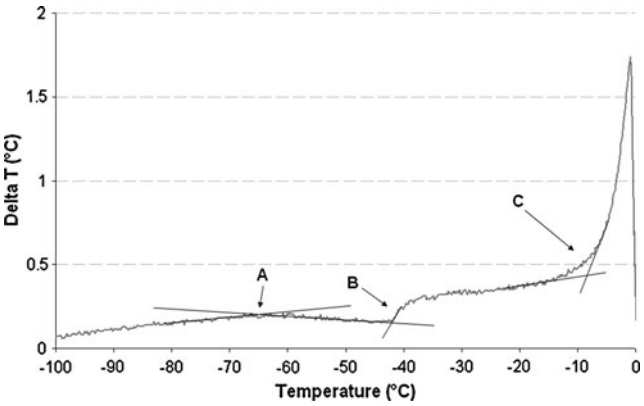

FIG. 7. Differential thermal analysis of the collagen–glycosaminoglycan suspension. Delta T represents the difference in temperature between the collagen–glycosaminoglycan suspension and the reference (AnalaR water). The following phenomena were observed: (A) hardening of the structure at −66°C, (B) softening of the frozen structure at −42°C, and (C) onset of ice melting at −6°C.

It was found that pore size decreased with T_f until −50°C, but reductions in T_f below this temperature did not result in a significant change in pore size. The size of ice crystals formed during freezing is dependant on both the structure of the crystals after nucleation and the subsequent growth through heat and protein diffusion within the frozen suspension. At the cooling rate used in this study, nucleation is complete before the cycle reaches the final T_f and therefore changes in T_f affect growth after nucleation and not nucleation itself. As T_f is reduced, the rate of heat and protein

Table 1. The Average Pore Size Produced at Different Freezing Temperatures and Annealing Times

	Average pore size				
	−10[a]	*−40*[a]	*−50*[a]	*−60*[a]	*−70*[a]
Pore size (μm)	324.9	120.0	91.3	84.6	95.8
Pore size SD (μm)	22.1	11.7	10.1	5.7	6.9
	0.25[b]	*6*[b]	*12*[b]	*18*[b]	*48*[b]
Pore size (μm)	135.6	112.5	143.8	191.4	190.6
Pore size SD (μm)	8.6	12.3	16.6	17.8	13.8

[a]T_f (°C).
[b]Annealing time (h).
T_f, freezing temperature; SD, standard deviation.

diffusion also decreases, reducing the size of the ice crystals formed. However, when the temperature drops below the T_g, the viscosity of the frozen suspension increases to the point where ice crystal growth is negligible.[15,28] DTA indicates an increase in viscosity at −42°C (Fig. 7, B), which correlates with the reduction in ice crystal growth between −40°C and −50°C found in the pore size data (Fig. 3). These results indicate that the T_g of the CG slurry used in this study is −42°C, and that reductions in T_f below this temperature have no effect on pore size because of increased viscosity. It should be noted that previous studies have found that pore size reduces with T_f as low as −80°C.[10,29] However, the cooling rates used in these studies are greater (>1°C/min) than the rate used in this study. This permits T_f to influence both the shape of the ice crystals as they nucleate and the subsequent growth through diffusion. The drawback of cooling rates above 1°C/min is a reduction in the homogeneity of the structures that are formed.[13]

Annealing increases the rate of ice crystal growth within a frozen suspension by causing a reduction in viscosity and a corresponding increase in heat and protein diffusion. Previous studies using nanocapsules and albumin have shown that ice crystal size increases with increasing annealing temperature.[16,18] In this study, an annealing temperature of −10°C was used to produce a large increase in growth rate while staying safely below the melting temperature of the frozen suspension. The effects of the introduction of this step and the length of the annealing time used were the focus of this study. Interestingly, annealing was found to cause an initial decrease in pore size, followed by an increase after 18 h of annealing. During annealing, a phenomenon known as Ostwald ripening occurs, which is the preferential melting of dispersed ice crystals that are smaller than a critical size. This is due to the relationship between radii of curvature and vapor pressure described by the Kelvin equation: smaller ice crystals have higher vapor pressures and will melt at lower temperatures.[15] Therefore, it is possible that the mean pore size would initially decrease as the diameter of these ice crystals contracts during melting, and once melting is complete the ice crystal size would increase because of the reduced viscosity at the annealing temperature and accretion of water from the melted ice crystals. This ripening may be a possible explanation for the initial decrease in pore size that can be observed up to an annealing time of 6 h and the subsequent increases in pore size as the remaining ice crystals grow because of the reduction in the viscosity of the suspension.

An interesting caveat to this study is that previously O'Brien *et al.*[4] reported a pore size of 150.5 and 95.9 μm for scaffolds fabricated at a T_f of −10°C and −40°C, respectively. Our results, however, show a pore size of 324.9 and 120.1 μm for scaffolds fabricated at the same temperatures. The discrepancy between these results can be accounted for by differences in the trays used to contain the CG suspension during freeze-drying. To prevent warping at low temperatures, the thickness of the stainless-steel trays used in this study was twice as that of the trays used by O'Brien *et al.* This change will decrease the cooling rate of the solution, which has been shown to increase pore size, thereby explaining the larger pore size found in our study. Analysis of the temperature of the slurry during freezing was carried out using thermocouples as has been described previously.[13] The result shows that the cooling rate is indeed reduced in comparison to the temperature plots of O'Brien *et al.* (Fig. 8). The tray thickness may have had a less effect at a T_f of −40°C, as ice crystal growth rate is slower at this temperature because of increased viscosity. However, this result shows that an alternative scaffold fabrication process might be to use vessels with a lower thermal conductivity (e.g.,

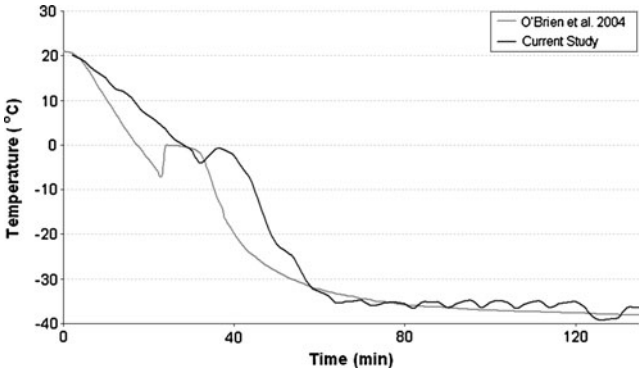

FIG. 8. Temperature maps illustrating the average temperature of the slurry during fabrication with a comparison between the freezing profile from this study and that of a previous study.[13] It can be seen that the cooling rate in this study is lower than that of O'Brien *et al.*[13] because of the thicker trays used during the fabrication process.

polymers) than the stainless-steel tray used during freeze-drying in this study. By combining a reduced cooling rate with the optimized freeze-drying methods developed in this study, further increases in the pore size of the scaffolds might be achieved.

Conclusion

Pore size has been previously found to be an important aspect of scaffold design.[1–4] The results of this study show that modifications to the freeze-drying cycle can be used to produce a variety of CG scaffolds with a wide range of mean pore sizes (85–325 μm). Altering the T_f was found to be the most effective method for varying pore size, although annealing may prove useful when combined with high T_f. In addition, DTA combined with pore size analysis indicates that the T_g of the CG suspension is -42°C. The scaffolds produced in this study provide a basis for the investigation of the effects of pore size on cellular activity.

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Disclosure Statement

No competing financial interests exist. Collagen materials were provided by Integra Life Sciences, through a material transfer agreement.

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