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Characterization of hyaluronan–methylcellulose hydrogels for cell delivery to the injured spinal cord

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Abstract: No effective clinical treatment currently exists for traumatic spinal cord injury. Cell replacement therapy holds promise for attaining functional repair. Cells may be delivered directly or near the injury site; however, this strategy requires a delivery vehicle to maintain cell viability. We have identified an injectable, biocompatible, and biodegradable hydrogel scaffold composed of hyaluronan (HA) and methylcellulose (MC) that may be an effective scaffold for therapeutic cell delivery. The purpose of the present study was to determine the effects of polymer concentration on HAMC mechanical strength, gelation time, and cell viability. The yield stress of HAMC, a measure of mechanical stiffness, was tunable via manipulation of MC and HA content. Measurement of the elastic and storage moduli as functions of time revealed that HAMC gels in less than 5 min at physiological temperatures. Human umbilical tissue-derived cells encapsulated in HAMC were homogenously and stably distributed over 3 days in culture and extended processes into the scaffold. Cell viability was stable over this period in all but the most concentrated HAMC formulation. Because of its strength-tunability, rapid gelation, and ability to maintain cell viability, HAMC is a promising vehicle for cell delivery and is being tested in ongoing *in vivo* studies. © 2012 Wiley Periodicals, Inc. J Biomed Mater Res Part A: 101A: 1472–1477, 2013.

Key Words: hydrogel, cell therapy, spinal cord injury, hyaluronan, cell scaffold

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INTRODUCTION

Traumatic compression of the spinal cord is a devastating injury, resulting in significant neural tissue damage and a dramatic loss of locomotor and sensory function. Unlike the peripheral nervous system, where injured axons can regenerate and re-establish functional connections, repair in the central nervous system is very limited. Current treatment options for spinal cord injury (SCI) are restricted to systemic delivery of methylprednisolone, decompressive surgery, and physical rehabilitation, all of which result in only minimal functional recovery.[1]

An emerging approach for achieving functional repair after SCI is exogenous cell transplantation. Transplanted cells can replace damaged tissue and provide trophic or cell-contact mediated support for neuroprotection and regeneration.[2,3] However, some recent reports have indicated that neural stem/progenitor cells showed significant cell death after bolus injection into the spinal cord.[4,5] Regardless of the cell therapy tested for spinal cord repair, the delivery vehicle must be selected carefully in order to support extended cell viability and therapeutic activity. We have identified a novel biodegradable and injectable hydrogel scaffold that may be used to deliver and encapsulate cells for spinal cord delivery.[6,7] Composed of a physical blend of hyaluronan (HA) and methylcellulose (MC), this HAMC hydrogel provides the cells with a three-dimensional microenvironment, which is an important factor in enhancing cell viability.[8,9] HA is a natural extracellular matrix polysaccharide that has demonstrated wound-healing properties,[10] while MC results in gel formation via thermally

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induced physical crosslinks.[11] Retinal stem/progenitor cells delivered to the subretinal space in HAMC were more evenly distributed than those delivered in traditional saline solutions.[6] Similar results were observed *in vitro* for neural stem/progenitor cells.[7] Consequently, HAMC possesses considerable potential as a cell delivery vehicle.

Previous work with a drug-delivery formulation of HAMC revealed that the mechanical properties of the material are strongly dependent upon the concentration of HA and MC used to formulate the hydrogel.[12] However, cell delivery applications of HAMC have focused almost exclusively on a single formulation composed of 0.5 wt % MC and 0.5 wt % HA. Because cell viability in three-dimensional culture conditions is known to be dependent upon scaffold stiffness,[9,13] our goal was to investigate how cells respond to various concentrations of HA and MC in the hydrogel. In addition, we were interested in understanding how the presence of cells impacts the mechanical properties of the material. Human umbilical tissue-derived cells (hUTC) were studied as they are known to secrete a variety of trophic factors such as hepatocyte growth factor, basic fibroblast growth factor, monocyte chemotactic protein 1 and interleukin 8, as well as the neurotrophic factors brain-derived neurotrophic factor and interleukin 6.[14] In addition, a small population of hUTC can differentiate to form neurons ($TuJ1^+$ cells).[14] For these reasons, transplanted hUTC have the potential to stimulate recovery in the injured spinal cord.

