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Gelatinized Copper–Capillary Alginate Gel Functions as an Injectable Tissue Scaffolding System for Stem Cell Transplants

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Abstract

In severe hypoxic–ischemic brain injury, cellular components such as neurons and astrocytes are injured or destroyed along with the supporting extracellular matrix. This presents a challenge to the field of regenerative medicine since the lack of extracellular matrix and supporting structures makes the transplant milieu inhospitable to the transplanted cells. A potential solution to this problem is the use of a biomaterial to provide the extracellular components needed to keep cells localized in cystic brain regions, allowing the cells to form connections and repair lost brain tissue. Ideally, this biomaterial would be combined with stem cells, which have been proven to have therapeutic potentials, and could be delivered *via* an injection. To study this approach, we derived a hydrogel biomaterial tissue scaffold from oligomeric gelatin and copper–capillary alginate gel (GCCAG). We then demonstrated that our multipotent astrocytic stem cells (MASCs) could be maintained in GCCAG scaffolds for up to 2 weeks *in vitro* and that the cells retained their multipotency. We next performed a pilot transplant study in which GCCAG was mixed with MASCs and injected into the brain of a neonatal rat pup. After a week *in vivo*, our results showed that: the GCCAG biomaterial did not cause a significant reactive gliosis; viable cells were retained within the injected scaffolds; and some delivered cells migrated into the surrounding brain tissue. Therefore, GCCAG tissue scaffolds are a promising, novel injectable system for transplantation of stem cells to the brain.

Keywords

Anisotropy; ionotropy; capillary; alginate; gelatin; tissue scaffold; biomaterial; stem cell; tissue engineering

1. Introduction

Tissue engineering is a primary focus in the field of regenerative medicine and stem cells are shown to offer a potential source to replace the tissue lost in neurologic disorders [1, 2]. A significant clinical challenge is the functional repair of complex tissue lesions, cysts and cavities resulting from wounding, disease, infection and/or ischemia [3, 4]. Ideally, clinicians would like to treat such voids as non-invasively as possible by filling them with some injectable composite mixture of stem–progenitor cells and/or growth factors, and biomaterial. Tissue engineers are, therefore, enlisted to provide biomaterial systems that are double-tasked to act as injectable delivery vehicles and tissue scaffolds. The fundamental belief underlying this prominent strategy is that delivering the stem–progenitor cells in a biomaterial system will localize them (perhaps in high number) and facilitate tissue formation, vascularization and integration; once engrafted, this mass could have tremendous potential and plasticity to effect functional repair [5]. This is of great appeal because even a small amount of tissue restoration could produce a large degree of functional recovery, especially in the case of brain and spinal cord injuries. Hence, simple, effective and economic injectable scaffold biomaterials are highly sought-after tissue-engineering commodities.

One such devastating brain injury that afflicts neonates is hypoxic–ischemic encephalopathy (HIE), the brain manifestation of systemic asphyxia [6], which occurs in about 20 of 1000 full-term infants and in nearly 60% of very low birth weight (premature) newborns [7–9]. In severe cases, cortical lesions/cavities result from the destruction of neurons, astrocytes and supporting extracellular matrix, and there is currently no way to ameliorate the long-term deficits in these neonates. Injection of neural stem cells combined with a tissue scaffold into severe HIE lesions could potentially be a therapeutic strategy to attenuate these long-term deficits.

A number of injectable biomaterial systems have been previously developed and investigated. For example, degradable poly(lactic-co-glycolic acid) microsphere suspensions have been used to construct injectable cartilage [10], adipose [11] and muscle tissue [12], as well as injectable scaffolds for neural stem cell transplantation into complex cystic brain lesions [2, 13]. Calcium sulfate–alginate gels in combination with chondrocytes or fibroblasts have been investigated for injectable soft tissue replacement [14, 15], and injections of chitosan–glycerol phosphate hydrogels loaded with embryonic stem cells into infarcted heart wall are reported to improve the lesion compared to controls after four weeks in the rat [16].

Peptide amphiphile (PA) molecules synthesized with alkylated peptide aminotermini *via* solid phase chemistry can self-assemble into promising hydrogels [17, 18]. Arginine–glycine–aspartic acid (RGD) sequences can be incorporated in the PA molecules and scaffolds derived from these formulations are reported to enhance the osteogenic differentiation of cultured rat mesenchymal stem cells (MSCs) [19, 20]. Injectable PA formulations can also be loaded with growth factors such as basic fibroblast growth factor (bFGF) [21, 22] or bone morphogenetic protein-2 (BMP-2) [23] and provide controlled release of the factor(s) *in vivo* to enhance angiogenesis and/or bone formation.

