

# Agarose and methylcellulose hydrogel blends for nerve regeneration applications

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## Abstract

Trauma sustained to the central nervous system is a debilitating problem for thousands of people worldwide. Neuronal regeneration within the central nervous system is hindered by several factors, making a multi-faceted approach necessary. Two factors contributing to injury are the irregular geometry of injured sites and the absence of tissue to hold potential nerve guides and drug therapies. Biocompatible hydrogels, injectable at room temperature, that rapidly solidify at physiological temperatures (37 °C) are beneficial materials that could hold nerve guidance channels in place and be loaded with therapeutic agents to aid wound healing. Our studies have shown that thermoreversible methylcellulose can be combined with agarose to create hydrogel blends that accommodate these properties. Three separate novel hydrogel blends were created by mixing methylcellulose with one of the three different agaroses. Gelation time tests show that the blends solidify at a faster rate than base methylcellulose at 37 °C. Rheological data showed that the elastic modulus of the hydrogel blends rapidly increases at 37 °C. Culturing experiments reveal that the morphology of dissociated dorsal root ganglion neurons was not altered when the hydrogels were placed onto the cells. The different blends were further assessed using dissolution tests, pore size evaluations using scanning electron microscopy and measuring the force required for injection. This research demonstrates that blends of agarose and methylcellulose solidify much more quickly than plain methylcellulose, while solidifying at physiological temperatures where agarose cannot. These hydrogel blends, which solidify at physiological temperatures naturally, do not require ultraviolet light or synthetic chemical cross linkers to facilitate solidification. Thus, these hydrogel blends have potential use in delivering therapeutics and holding scaffolding in place within the nervous system.

## 1. Introduction

The irregularity of the cavity caused by central nervous system trauma complicates the provision of scaffolding for nerve regeneration therapies [1]. This has led to the creation of various polymer scaffolding that can bridge the gap caused by injury [2–11]. The use of hydrogels in biomedical applications has been studied in cartilage [12], skeletal tissue [13], smooth muscle [14], wound dressings [15, 16], drug delivery [17] and the spinal cord [18, 19]. Hydrogel materials that are supportive of nerve survival, injectable and able to conform to the geometry of the injury site are being investigated [5–10].

Hydrogel materials are ideal for nerve regeneration applications because of their potential ability to release therapeutic drugs and anchor nerve guidance devices. It is important that these hydrogels be injectable in the liquid state and solidify once subjected to physiological temperatures. After gelation, they should continue as a solid and remain in the injury site while healing and regeneration occur. Also, due to the delicate nature of the nervous system, it is necessary to develop hydrogel systems that solidify naturally without chemically manipulating the structure of the material.

Low gelation temperature (8–17 °C) agarose has been used in nerve regeneration applications previously [5, 20].

Agarose is a purified linear galactan hydrocolloid derived from marine algae that consists of repeating agarobiose disaccharide units [21]. Low gelation temperature agarose solidifies at 8–17 °C and stays solid until melting around 37 °C, thus demonstrating a high degree of thermal hysteresis [22]. It is injectable, biocompatible and can be linked with proteins to encourage axonal extension [23–26]. Although low gelation temperature agarose does foster axonal outgrowth *in vitro* [20], the low melting point of agarose makes it difficult to use *in vivo*. It may be possible to maintain the growth promoting properties of agarose, while changing its gelation properties so that the hydrogel remains solid at physiological temperatures. Experiments have been conducted to cool low gelation temperature agarose after injection [5], yet blowing cold gases onto nervous system tissue could potentially be harmful to neurons that survived the initial injury.

Other forms of agarose with different chemical properties are also available that produce varied thermal characteristics, based on the degree of methoxyl substitution [27]. These forms of agarose, commercially known as Metaphor<sup>®</sup> and NuSieve 3:1<sup>®</sup>, have not been tested and both have higher gelation temperatures than the low gelation temperature agarose known as SeaPrep<sup>®</sup>. Since all forms of agarose must be cooled from a higher temperature to form a solid hydrogel, injecting heated liquid into an injury site is dangerous because high temperatures could also initiate further injury. Thus, there is a need to augment the gelation properties of agarose.

Methylcellulose is a food additive used as a thickening agent and has gelation properties opposite to that of agarose. Methylcellulose is unique because of its thermoreversibility characteristics. It exhibits low viscosity at low temperatures and a higher viscosity at higher temperatures as the polymer chains dehydrate and interact with each other. Methylcellulose is biocompatible and its gelling properties have been well studied [6, 10, 28–30]. At room temperature it can be injected through a common syringe *in vivo*. Methylcellulose has been proven to support some nerve regeneration [7] and can also be linked with proteins which encourage axonal extension [31]. Mixtures of methylcellulose with hyaluronan, a substance with gelling properties similar to agarose, have recently been shown to change the properties of methylcellulose and provide a new hydrogel with regenerative abilities [10].

Currently, no studies have been published on the effects of combining agarose and methylcellulose. Agarose's ability to support nerve growth and the unique gelling characteristics of methylcellulose lead us to believe that the combination of these two biocompatible injectable hydrogels would create a novel three-dimensional material that could be injected directly into the damaged nervous system or in close proximity to the nervous system, solidify *in situ*, and hold and release therapeutics into the injury site. Experimentation with multiple forms of agarose will allow for determination of the optimum agarose and methylcellulose blend.

## 2. Materials and methods

### 2.1. Creation of agarose/methylcellulose blends

5%, 7% and 9% (wt/vol) methylcellulose hydrogels were prepared in phosphate buffer saline (PBS) and tested for gelation temperature. It was observed that 5% methylcellulose required temperatures higher than physiological temperatures to solidify while 9% methylcellulose was highly viscous causing the hydrogel to solidify at temperatures much lower than 37 °C. 7% methylcellulose solidified at approximately 37 °C and was chosen to be the concentration used in combination with agarose.