MATERIALS AND METHODS

Material preparation

HA was purchased from Novamatrix (1500 kDa; Drammen, Norway) and MC was purchased from Shin-Etsu (300 kDa; Tokyo). HA and MC were sterilized via dissolution in ddH_2O, filtration through a 0.22-μm poly(ether sulfone) membrane, and lyophilized to recover the solid polymer. Sterile HAMC was prepared by dissolving HA and MC in hUTC media [Dulbecco's modified Eagle medium (Gibco) with penicillin-streptomycin (PenStrep, Sigma-Aldrich) and GlutaMAX™ (Gibco)] overnight at 4°C. hUTC (provided by Advanced Technologies and Regenerative Medicine LLC, ATRM) in a media suspension (or an equivalent volume of media alone for noncell controls) were physically mixed into the hydrogel at 1:9 cell suspension:hydrogel ratio. HAMC blends with the following HA:MC weight percent ratios were produced: 0.25:0.25, 0.50:0.50, 0.75:0.75, 1.0:0.75, and 1.0:1.0.

Rheological characterization of HAMC blends

All rheological data were collected using a TA Instruments AR1000 rheometer (New Castle, DE) equipped with a 60 mm, 1° acrylic cone. Temperature was controlled using an integrated Peltier plate, and sample evaporation was minimized using a solvent trap. HAMC yield stress (τ_y) was characterized via stress-controlled steady-state experiments at 37°C. To allow for thermal equilibration, samples were conditioned for 20 min at 37°C before shear. Shear rates were then recorded for shear stresses ranging between 0.01 and 20 Pa. The gelation points of the HAMC blends were characterized via measurement of the storage (G') and loss (G'') moduli as functions of time. To simulate *in vivo* injection, the temperature of the Peltier plate was changed from 4 to 37°C at time zero, and the moduli were recorded periodically for 40 min at an angular frequency of 1 Hz and 1% strain (confirmed to lie within the linear viscoelastic regions of the HAMC blends).

In vitro characterization of hUTC viability in HAMC

Viability of hUTC was studied in the four HAMC blends immediately (day 0) and 3 days after seeding. Cells were fluorescently labeled using CellTrace™ CFSE dye (Invitrogen) and ethidium homodimer-1 (EthD1, Invitrogen). The labeled cells were trypsinized and resuspended at a concentration of 1×10^4 cells/500 mL of HAMC. Viability was assayed using confocal imaging (Olympus Fluoview FV1000) and single cell counting, where $CFSE^+$ $EthD1^-$ cells were classified as live and $CFSE^+$ $EthD1^+$ were classified as dead.

Statistics

All statistical analyses were performed using Prism 5.0 (GraphPad Software). Differences between two groups were assessed by paired *t*-tests, while differences between three or more groups were assessed by one-way ANOVA with Bonferonni correction where appropriate. Significance levels were indicated by $p < 0.05$ (*), 0.01 (**), and 0.001 (***).

RESULTS

Rheological characterization of HAMC without hUTC

Rheological testing was used to characterize the yield stress of five HAMC blends (0.25/0.25, 0.5/0.5, 0.75/0.75, 1.0/0.75, and 1.0/1.0) without the inclusion of cells. Previous work has shown that 0.5/0.5 HAMC possesses a nonzero yield stress,[7] meaning that it will not deform in response to shear until a certain minimum amount of stress is applied. Yield stress magnitude was used in this study as a measure of overall hydrogel strength. Figure 1 displays shear stress versus shear rate traces for the five HAMC blends without hUTC, where the yield stress is given by the vertical intercept. With the exception of 0.25/0.25 HAMC, yield stress increased with total polymer content in the hydrogel, ranging from 1.6 Pa for 0.5/0.5 to 4.3 Pa for 1.0/1.0. This demonstrates that the gel is strengthened upon addition of both MC, which comprises the physical gel-forming crosslinks, and HA, which enhances gelation via viscosity and salting-out effects.[15] The zero yield stress of 0.25/0.25 HAMC signified that it cannot resist deformation in response to shear and thus does not form a gel. Consequently, it was not examined further.