Commercially available injectable collagenous microbeads (Cultispher®) have been shown to support both adipogenic and osteoblastic differentiation of cultured human adipose-derived stem cells [24]. Injectable fibrin gel formulations (Tissucol® and Tisseel®) have been shown to influence human mesenchymal stem cell proliferation and differentiation [25–27]. Platelet-rich plasma/fibrin formulations loaded with autologous bone marrow-derived stromal cells have been used clinically for bone grafting/regeneration [28], and

human extracellular matrix derived from adipose tissue aspirates has been developed for injectable adipose tissue engineering [29]. There is, however, no broad consensus on which particular injectable system is best for any particular application. Hence, refinement and optimization of existing systems, as well as development of new injectable biomaterial systems, remain active areas of research.

Capillary alginate gel (CAG) tissue scaffolds are inexpensive, self-assembled biomaterials that can be derived from a reaction of alginate with a divalent metal cation in water [30–35]. Here we describe a novel method for producing injectable tissue scaffolds using the copper–capillary alginate gel (CCAG) system plus oligomeric gelatin. These new hydrogel biomaterials (GCCAGs) are chemically cross-linked, water-swollen networks of alginate and low- M_w gelatin that possess uniaxial, parallel capillary channels with a tight range of diameters. We show that GCCAG scaffolds functioned effectively as 3-D culture substrates and maintained multipotent astrocytic stem cells (MASCs), a neural stem cell population, for up to two weeks *in vitro*. MASCs harvested from the GCCAG scaffolds after 12 days in scaffold culture still possessed neurogenic potential. We further demonstrate that mixtures of GCCAG and MASCs could be microinjected into neonatal rat brains. After one week survival time, viable transplanted cells and host cells were observed in the GCCAG scaffold material at the injection site.

2. Materials and Methods

2.1. Generation of MASCs

Our method for generating MASCs cultures has been previously described [36]. In brief, subependymal zones (SEZs) were dissected from the brains of either C57/BL6 or green fluorescent protein (GFP) transgenic neonatal mice (postnatal day 1–6) (The Jackson Laboratory). Tissue chunks were dissociated into a single cell suspension using a series of descending diameter glass pipettes. They were then washed several times in medium and plated in culture flasks in DMEM/F-12 medium (Invitrogen) supplemented with 5% fetal bovine serum (FBS, Atlanta Biologicals), N-2 supplement (Sigma), recombinant-human-EGF (20 ng/ml, Sigma) and recombinant-human-FGF (10 ng/ml, Sigma). A portion of the cell suspension used for following 3-D culture and transplantation was plated onto polyornithine/laminin-coated glass coverslips to characterize their phenotypes. Most of the cells in the monolayer cultures (95–100%) expressed the astrocyte marker GFAP, none expressed neuronal marker β -III tubulin, and a small percentage (<5%) expressed the microglial-specific surface protein CD11b.

2.2. Preparation of Biomaterial

All alginate used was Keltone LV CR sodium alginate donated by ISP Alginates. Copper sulfate pentahydrate ACS grade was purchased from Acros Organics. Gelatin type A (Sigma-Aldrich), N-hydroxysulfosuccinimide (Sulfo-NHS, Pierce) and N-ethyl-N'-(3-dimethylamino propyl) carbodiimide hydrochloride (EDC, Sigma-Aldrich) were used as received without further purification.

2.2.1. Oligomeric Gelatin—A MilliQ water solution containing 10% (w/v) gelatin and 1% (v/v) 1 M NaOH was transferred to a glass Erlenmeyer flask and covered with aluminum foil; the solution was then heated in an oven at 80°C for 72 h. The solution was mixed regularly by hand over the heating time. After heating, this stock solution was stored at 4°C for future use.