Three agaroses were chosen to be tested for combination with methylcellulose. SeaPrep<sup>®</sup> agarose was chosen because of its known ability to support nerve growth [5]. NuSieve 3:1<sup>®</sup> and MetaPhor<sup>®</sup> (Cambrex Bio Science Rockland, Inc., Rockland, ME) have never been tested and were chosen because of novelty and because their gelation properties differ from those of SeaPrep<sup>®</sup> agarose. A type of methylcellulose called METHOCEL<sup>®</sup> A15 Premium LV (Dow Chemical Company, Midland, MI) was used because of its previous success when used within the central nervous system [10].

The concentrations of agarose to be used were chosen based on gelation tests with mixtures containing 7% methylcellulose and varying amounts of agarose. For each type of agarose, the concentrations of the hydrogel that exhibited the fastest gelation time and a gelation temperature of approximately 37 °C were chosen to be tested further. This resulted in 1.5% (wt/vol of PBS) MetaPhor<sup>®</sup> and SeaPrep<sup>®</sup> blends, and 0.75% (wt/vol of PBS) NuSieve 3:1<sup>®</sup>.

To create each blend, the desired masses were first measured. The agarose was then deposited into a beaker and mixed with 7.4 pH sterile PBS. The top of the beaker was covered and the beaker was heated with constant stirring until the mixture was clear and colorless. After the foil was removed, the solution was measured carefully to check for evaporation. Water was mixed into the hydrogel to ensure accurate concentration and osmolarity. While covered, the mixture was heated before being filtered into a sterile beaker using a Millex 0.22 µm filter (Millipore, Bedford, MA). Methylcellulose powder was sterilized using ethylene oxide gas. The methylcellulose addition utilized a previously published method for mixing methylcellulose [6]. The agarose hydrogel solution was heated in the sterile hood until it reached over 80 °C. When the PBS is above this temperature, the methylcellulose cannot dissolve and can easily be dispersed throughout the liquid [32]. While still on the hot plate, methylcellulose was added. The mixture was then stirred until all the particles were dispersed. When a cloudy uniform liquid had formed, the beaker was moved to an ice bath where cold (~4 °C) sterile PBS was added. The mixture was stirred for 10 min and then centrifuged to remove air bubbles that formed during stirring.

### 2.2. Gelation temperature test

It was important to know the gelation temperature characteristics of the agarose hydrogels alone and when they

are mixed with methylcellulose. Gelation temperature was measured using the inverted test tube method [10]. The three agarose solutions (1.5% MetaPhor<sup>®</sup>, 0.75% NuSieve 3:1<sup>®</sup> and 1.5% SeaPrep<sup>®</sup>) were melted and then equilibrated at 37 °C for at least 3 h prior to temperature testing. Three samples of each of the hydrogels ( $n = 3$ ) were submerged in a 50 °C water bath for 1 min, and then the temperature of the bath was lowered 1 °C min<sup>-1</sup> until gelation occurred. When the mixture no longer flowed while inverted, it was considered solid, and the temperature was recorded.

Agarose and methylcellulose have inverse thermal gelling properties. Consequently, the blends solidify at higher temperatures than do the agarose hydrogels alone [22]. Triplicate samples ( $n = 3$ ) of each of the blends were started at 15 °C in the water bath where they equilibrated for 5 min, and the water temperature was increased 1 °C min<sup>-1</sup> until gelation occurred.

### 2.3. Gelation time test

To begin the gelation time test, three 1 ml samples of each of the liquid hydrogels ( $n = 3$ ) were pipetted into a 1.5 ml micro-centrifuge tube. The tube was allowed to equilibrate to room temperature for 20–30 min. It was then placed in a 37 °C water bath and checked every minute by a blinded observer to see if the liquid solidified. Time was stopped when the hydrogels were out of the water and was restarted when they were put back into the water. The gelation point was determined by the inverted test tube method [10]. Hydrogels were inverted and firmly shaken twice to be sure that there was no adhesion to the side of the micro-centrifuge tube before declaring each a solid. The time required for the liquid to change to a solid was recorded, and this value was denoted as the gelation time.

### 2.4. Rheometry

Small amplitude oscillatory rheometry was conducted using a Bohlin C-VOR Rheometer (Malvern Instruments Ltd, Malvern, Worcestershire, UK) and provided quantified values for gelation time. Frequency sweeps from 10 to 100 radians were repeated isothermally at both 30 °C and 37 °C for 45 min. Each of the hydrogel samples, in triplicate ( $n = 3$ ), were heated using a convection oven, utilizing heated nitrogen air to maintain the temperature during the test.

### 2.5. Quantification of the force required to inject hydrogel

The hydrogels of this study were created to be injected into an injury site. Once the hydrogels were mixed, the hydrogels had visibly high viscosity. To quantify the injection force for each hydrogel, a force-sensing injection pump experiment was designed. The injection force was measured using a NE-1600 syringe pump (New Era Pump Systems, Farmingdale, NY) and a Vernier Dual Range Force Sensor (Vernier Software and Technology, Beaverton, OR) in triplicate ( $n = 3$ ). The force measurement system was set up to measure compression. 28-gauge 100 cc micro-fine insulin needles (BD Medical, Franklin Lakes, NJ) were filled with 20 cc of each hydrogel. 20 cc is greater than the eventual clinical amount of hydrogel that

will be used, but allowed sufficient time to record the forces generated by extruding the hydrogel through the syringe. The syringe was placed in the syringe pump and injected while the compressive force was being measured. The highest force recorded by the measurement system was recorded. The force was measured at injection rates of 0.1 ml min<sup>-1</sup>, 0.2 ml min<sup>-1</sup> and 0.3 ml min<sup>-1</sup>.

### 2.6. Structural characterization

For evaluation of potential drug release characteristics, the pore sizes of the blends and plain methylcellulose were evaluated using scanning electron microscopy (SEM). For preparation, a disc of each of the hydrogels was solidified in a mold ( $n = 3$ ). The samples were snap-frozen using liquid nitrogen and freeze-dried. Next, the samples were coated with platinum/palladium using a Hummer 6.2 Sputter Coater (Anatech Ltd, Denver, NC) and analyzed with a Hitachi S4700 field emission scanning electron microscope. Four images were obtained at a magnification of 250× and four images were obtained at a magnification of 900× for each hydrogel sample. The pore sizes from all images were measured using Adobe Photoshop and the scale recorded from the SEM. The pore size was counted as the diameter of the pore. For oblong pores, the shortest diameter was recorded because it was taken to be the limiting diameter.