The gelation points of the HAMC blends without the inclusion of hUTC were characterized via measurement of the storage (G') and loss (G'') moduli as functions of time. The gelation point is defined as the time in which G' becomes equal to G''. As shown in Figure 2, gelation time and moduli at the gelation point tended to increase with total polymer content. This means that the gel, although it takes longer to form, is stronger when there is more MC

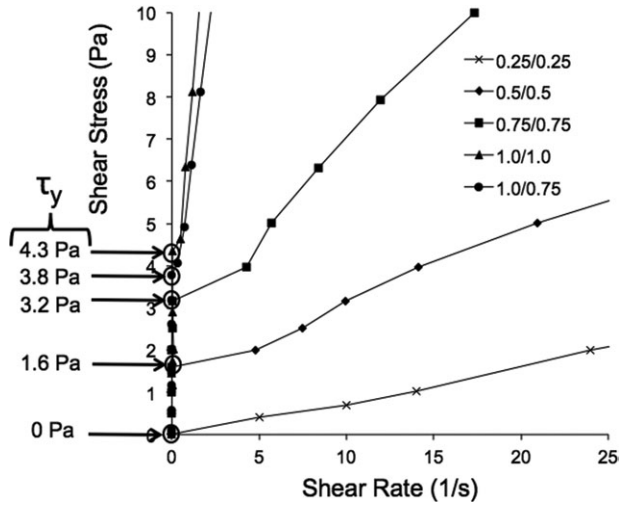

FIGURE 1. Shear stress versus shear rate relationships for five HAMC blends without cells (0.25/0.25, 0.5/0.5, 0.75/0.75, 1.0/1.0, and 1.0/0.75) demonstrate that yield stress (τ_y) increases with total polymer content.

and HA in the blend, which is in agreement with the yield stress data presented in Figure 1. Significantly, all blends formed a gel rapidly, as the slowest gelling blend required only 5 min to reach its gelation point.

HAMC rheology with hUTC

As shown in Figure 3, the addition of hUTC (at a loading of 10 million cells per mL) reduced the yield stress of all four blends. This indicates that dispersion of cells throughout the hydrogel matrix reduces its strength. In addition, the presence of cells slows gelation, as displayed in Figure 4 for the 0.75/0.75 formulation. Specifically, it takes ~1.4 more minutes for G' to intercept G'' when hUTC are included in the hydrogel. Although the equilibrium values of G' are similar with and without cells, the difference between the equilibrium G' and G'' values is smaller with the inclusion of cells, which is indicative of a weaker gel and thus corroborates the yield stress data in Figure 3.

hUTC viability in HAMC

CFSE-labeled hUTC were dispersed in each of the four HAMC blends, and their distribution was studied using confocal reconstructive imaging (Fig. 5). hUTC were homogenously distributed within the HAMC matrix immediately after mixing (day 0), and this distribution was stably maintained after 3 days of culture in all four blends. Interestingly, the initial rounded morphology of the cells observed on day 0 transitioned to a more extended morphology after 3 days, and the extent of this cellular extension tended to decrease with total polymer content in the scaffold. As shown in Figure 6, the population of live cells ($CFSE^+$ $EthD1^-$) was similar across all formulations immediately after seeding (day 0). On day 3, the only significant decrease in live cells was observed in 1.0/1.0 HAMC both in comparison with 1.0/1.0 on day 0 [Fig. 6(A)] and all other blends on day 3 [Fig. 6(B)]. The maintenance of live cell numbers in 0.5/0.5, 0.75/0.75, and 1.0/0.75 HAMC after 3 days of culture demonstrates their suitability as a scaffold for the delivery of hUTC.

