GELLING TEMPERATURE AND GEL CONCENTRATION EFFECTS ON TISSUE DEVELOPMENT IN CHONDROCYTE-SEEDED AGAROSE HYDROGELS

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INTRODUCTION

Agarose is a clear, thermoreversible hydrogel that has found application in numerous studies aimed at cartilage tissue engineering [1-3]. This hydrogel is supportive of the chondrocyte phenotype [4] and allows for the accumulation of a functional extracellular matrix with time in culture [2]. Agarose is neutrally charged, and forms solid gels at room temperature. These gels exhibit significant thermal hysteresis, in that the viscosity profile with heating is much different than that with cooling [5]. Additionally, the initial strength of the gel is dependent on the rate of gelling, which in turn is dependent on the ambient temperature [5]. Gel strength is also strongly dependent on the concentration of the gel in solution [3,6]. The mechanical strength of agarose originates form the entangling of long polysaccharide chains, and both gel rate and concentration play a significant role in this process. Basic science studies involving agarose gel formation have also demonstrated that rapid cooling leads to a decreased, more homogeneous pore size [5,7]. Increasing the gel concentration additionally decreases gel pore size and permeability [8]. A number of studies have used agarose for the investigation of chondrocyte growth and response to mechanical stimuli. These studies have been carried out with a range of agarose concentrations (1%-3% wt/vol) and with different gelling temperatures [2-4,9,10]. The interpretation of results stemming from these studies is at least in part dependent on the physical properties of the gel utilized. Furthermore, as agarose becomes more widely used for tissue engineering studies, a careful consideration of these factors on gel formation must be addressed. In this study, the influence of two different parameters, ambient gelling temperature and bulk gel concentration, on the long-term growth of chondrocyte-seeded agarose hydrogels was investigated.

MATERIALS AND METHODS

Cell Culture: Articular cartilage was harvested from the carpometarcarpal joint of four 3-6 month old bovine calves, and chondrocytes isolated as described previously [3]. Cells were sedimented at 1000 x *g*, and were resuspended in high glucose DMEM supplemented with 20% fetal bovine serum with buffers, amino acids, antibiotics, antimycotics, and 50 μg/ml fresh ascorbic acid. In the first study, gels were formed with 2% agarose (type VII) in PBS at 60 x 10^6 cells/ml and cast at a thickness of 2.25 mm. Gelling was allowed to proceed at ambient room temperature (25°C) or in the freezer (-20°C) for 15 minutes. In the second study, gels containing a final agarose concentration of either 2% or 3% with cells at 60 x 10^6 cells/ml were gelled at room temperatures for 15 minutes. In both studies, after gelling, disks (∅ 4.76 mm x 2.25 mm thick) were cored and cultured (15-20 constructs) in 30 ml of fully supplemented DMEM (as above). Media, with fresh ascorbic acid, were changed thrice weekly. **Mechanical Testing**: Each week for a month, 3-4 samples were removed from culture, measured for thickness and diameter, and tested in unconfined compression between impermeable platens. Testing consisted of a ramp and hold of 10% of the measured gel thickness. After equilibrium was reached, a dynamic displacement of 40 μm was applied at frequencies ranging from 0.005-1.0 Hz. The Young's and dynamic modulus were calculated from the load/deformation profiles and the specimen geometry. **Biochemical Composition**: After testing, constructs were rehydrated in PBS, weighted wet, and digested with papain. GAG content of digests was determined using the DMMB dye assay [11], scaled for microplates. **Statistics**: Multiple comparisons between groups were made with unpaired t-tests assuming unequal variance using the Microsoft Excel analysis tool pack. All data are reported as the mean ± SD of 3-4 samples.

RESULTS

Temperature Effects: On day 0, agarose hydrogels exhibited higher moduli for those gelled more rapidly (at –20°C ambient temperature). Rapid gelling led to an increase in both the Young's ($p<0.025$) and dynamic modulus (at 1 Hz) compared to gels formed at room temperature (**Figure 1, 2**). GAG content of the two gels was not significantly different on day 0 (**Figure 3**). Over the first two weeks of culture, these initial findings inverted, with gels formed at room temperature achieving higher mechanical properties and GAG content. By day 28, gels formed at room temperature had significantly higher Young's modulus ($p<0.05$), dynamic modulus ($p<0.05$), and GAG content ($p<0.01$) compared to gels formed at –20°C.

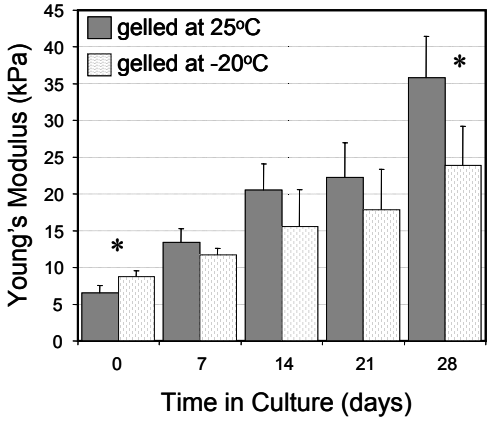

Figure 1 – Young's modulus over time for constructs gelled at two different temperatures. *indicates significant difference between groups (p<0.05, n=3).