2.2.2. CCAG Scaffold Production—Briefly, an alginate-coated Petri dish was filled with a solution containing 2% (w/v) sodium alginate and 2.6% (w/v) degraded gelatin. A

Kimwipe® (Kimberly-Clark) soaked with 0.5 M CuSO₄ solution was brought down on top of the filled Petri dish. After 5 min, the soaked Kimwipe® was removed and the Petri dish was transferred to a plastic container with a lid allowing for a 3-mm submersion in 0.5 M CuSO₄ solution. The copper sulfate-filled container was covered and left undisturbed for 36–48 h. The gel-containing Petri dish was then removed and the gel carefully separated from the dish with a thin spatula. The parent gels were sectioned into 5-mm-thick strips, submerged in 4°C water and stirred on a shaker overnight. The water was then completely exchanged and the samples placed back on the shaker overnight. The strips were then cut parallel to the capillary long axis into 3-mm-thick rectangular blocks; the top and bottom of the blocks were then cut off perpendicular to the capillary long axis to yield a gel block with approximate length (parallel to the capillary long axis), width and thickness dimensions of 5 × 5 × 3 mm³, respectively; the bottom corners of the blocks were also cut off. All of the cut blocks were placed into a 50-ml conical tube (Costar), submerged into 4°C water and stored at 4°C for future use.

GCCAG blocks were placed on a Kimwipe for 1 min to dry and then put into a 15-ml conical tube (Costar). The weight of GCCAG plus the tube was measured and the weight of GCCAG calculated. GCCAG was approximated to be 94% water in mass; the amounts of EDC and Sulfo-NHS reagents used were calculated based on the further approximation that the remaining 6% ‘dry’ mass was totally alginate. Based on this approximation a mole ratio of alginate/EDC/Sulfo-NHS of 1:2:2 was used for the cross-linking reaction. Sulfo-NHS was dissolved in 10 ml PBS at 4°C in a 15 ml tube; scaffolds were then soaked in the Sulfo-NHS solution for 1 h on a shaker at 4°C before the EDC was added. The tubes were then shaken at 4°C for 21 h. Newly cross-linked GCCAG scaffolds were then rinsed several times with small volumes of PBS and rinsed in large volumes of MilliQ water over 24–48 h. Samples were then stored at 4°C.

2.2.3. Scanning Electron Microscopy–Energy Dispersive Spectroscopy (SEM–EDS)—Freeze-dried samples of 2/2.6/0.5 GCCAG were mounted using double-sided carbon tabs (SPI Supplies) on an aluminum stub. The mounted specimens were then carbon-coated (Ion Equipment) and stored until analyzed. Imaging and X-ray analysis were performed with a JEOL JSM-6400 SEM equipped with an Oxford energy dispersive spectroscopy (EDS) system and a LINK ISIS software package version 3.35 (Oxford Instruments, USA). All samples were analyzed at 15 keV accelerating voltage.

2.2.4. Scaffold Culture—Sterile scaffolds were conditioned in DMEM/F-12 medium supplemented with 5% FBS and N-2 supplement but not growth factors, prior to cell seeding. Scaffolds were placed in ultra-low-attachment 6-well plates (Costar), one scaffold per well; 3 ml medium was added to each well and the dish placed in a 37°C cell-culture incubator for 24–48 h. Medium was completely exchanged at least twice over the conditioning period. After conditioning, scaffolds were placed individually into 1.5-ml microcentrifuge tubes, cut corners end down. Adapting a method originally developed for cardiomyocyte seeding [37], scaffolds were charged with cells by first gently pipetting MASC suspension (approx. $3.3 \times 10^6/\mu\text{l}$) atop each scaffold; the 1.5-ml tubes caps were then removed with scissors, and the tubes placed singly into 15-ml conical tubes. These conicals were then centrifuged in a swinging bucket centrifuge (Eppendorf 5702) at 500 rpm for 2 min. The process was repeated (if necessary) to ensure sufficient charging of the scaffolds with MASCS. Each charged scaffold was then cut parallel to the capillary long axis approx. 4 times with a flame-sterilized razor blade to produce thin slices; each slice was then halved parallel to the capillary long axis, yielding thin, MASC-charged scaffold strips approx. $1 \times 1 \times 5 \text{ mm}^3$. These strips were maintained in the culture medium and growth factor identical to those described above. The media were exchanged and growth factors

were added every other day for scaffold cultures; representative scaffolds were collected and processed (below) at 5, 9 and 14 days in culture.

2.3. Immunostaining

GCCAG scaffolds were transferred from culture plate wells to glass scintillation vials and washed with PBS with Ca^{2+} , Mg^{2+} (Hyclone). Paraformaldehyde (PFA, 4%, Sigma-Aldrich) in PBS solution (5 ml) was then added and the scaffolds gently shaken overnight at room temperature. The fixative was then removed and the samples washed with PBS to remove residual fixative solution. Scaffolds were then submerged in 5 ml of 30% (w/v) sucrose in PBS and placed at 4°C for at least 24 h.