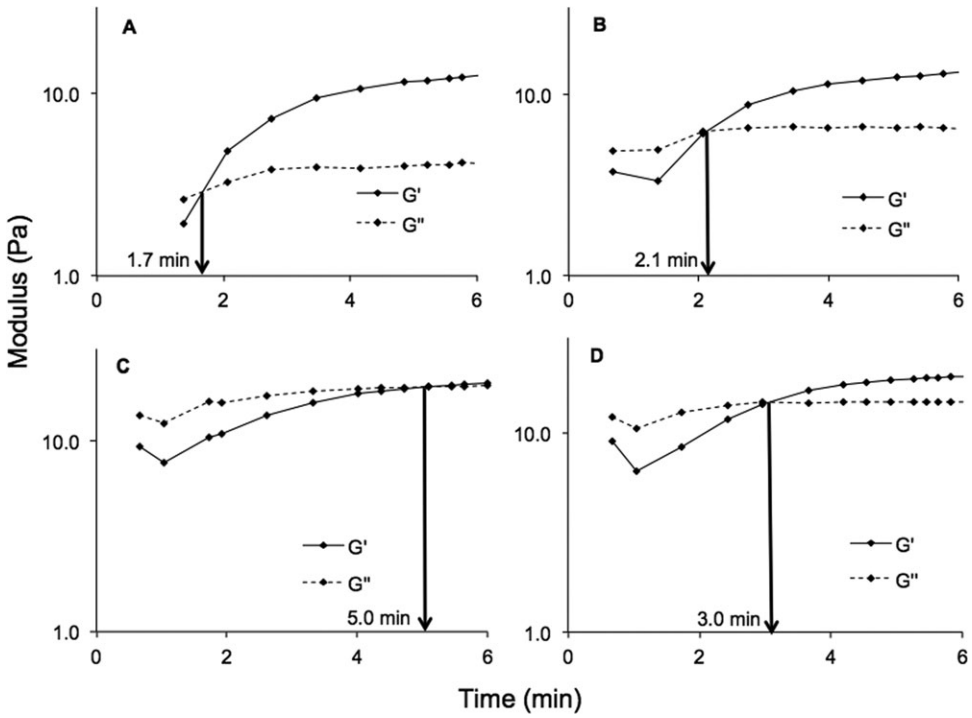

FIGURE 2. Gelation point of (A) 0.5/0.5, (B) 0.75/0.75, (C) 1.0/1.0, and (D) 1.0/0.75 HAMC. Storage (G') and loss (G'') moduli were measured over time after temperature adjustment from 4 to 37°C at time zero, simulating *in vivo* injection. Gelation time and moduli at the gelation point tended to increase with total polymer content, but all blends gelled in 5 min or less.