### 2.7. In vitro dissolution

For nerve regeneration applications, it is important that the injected hydrogel persist in the injury cavity to allow for sustained release of therapeutics and to hold scaffolds in place. To study dissolution, a simple test [10] involving the biomaterial bathed in PBS at 37 °C is able to provide an *in vitro* simulation of hydrogel dissolution. The masses of eighteen 1.5 ml micro-centrifuge tubes were pre-recorded. 100 μl of each hydrogel solution was injected into the bottom of a centrifuge tube in triplicate ( $n = 3$ ). The solutions were incubated for approximately 1 h at 37 °C to ensure solidification. For each blend, the mass of the solution in the tubes was calculated by subtracting the pre-recorded mass of the tubes from the total mass with the hydrogel inside. Three of the tubes were freeze-dried immediately and the freeze-dried masses were recorded. 800 μl of PBS was then added into the fifteen remaining micro-centrifuge tubes on top of the solidified hydrogel mixture. At the same time every day, PBS was poured off and new PBS was added. At 1, 4, 7, 14 and 28 days, three of the tubes were removed, freeze-dried and massed for dissolution values. The percentage of dissolution was calculated using the following equation:

$$\%Dissolution(t) = 100 - \left( \frac{W_d(t)/W_w(0)}{W_{d1}(0)/W_{w1}(0)} \times 100 \right)$$

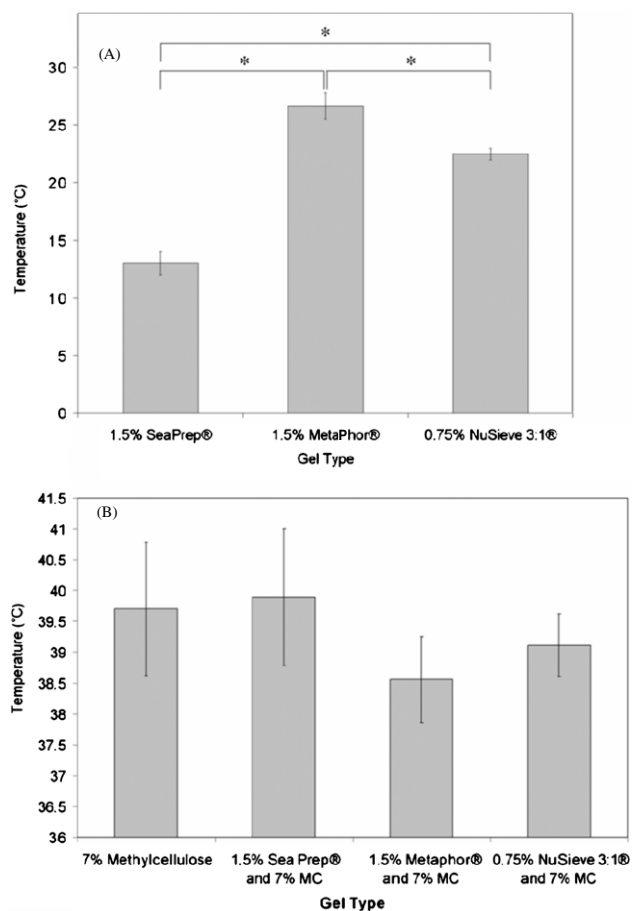
where  $W_{d1}(0)$  is the mass of dry control hydrogel polymer,  $W_{w1}(0)$  is the mass of wet control hydrogel,  $W_d(t)$  is the mass of the dry polymer after dissolution and  $W_w(0)$  is the initial mass of the hydrogel before dissolution.

2.8. Neuronal response to hydrogels *in vitro*

The hydrogel fabricated would be a viscous liquid at refrigerated temperatures upon application. Once presented to a physiological environment, the hydrogel would then solidify. Thus, it is important to determine whether the application of a chilled hydrogel to neurons and subsequent solidification affects neuronal viability and morphology. Therefore, dorsal root ganglion cells were isolated from embryonic stage nine chick embryos using techniques approved by the Institutional Animal Care and Use Committee at Michigan Technological University. Gathered ganglia were placed into Hank's Balanced Salt Solution (HBSS) (Media Tech, Herndon, VA), pipetted into a conical tube and centrifuged. The HBSS was removed and 1 ml of 0.25% trypsin solution added. The trypsin were incubated with the cells for 20 min, and then centrifuged and removed. 2 ml of growth medium (88% Dulbecco's Modification of Eagle's Medium (Mediatech Inc., Herndon, VA), 10% fetal bovine serum (FBS) and 2% penicillin streptomycin) was added to the cells and a fire-blown Pasteur pipette was used to dissociate the cells. The solution was then centrifuged, the growth medium removed and 1 ml of neural growth media (neurobasal medium l-glutamine, B-27, and penicillin/streptomycin) (Invitrogen, Carlsbad, CA) was added. The cells were then placed into a small culture dish and allowed to incubate for 30 min. After 30 min, the culture dish was agitated and the neural basal media consisting of neurons was removed and placed back into a conical tube. 10  $\mu$ l of the cell suspension was then stained with Trypan Blue, to account for cells that may have died during the dissociation process.

Neurons were plated onto laminin coated 6-well plates (BD Biocoat, San Jose, CA) and allowed to attach for 48 h in a tissue culture incubator in the presence of neural growth medium supplemented with NGF (Calbiochem, San Diego, CA) for a final concentration of 50 ng ml<sup>-1</sup>. After 48 h, the neural growth medium was removed and approximately 50  $\mu$ l of chilled hydrogel (refrigerated temperature) was placed on top of the neurons. The cells with hydrogel were then placed into a tissue culture incubator for 15 min to allow for hydrogel solidification, after which neural growth medium supplemented with NGF was again added. The cultures were then allowed to persist for 48 h and then the cultures were stained.