Gel Concentration Effects: On day 0, 3% chondrocyte-seeded agarose hydrogels exhibited a higher Young's modulus ($p<0.05$) compared to similarly seeded 2% gels, with no significant difference in either dynamic modulus or GAG content (**Figure 4**). With time in culture, significant increases were observed in GAG content and the Young's and dynamic modulus for all groups ($p<0.05$) except for the Young's modulus of 3% gels. By day 28, however, 3% gels had increased in diameter ($p<0.05$) and thickness ($p<0.005$) compared to 2% gels at the same time point. Interestingly, by this time point, the Young's ($p<0.05$) modulus was higher for 2% gels, while the GAG content and dynamic modulas were not significantly different between groups (**Figure 4**).

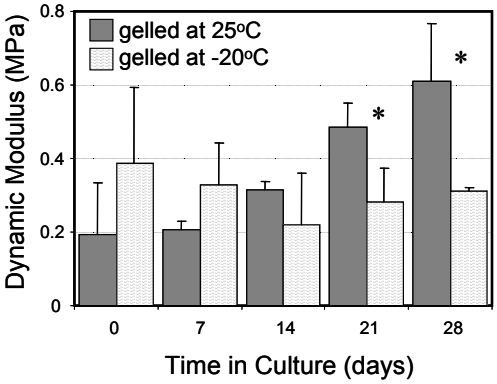

Figure 2 – Dynamic modulus (at 1 Hz) over time for constructs gelled at two different temperatures. *indicates significant difference between groups (p<0.05, n=3).

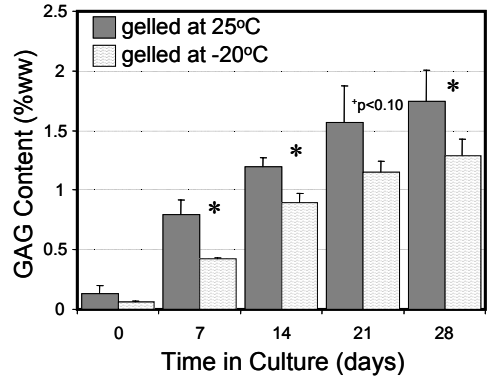

Figure 3 – GAG content (normalized to wet weight) for constructs gelled at two different temperatures. *indicates significant difference between groups (p<0.05, n=3).

DISCUSSION

While agarose provides a simple environment in which to encapsulate cells, significant differences in gel properties may arise from alterations in the gelling composition and environment. This study demonstrates that variation of two parameters, ambient gelling temperature and gel concentration, can affect the developing tissue properties of chondrocytes embedded within this 3D environment. In this study, gelling at room temperature (25°C) resulted in softer gels than those formed under rapid gelling conditions (-20°C). Interestingly, these findings were short-lived, with gels formed at room temperature quickly developing material properties superior to the initially stiffer gels formed by rapid cooling. Similarly, an increase in gel concentration (to 3%) resulted in gels with a greater initial stiffness, though 2% chondrocyte-seeded agarose constructs showed better mechanical properties, with less change in construct dimensions, over long term culture. While the findings of these two studies are qualitatively similar, subtle differences do arise from the differing gelling conditions, in particular with regard to the final GAG concentration in the gels. As described above, the increases in gelling rate and concentration of agarose lead to decreases in pore size. These decreases may affect the partitioning and diffusion of large molecules (such as growth factors) into the gel [8], altering chondrocyte production of proteoglycans and other molecules (such as collagens). The local density of agarose may also restrict the diffusion of matrix constituents, limiting the formation of a tissue spanning structural network. Precise control of the local concentration and gelling properties of chondrocyte-seeded agarose hydrogels may be used to direct the development of inhomogeneous material properties in these constructs.

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	day	diameter (mm)	thickness (mm)
2%	0	4.76 ± 0.00	2.33 ± 0.04
	28	5.10 ± 0.12	2.81 ± 0.08
3%	0	4.76 ± 0.00	2.36 ± 0.01
	28	5.63 ± 0.26**	3.59 ± 0.16**

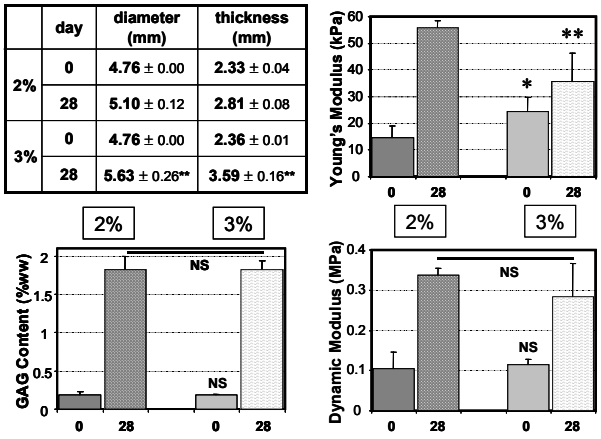

Figure 4 – Dimension, GAG content, Young's modulus, and dynamic modulus (at 1 Hz) for 2 or 3% agarose constructs. * and ** indicate significant difference between 2% and 3% groups on day 0 or day 28, respectively (p<0.05, n=3).