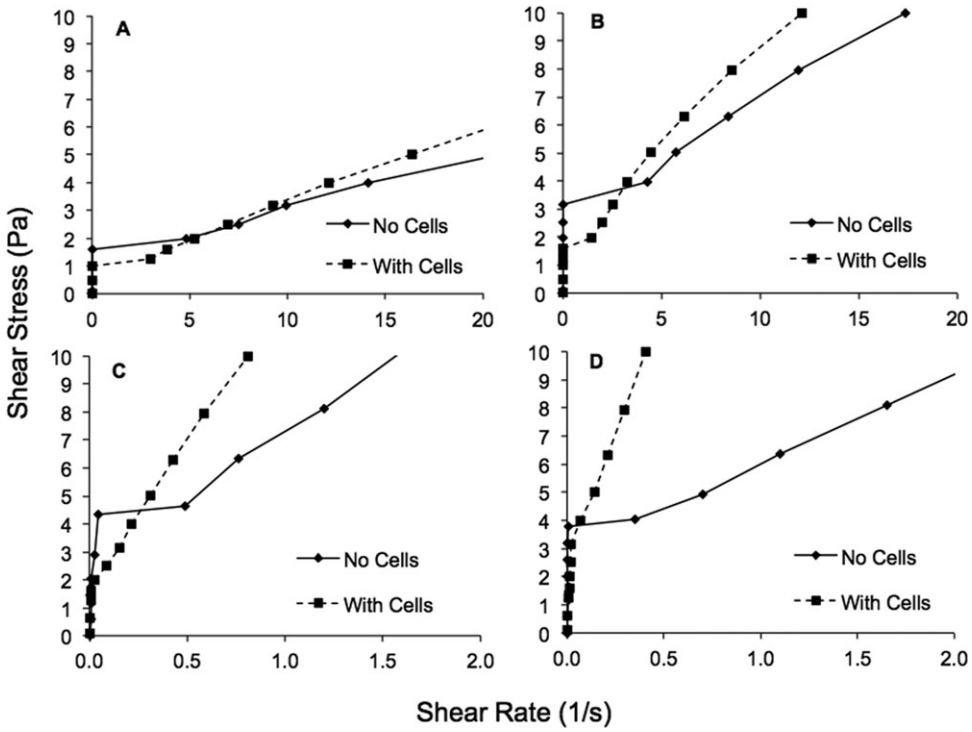

FIGURE 3. Comparison of shear stress versus shear rate relationship for (A) 0.5/0.5, (B) 0.75/0.75, (C) 1.0/1.0, and (D) 1.0/0.75 HAMC without cells and with 10 million cells per mL. For all blends, the presence of cells reduces, but does not eliminate, the yield stress. Note that the *x*-axes of (C) and (D) are different from those in (A) and (B).

DISCUSSION

The efficacy of therapeutic cell delivery to the injured spinal cord requires an appropriate delivery vehicle or scaffold to support maximal cell viability and persistence in the injured tissue. HAMC, a physical hydrogel that is injectable and biodegradable, has been shown to enhance the survival and distribution of retinal stem/progenitor cells[6] and neural stem/progenitor cells.[7] Consequently, HAMC is a promising vehicle for the delivery of hUTC to the spinal cord. However, previous studies have been limited to a single HAMC blend with a 0.5:0.5 HA:MC ratio by weight. Accordingly, the aim of this study was to analyze the effect of polymer composition on gel mechanical properties and cell survival. The five HA/MC weight percentages examined were selected, because they surround the previously successful 0.5/0.5 wt % blend.[6] Higher polymer concentrations were postulated to increase gel strength, but possibly hinder cell survival, while less-concentrated blends were expected to be more permissive to cellular growth, but weaker mechanically. All gels matched the modulus estimated for the spinal cord ($<$300 Pa).[16]

It was shown that gel strength could be tuned through simple adjustment of the MC and HA contents in the gel. Specifically, increasing the total polymer content in the scaffold resulted in an increase in yield stress and equilibrium storage modulus. This tunability is significant as the mechanical properties for optimal cell viability are dependent upon the particular cell population of interest.[17,18] Interestingly, dispersion of hUTC into the hydrogels caused a reduction in the yield stress compared to noncell controls. One possible explanation is that cells scattered throughout the polymer matrix physically impede the formation of hydrophobic junctions between MC chains. However, it should be emphasized that the strength reduction is modest, as even

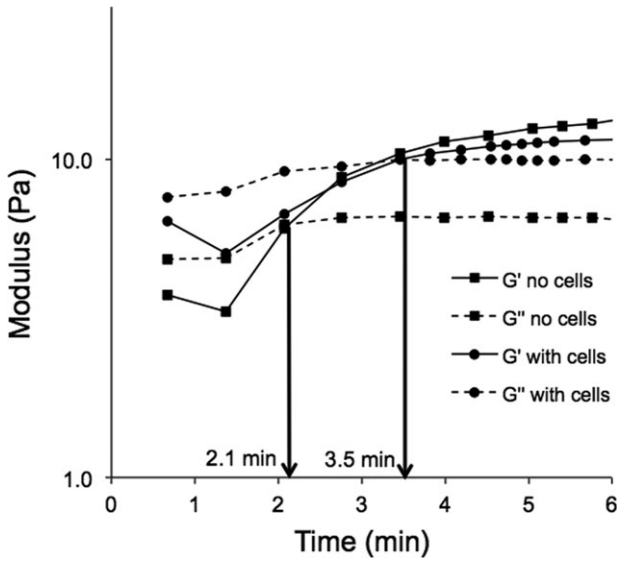

FIGURE 4. Gelation point of 0.75/0.75 HAMC without cells and with 10 million cells per mL. Storage (G') and loss (G'') moduli were measured over time after temperature adjustment from 4 to 37°C at time zero, simulating *in vivo* injection. The presence of cells slows gelation by roughly 1.4 min.