Neural growth medium was removed from the hydrogels and the hydrogels were washed with PBS. Calcein-AM stock solution was made by dissolving 1 mg of calcein-AM (Sigma, St Louis, MO) into 250  $\mu$ l of DMSO. 5  $\mu$ l of the calcein-AM stock was added to 10 ml of PBS. The PBS/calcein-AM solution was added on top of each hydrogel. We found that subjecting the hydrogels to a room temperature environment for an extended period of time caused the solidified hydrogels to dissolve. To prevent dissolution, the hydrogels were placed back into the incubator to allow the stains to interact with the cells. The stain was removed after 30 min and the hydrogels were again washed with PBS for 15 min. The cell cultures were analyzed using a Zeiss Axiovert 200M inverted fluorescence microscope with Apotome. The hydrogels were analyzed to confirm that the application of the hydrogel did

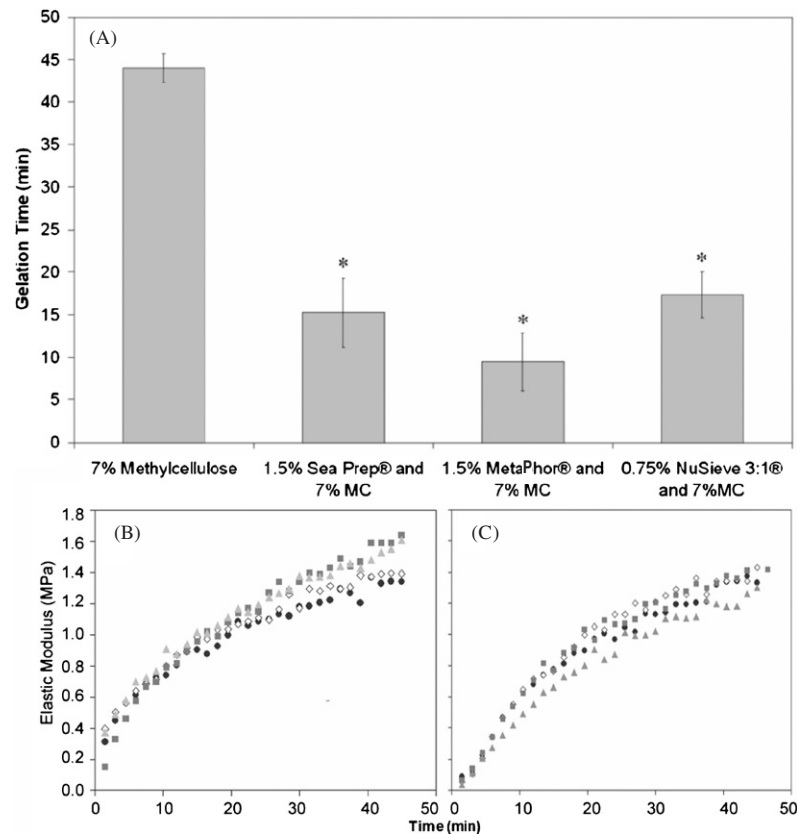


**Figure 1.** (A) Gelation temperatures of three agaroses when not mixed with methylcellulose. These temperatures were found by lowering the temperature at a constant rate rather than raising it at a constant rate. All agaroses had statistically significant gelation temperatures using the Tukey–Kramer HSD test ( $n = 3$ ). (B) Temperatures at which mixtures of agarose (1.5% SeaPrep®, 1.5% MetaPhor®, 0.75% NuSieve 3:1®) and 7% methylcellulose solidified compared to solely 7% methylcellulose hydrogel. ‘\*’ denotes statistical significance when comparing the plain agarose hydrogels to each other using the Tukey–Kramer HSD test ( $n = 3$ ).

not alter neuronal viability or morphology. Each of the hydrogels were tested three times ( $n = 3$ ), meaning that each hydrogel was added to cultures prepared on three separate days.

## 2.9. Statistical analysis

ANOVA was run to determine statistical significance between the temperatures when agarose gelled, the time it took the hydrogels to blend, and the per cent of hydrogel that had degraded. The Tukey–Kramer HSD test of all pairs was used to determine statistical significance between individual experimental groups. These calculations were performed using JMP IN 5.1 software with a significance level of  $\alpha = 0.05$  (SAS Institute Inc., Cary, NC). All data are reported as the mean plus or minus the standard deviation.



**Figure 2.** (A) Time required for mixtures of agarose (1.5% SeaPrep<sup>®</sup>, 1.5% MetaPhor<sup>®</sup>, 0.75% NuSieve 3:1<sup>®</sup>) and 7% methylcellulose to solidify in a 37 °C water bath. \*<sup>\*</sup> denotes statistical significance when comparing each of the blends to plain methylcellulose using the Tukey–Kramer HSD test ( $n = 3$ ). (B and C) Elastic modulus of the hydrogels (( $\diamond$ ) 1.5% SeaPrep<sup>®</sup> with 7% MC, ( $\blacksquare$ ) 0.75% NuSieve 3:1<sup>®</sup> with 7% MC, ( $\blacktriangle$ ) 1.5% MetaPhor<sup>®</sup> with 7% MC, ( $\bullet$ ) base 7% MC) during an isothermic frequency sweep from 10 to 100 radians. Temperature was kept at 37 °C (B) and 30 °C (C). In all of the rheology tests, three samples of each hydrogel were tested at 37 °C (B) and 30 °C (C). Rheology experiments were conducted in triplicate. The data presented represent one trial for each hydrogel since there was little variability between samples in each hydrogel group.

### 3. Results

#### 3.1. Gelation temperature

The gelation temperatures of the plain agarose hydrogels were below physiological temperatures. 1.5% SeaPrep<sup>®</sup> proved to have the lowest gelation temperature, 0.75% NuSieve 3:1<sup>®</sup> had the next highest and 1.5% MetaPhor<sup>®</sup> had the highest gelation temperature among the unblended agaroses. The plain agaroses all had significantly different gelation temperatures when compared to each other (figure 1(A)).

Figure 1(B) shows the results of the gelation temperature tests used in deciding the gelation temperature and the concentrations of agarose to be used in the remainder of the study. The agarose percentages chosen (1.5% SeaPrep<sup>®</sup>, 1.5% MetaPhor<sup>®</sup> and 0.75% NuSieve<sup>®</sup>) when mixed with 7% methylcellulose all gelled at approximately 37 °C when subjected to the temperature test.