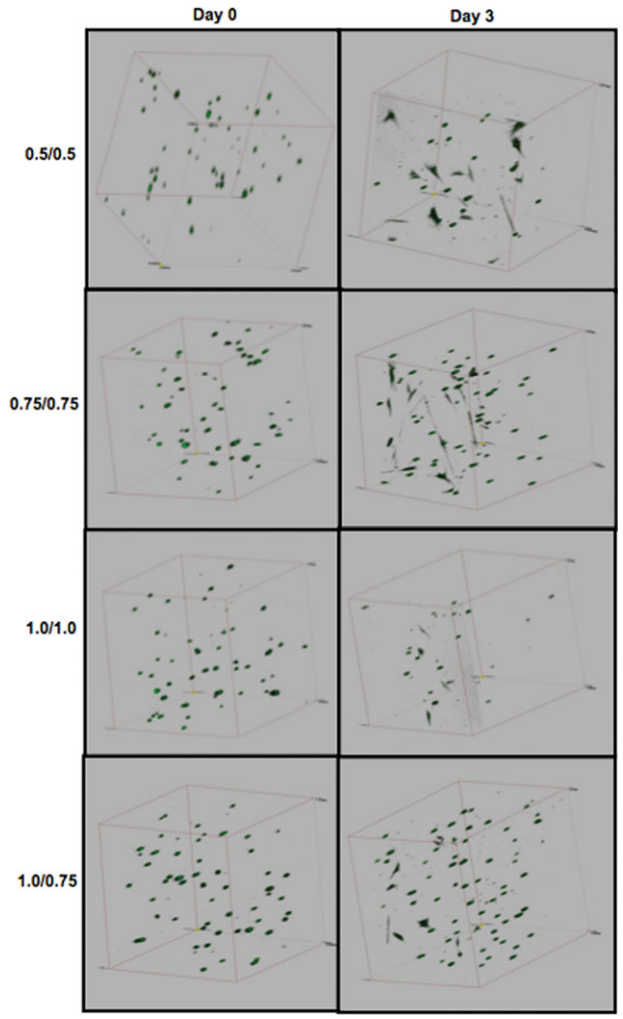

FIGURE 5. Confocal reconstructions of $CFSE^{+}$ hUTC suspensions immediately (day 0) and 3 days after seeding in 0.5/0.5, 0.75/0.75, 1.0/1.0, and 1.0/0.75 HAMC illustrating random cellular distribution and inhibition of cellular aggregation and settling. Cells assume a more extended morphology after 3 days in the gel. Boxed region is 1.7 × 1.7 × 1.7 mm. [Color figure can be viewed in the online issue, which is available at wileyonlinelibrary.com.]

the weakest HAMC blend remains a gel (i.e., it has a nonzero yield stress) upon the addition of cells.

In addition to gel strength, gelation time is important to the success of HAMC as a cell delivery scaffold. Because of the inverse thermal-gelling properties of MC, HAMC acts like a viscous liquid at ambient temperature ($G' < G''$) but gels upon exposure to physiological temperatures ($G' > G''$). Rapid gelation upon injection into the body is thought to positively contribute the longevity of the scaffold. Although gelation time upon simulated *in vivo* injection was observed to increase with total polymer content, all blends were confirmed to gel in 5 min or less, which is sufficiently fast for hUTC delivery. Addition of cells to the 0.75/0.75 hydrogel delayed gelation, but only by roughly 1.4 min. It should be noted that a difference in testing methodology resulted in the HAMC gelation times reported herein to be much faster than those reported previously.[6] In contrast to the observation-based inverted tube test method used in previous work, the G'/G'' time sweep method uses precise quantitation of viscoelastic behavior to determine the point in which a gel network has formed and so is considered a more accurate technique.[19]

Relevant to the ultimate application of HAMC for cell delivery, we tested whether the difference in rheological properties of the four HAMC blends impacted the morphology and survival of encapsulated hUTC. Cells were evenly distributed throughout the gel immediately after formulation, and this was maintained for 3 days in culture. HAMC thus prevents cellular aggregation and allows the cells to exist in a more natural three-dimensional arrangement. Another feature important to the viability of anchorage-dependent cells like hUTC is the ability to extend processes into the scaffold. Adhesion to the substrate in this manner prevents anoikis and so enhances the survival of transplanted cells.[9,20] The presence of cell processes extending into the matrix after 3 days in all HAMC blends reflects positively on the utility of the hydrogel as a cell delivery scaffold. The mechanism of cell adhesion to the material is undefined; however, we postulate that HA is mediating the