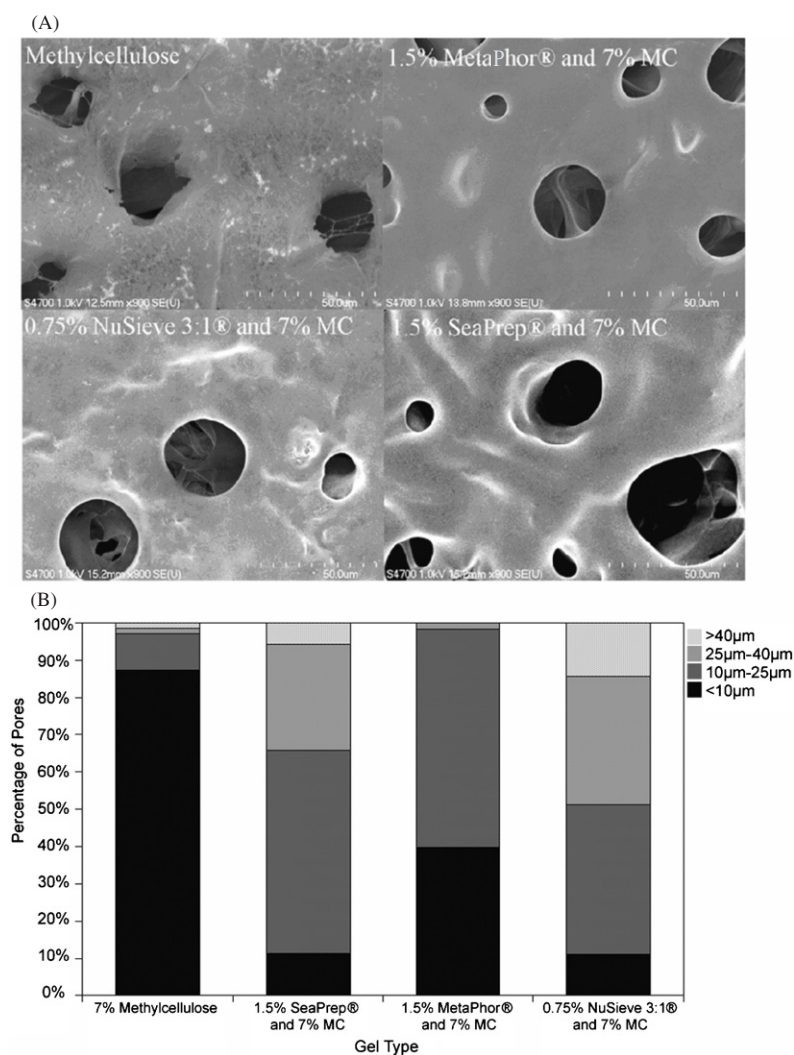
#### 3.2. Gelation time

Significant differences were seen in the time required for methylcellulose to solidify versus the gelation time of the

three blended hydrogels using the inverted test tube method as shown in figure 2(A). The hydrogel that solidified fastest was the 1.5% MetaPhor<sup>®</sup> with 7% methylcellulose which solidified on average after 9.5 min. The longest gelation time for any of the blended hydrogels was 0.75% NuSieve 3:1<sup>®</sup> with 7% methylcellulose which took an average of 17.3 min to solidify. 1.5% SeaPrep<sup>®</sup> with 7% methylcellulose took an average of 15.2 min to solidify. These were significantly lower than the 7% methylcellulose which averaged 44 min to solidify at 37 °C.

#### 3.3. Rheometry

The rheological testing showed that the elastic modulus of the hydrogels increased immediately once placed into a heated environment. The modulus then continued to gradually increase over the course of 15 min. All of the hydrogels had similar increasing modulus curves. Figures 2(B) and (C) compare the four tested hydrogels at 37 °C and 30 °C, respectively, at the frequency of 100 radians. The chart plots the rising modulus of the gels over the course of thirty iterations of a frequency sweep from 10 to 100 radians lasting 1.5 min each. The modulus values at 100 radians are shown here (all



**Figure 3.** (A) SEM microscope images showing pore size and morphology of base methylcellulose (MC), 1.5% MetaPhor® agarose and 7% methylcellulose blend, 0.75% NuSieve 3:1® agarose and 7% methylcellulose blend, 1.5% SeaPrep® agarose and 7% methylcellulose blend. Three samples of each hydrogel were analyzed. (B) Pore size distribution of all pores imaged within each of hydrogel species.

frequencies showed similar results). All hydrogels blends and plain methylcellulose at 37 °C had a modulus value of 1.0 MPa after residing in the heated chamber for 15 min. Each hydrogel was tested three times. However, since there was little variability seen within a particular hydrogel group, no error bars are reported.

### 3.4. Ease of injection

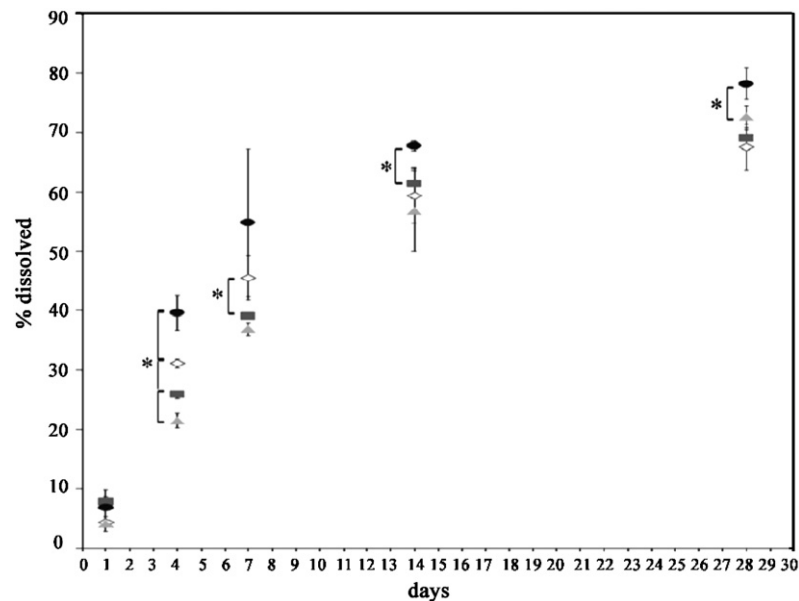
7% methylcellulose proved to require less force to inject than any of the agarose and methylcellulose blends. The force required to inject the hydrogel blends was noticeably larger than the force needed to inject methylcellulose, but all hydrogels could be injected through a 28-gauge syringe. The hydrogels requiring the most force to inject were the MetaPhor® and SeaPrep® blends. Table 1 shows the maximum forces recorded at the highest injection rate tested (0.3 ml min<sup>-1</sup>).

**Table 1.** Maximum force applied when injecting polymers at a rate of 0.3 ml min<sup>-1</sup>.

7% Methylcellulose	15.37 ± 1.38 N
1.5% SeaPrep® and 7% MC	19.83 ± 2.48 N
1.5% MetaPhor® and 7% MC	22.08 ± 2.12 N
0.75% NuSieve® and 7% MC	20.43 ± 1.62 N

### 3.5. Structural characterization

SEM images were captured of each of the hydrogels. Figure 3(A) contains representative images of each of the hydrogels. Characterization of the pore sizes within the different hydrogels (figure 3(B)) showed that the 7% methylcellulose hydrogel had the highest percentage of pores less than 10 µm in diameter. The addition of agarose to the hydrogel created larger pores. Of the hydrogel blends, the 1.5% MetaPhor®/7% methylcellulose hydrogel blend had a pore size distribution most similar to that of 7%



**Figure 4.** Per cent of hydrogel that is dissolved measured on day 1, 4, 7, 14 and 28. (◇)1.5% SeaPrep<sup>®</sup> with 7% MC, (■) 0.75% NuSieve 3:1<sup>®</sup> with 7% MC, (▲) 1.5% MetaPhor<sup>®</sup> with 7% MC, (●) base 7% MC. Dissolution studies for each hydrogel were run in triplicate ( $n = 3$ ). ‘\*’ denotes statistical significance when comparing the agarose/methylcellulose blends to each other and to plain methylcellulose using the Tukey–Kramer HSD test.

methylcellulose. The 1.5% SeaPrep<sup>®</sup>/7% methylcellulose blend and 0.75% NuSieve 3:1<sup>®</sup>/7% methylcellulose blend had more pores of larger diameter than the 1.5% MetaPhor<sup>®</sup>/7% methylcellulose blend and the methylcellulose control.

### 3.6. *In vitro* dissolution

Plain 1.5% SeaPrep<sup>®</sup>, 0.75% NuSieve 3:1<sup>®</sup> and 1.5% MetaPhor<sup>®</sup> agarose all dissolved completely when left in PBS in a 37 °C incubator for only 7 days, making it difficult to record the percentage dissolved due to hydrogel instability (data not shown). As seen in figure 4, the three blends have markedly similar dissolution trends. Each hydrogel blend was approximately 70% dissolved by the end of 28 days in the cycled PBS solution. Base 7% methylcellulose proved to be a weaker hydrogel that dissolves more rapidly than the hydrogel blends, dissolving over 75% in 28 days.

### 3.7. Cell culturing

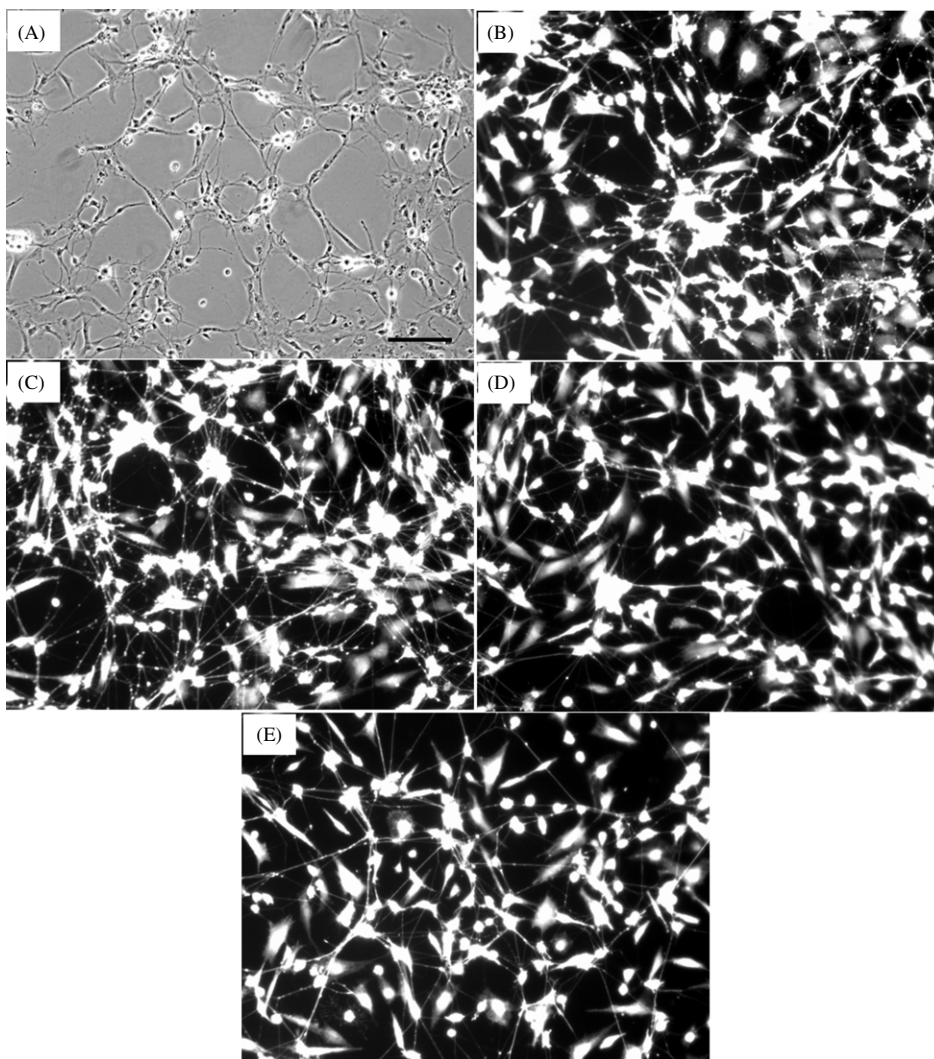
Dissociated DRG cultures were analyzed prior to administration of each of the hydrogels (figure 5(A)). Cultures in which 7% methylcellulose (figure 5(B)), 7% methylcellulose/1.5% SeaPrep<sup>®</sup> (figure 5(C)), 1.5% MetaPhor<sup>®</sup>/7% methylcellulose (figure 5(D)) or 7% methylcellulose/0.75% NuSieve 3:1<sup>®</sup> (figure 5(E)) were added show that neurons maintained their morphology after addition of the hydrogel. Even though representative images showing dissociated neurons in the presence of hydrogel contained different amounts of cells (due to the uneven distribution of cells on the culture dish), all images showed that even after administration of hydrogel neurons remained attached to the culture dish and had axons that were connected