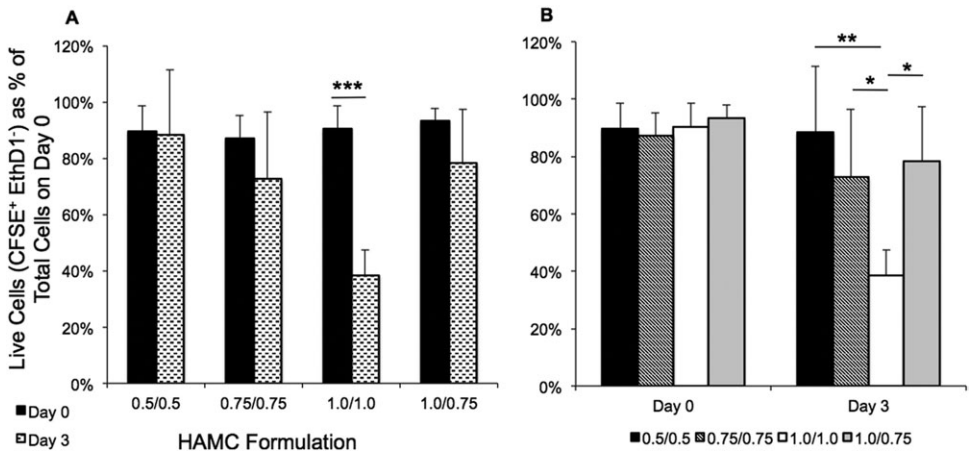

FIGURE 6. Percent live hUTC immediately (day 0) and 3 days after seeding in 0.5/0.5, 0.75/0.75, 1.0/1.0, and 1.0/0.75 HAMC. Panel (A) depicts statistics comparing day 0 and 3 for each blend, while panel (B) depicts statistics comparing different blends on each day. ($n = 6$ per group, mean ± standard deviation).

process. HA interacts with cells via the CD44 cell-surface glycoprotein, which is expressed in the majority of mammalian cells, including hUTC.[14] However, the length and abundance of these extensions tended to decrease with total polymer content. This could be due to a reduction in gel permeability limiting molecular transport, but it is known that HAMC formulations as high as 1.0/2.0 wt % permit the rapid Fickian diffusion of large proteins.[21,22] Consequently, limitations in waste removal and nutrient provision are not likely the cause of the reduction in cell extensions at higher polymer concentrations. It is more likely that the increased stiffness of the hydrogel acts as a physical barrier to cellular elongation. The consequences of this impediment to the extension of processes were observed most acutely in 1.0/1.0 HAMC, as live cells (as a percent of total cells on day 0) dropped from 90.4% $\pm$ 8.2% on day 0 to 38.5% $\pm$ 9.0% on day 3. Importantly, a significant decrease in live cells on day 3 was not observed in the three other blends, meaning that the stiffness of these hydrogels was appropriate for the maintenance of cell viability.

CONCLUSIONS

HAMC hydrogels designed for localized, minimally invasive cell delivery to the injured spinal cord were characterized in terms of mechanical strength, gelation time, and cell viability. Mechanical strength of the scaffolds as measured through yield stress and elastic modulus was tunable through simple adjustment to the concentration of constituent polymers and viscous HAMC solutions gelled rapidly upon heating to physiological temperatures. hUTC cultured in HAMC were homogenously and stably distributed throughout the scaffold and were able to adopt an extended, adherent morphology. Live cell numbers were stable over 3 days in all blends except the most concentrated, 1.0/1.0 HAMC. Consequently, HAMC holds considerable potential as a scaffold for cell transplantation therapy. Ongoing studies are examining the efficacy of 0.5/0.5 HAMC for the delivery of hUTC to the injured rat spinal cord.

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