to each other. This shows that cultures remained viable after the addition of the hydrogel.

## 4. Discussion

The data presented here suggest that blends of agarose and methylcellulose solidify more quickly at physiological temperatures than plain methylcellulose. The combination of agarose with methylcellulose created blends that were viscous liquids at refrigerated temperatures, less viscous liquids at room temperature and solid species at physiological temperatures. These properties are most similar to methylcellulose, yet the new hydrogels did display unique and beneficial characteristics. The viscosity of plain methylcellulose and the blends, as seen by the rheometry data, increases within the first few minutes of temperature increase. As temperature increases, the hydrogels remain pliable for approximately 15 min. It should be noted that while the modulus continues to increase over time, the solidification occurs in the blends in 10 min, where the curves decrease in slope (figures 2(B) and (C)). It should also be noted that the blends all solidify at 30 °C as well, but the solidification time increased to 15–20 min. Hydrogel solidification occurs naturally, without exposing tissues to ultraviolet light or chemical cross linkers. While studies have shown that continued ultraviolet light exposure to cortical neurons induces apoptosis [33], brief exposure may also negatively influence neuronal viability in environments where the inflammatory response is already negatively impacting neuronal health. Cross linkers could also negatively impact neurons spared following central nervous system injury.

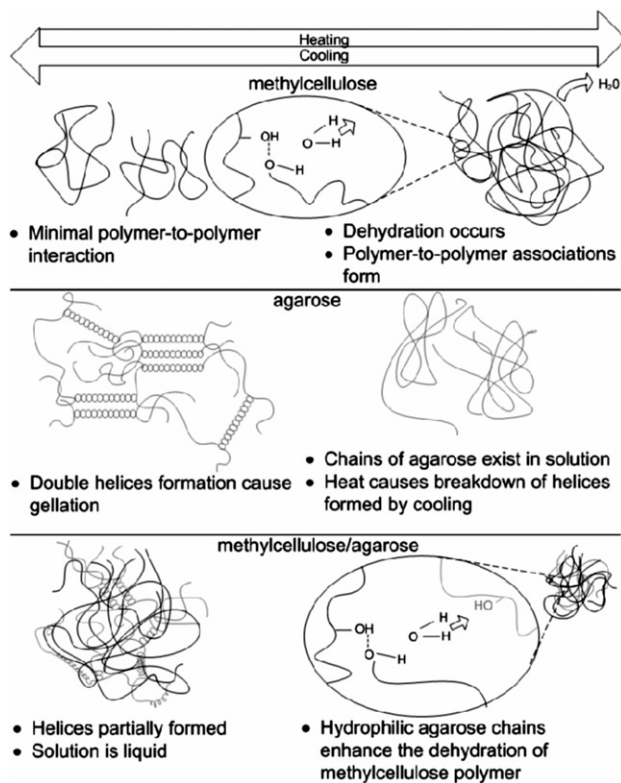
While the rheometry tests also showed gelation of the plain methylcellulose hydrogel to be similar to the blends,



**Figure 5.** Viability and morphology of neurons in the presence of the hydrogel blend. (A) Phase contrast image of dissociated culture before addition of hydrogel. (B) Dissociated culture in the presence of 7% methylcellulose. (C) Dissociated culture in the presence of 1.5% SeaPrep<sup>®</sup> with 7% methylcellulose. (D) Dissociated culture in the presence of 1.5% MetaPhor<sup>®</sup> with 7% methylcellulose. (E) Dissociated culture in the presence of 0.75% NuSieve 3:1<sup>®</sup> with 7% methylcellulose. The scale bar is equivalent to 100  $\mu\text{m}$ .

the inverted test tube experiment demonstrates the relative weakness of this hydrogel and its potential to run and escape the injury site even up to 40 min after injection. The Bohlin C-VOR rheometer available uses convection oven heating which heats the hydrogels in a manner unlike physiological conditions. Instead of air blowing to heat the hydrogels when injected *in vivo*, they will be surrounded by tissue at physiological temperatures. The inverted test tube test is able to better model the actual gelation time of the hydrogels in an environment similar to that seen in the damaged tissue. The rheological data are important, however, because they are able to show that the viscosity of the hydrogel increases over time. There is no distinct gelation point observed from the rheometry data, but rather a gradual increasing modulus, even at 30 °C, which shows that the hydrogels may even have a quicker gelation time if injected slowly or heated slightly before injection.

This more rapid gelation might be explained by examining the gelation properties of each individual hydrogel. When methylcellulose is kept at refrigerated temperatures its structure consists of a long polymer chain with water molecules embedded throughout, forming a viscous fluid. As methylcellulose heats up, it loses water molecules from within its polymer chain matrix. This initially decreases the viscosity as the polymer chains are allowed to freely move about, but eventually the polymer chains are forced to interact and a solid hydrogel is formed [32]. Agarose hydrogels solidify in a very different manner. When cooled, agarose chains form hydrogen bonds and hydrophobic relationships that aid toward the creation of double helices. It is the generation of these double helices that is assumed to create the formation of a solid agarose hydrogel [27]. The gelation mechanisms of these two hydrogels and a proposed mechanism of the hydrogel blends are shown in figure 6.



**Figure 6.** A macromolecular schematic description of how hydrogels solidify. Methylcellulose [32] and agarose [27] gelation mechanisms are both shown separately, and then the hypothesized gelation mechanism of the hydrogel blends is shown at the bottom.

The new hydrogel combinations have characteristics of both agarose and methylcellulose. It is possible that within the blend, agarose chains begin to form helices at lower temperatures, but helix formation is hindered due to the high concentration of interfering methylcellulose chains. Thus, at lower temperatures, the solution remains a liquid. When the hydrogel is heated, water escapes from the methylcellulose, and the agarose chains further unravel, causing a significant decrease in viscosity. When approaching gelation temperatures, the water escaping from the methylcellulose chains is expedited by its attraction to the agarose chains interpenetrating the methylcellulose. The interpenetrating blends also have more polymer matrix since the hydrogels have both methylcellulose and agarose chains. This creates a higher density hydrogel and increases the likelihood of polymer-to-polymer interaction. These could be reasons for the rapid gelation of the hydrogel blends.

Plain methylcellulose had a high number of small pores, due to the polymer-to-polymer gelling characteristic of this hydrogel. Interestingly, while NuSieve 3:1<sup>®</sup> and SeaPrep<sup>®</sup> agarose modified the hydrogel to have a broader range of pore sizes, MetaPhor<sup>®</sup> did not. The mechanism by which this occurs is not understood. The broad pore size distribution and this characterization of pore size will allow for greater understanding and control of therapeutic agent release. The polysaccharide chains of agarose and methylcellulose will affect release curves in a unique way due to their high

concentration of negative charges. This characteristic will likely cause slower release of positively charged peptides and a faster release of negatively charged species, allowing for more control over hydrogel therapeutic release.

The elastic modulus of the spinal cord has been measured at around 5 kPa, while the modulus of the spinal pia mater has been tested at 2.3 MPa [34]. Since the modulus of our material is much greater than the modulus of the spinal cord, it is not an ideal candidate for solely supporting axonal extension across an injury site, but since its modulus is less than the modulus of the innermost sheath surrounding the spinal cord, it is appropriate for use as an anchoring material for guidance devices and as a releasing mechanism for therapeutics.

All hydrogel blends dissolved in a similar manner. This could be expected since the concentration of polymer in the hydrogels was comparable. The three hydrogel blends dissolved more slowly in 28 days than did the plain methylcellulose, which increase its validity as a candidate material for central nervous system regeneration applications since longer dissolution times may be necessary for regeneration to occur within the injured central nervous system. It is important that the hydrogel persist for up to 14 days, since the blood-brain barrier (BBB) remains porous up to 14 days after injury [1]. A hydrogel persisting up to 14 days should then be able to deliver therapeutic over that time period. The blended hydrogels' ability to last for 28 days and then dissipate makes them ideal candidates for use as drug delivery devices. While the hydrogels dissolved slowly, the hydrogels also showed very minimal swelling in the PBS solution, holding at or below their original weight of water throughout the 28 days (data not shown).

Adding chilled hydrogel to established dissociated DRG cultures, allowing the hydrogel to solidify and allowing the hydrogel/cell interaction to continue over a period of 2 days did not affect neuronal viability and morphology (shown by the presence of neuronal attachment and the neurons maintaining their axonal processes) in all of the hydrogel groups tested. Thus, this suggests that the material and the process by which it would be applied *in vivo* would not negatively affect those neurons spared by initial injury. Since all three blends did not noticeably affect neuronal viability and morphology, particular blends could be used for different purposes based on gelation time or drug release requirements.

Since the addition of agarose to methylcellulose has been shown to reduce gelation time, affect pore size and not affect neuronal viability and morphology *in vitro*, the hydrogels have been injected within a rat spinal cord injury contusion model. Currently, different therapeutics are being loaded into the 1.5% SeaPrep<sup>®</sup> agarose/7% methylcellulose blend to characterize the release of these species from the hydrogel. The 1.5% SeaPrep<sup>®</sup> agarose/7% methylcellulose blend has the ability to dissolve, yet dissolves slowly enough to exist for up to 28 days post-injury. The blend also has the ability to solidify more quickly than plain methylcellulose when placed within a 37 °C environment. These studies have shown that the methylcellulose and agarose mixtures are materials that can be used in combination to potentially hold in place nerve

guidance channels and control the release of therapeutics in a more complex, multi-faceted approach aimed at enhancing regeneration and functional recovery within the damaged nervous system.

## 5. Conclusion

Because of unique polymer-to-polymer interactions, liquid agarose and methylcellulose hydrogel combinations polymerize once exposed to physiological temperatures naturally. The solidification of these hydrogels was shown to occur more rapidly than plain methylcellulose. The type of agarose affected the pore size character, gelation time and gelation temperature of the blend. Furthermore, *in vitro* experiments where the hydrogel was applied to neurons as a chilled liquid, allowed to solidify and then remained in contact with the neurons for 2 days suggest that the application of the hydrogel would not negatively affect neurons spared from the initial injury. Therefore, these hydrogel blends could prove to be beneficial as a component of a multi-faceted neuronal treatment, through providing a mechanism for drug delivery and anchoring scaffolding for directed regenerating of neurons through an injury site.

Due to SeaPrep<sup>®</sup> agarose's low gelation temperature, the larger pore size character of the blend containing SeaPrep<sup>®</sup> agarose and the ability of the blend that contained SeaPrep<sup>®</sup> agarose to be manipulated following gelation, the SeaPrep<sup>®</sup> agarose blend is recommended as the blend best suited for future experimental applications. The ease of creation, simplicity of delivery and use of natural biocompatible components that solidify naturally without the use of ultraviolet light or cross linkers add to the advantages of utilizing this blend.

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