Fixed scaffold strips equilibrated in 30% (w/v) sucrose/PBS were placed parallel to each other in square plastic molds. Each mold contained strips from a single time point, yielding a total of 4–5 strips for each mold. The molds were then filled with Tissue-Tek® O.C.T. compound (Sakura Finetek USA) solution and frozen quickly in methylbutane cooled with dry ice. Blocks were then stored in aluminum foil at –80°C until sectioned (40–60 μm sections on Superfrost Plus (+) slides, 2–3 sections per slide). Slides were stored at –20°C for further analysis.

Slides were dehydrated in cold acetone, air dried at room temperature (RT) and then blocked in normal FBS at RT for 20 min. Excess serum was blotted off, and slides were then incubated overnight at 4°C with rabbit anti-GFP (1:1000, Invitrogen) together with either mouse anti- β -III tubulin (1:1000, Promega) or with mouse anti-GFAP (1:10, Shandon), diluted in PBS plus 10% FBS. Slides were washed with 1 $\times$ PBS plus 0.1% Triton X-100 (PBSt) for 3 $\times$ 5 min and then incubated with species-specific secondary antibodies (1:500) raised in donkey (GFP: AlexaFluor-488, β -III tubulin and GFAP: AlexaFluor 594 (Molecular Probes) for 1 h at RT. After a final wash in 1 $\times$ PBSt for 3 $\times$ 5 min, slides were coverslipped with Vectashield w/DAPI (Vector Laboratories).

2.3.1. Optical Microscopy—Representative confocal image series were collected at each time point using an Olympus DSU spinning-disk confocal microscope running Slidebook ver. 1.3. Max projection over Z images were generated from 40 $\times$ Z-stacks collected at the ‘optimal’ step size calculated by the Slidebook software. All image processing was performed using NIH ImageJ ver. 1.43. Phase contrast and fluorescence microscopy were performed with an IX-70 Olympus/C Squared equipped with a MagnaFire digital camera system and software package ver. 2.4 (Optronics). Processed images of phase contrast micrographs of GCCAG cut perpendicular to the capillary long axis (4 different fields, 750 total capillaries) were produced with the Dynamic Threshold 1b plugin written by Gary Chinga Carrasco; these images were then analyzed using the built-in ImageJ particle counting/sizing features yielding the number of capillaries and their diameters.

2.3.2. Rat Hypoxic–Ischemic Model and Transplantation of MSCs within GCCAG Scaffolds—A pilot transplant study using neonatal rat pups was performed to establish that animals can survive with injected MSCs–GCCAG brain transplants and to evaluate the local reactive gliotic response to these implants and affirm GCCAG biocompatibility. All procedures and anesthetics on the animals were performed in accordance with University of Florida and NIH regulations governing the ethical care and handling of laboratory animals. Unilateral common carotid artery ligation followed by a hypoxic insult was performed in neonatal rats using the Rice–Vannucci model [38] as previously described [39]. Briefly, the 7-day-old rat pups ($n = 10$) were anesthetized using isoflurane. The right common carotid artery was isolated through a small incision in the neck using a dissecting microscope and electrocauterized upon isolation. The incision was

closed using Vetbond (Webster Veterinary Supply), and the pups were allowed to recover for one hour in a thermoregulated (37°C) environment using a gel pad (Deltaphase Isothermal Pad, Braintree Scientific). After the recovery period, the pups were placed in a Billups–Rothenburg chamber perfused with a humidified gas mixture (8% oxygen, 92% nitrogen) for 20 min. The pups were kept at a constant temperature (37°C) while in the chamber, using a gel pad. After the hypoxic exposure, the pups were returned to their dam. The sham-operated group ($n = 5$) underwent anesthesia followed by a small incision that was immediately closed.

Twenty-four hours after the injury, the pups were anesthetized for the transplant using isoflurane and placed in a clay mold. Small pieces of GCCAG conditioned in media lacking serum or growth factors were premixed with MASCs ($(3-5) \times 10^6$) and loaded into a large-barrel glass syringe (27 gauge needle, Hamilton); this cell–scaffold combination was then injected into the fine glass barrel of the transplant syringe (30 gauge needle, Hamilton). The heads of each pup were trans-illuminated under a dissection microscope, and the Hamilton syringe freshly loaded with the cell–scaffold combination was lowered through the scalp and skull into the injured hemisphere. Approximately 1 μ l of the mixture was slowly injected into the target areas. The animals were allowed to recover and returned to their dam post injections. A series of control injections was also performed. Selected HI ($n = 1$) and sham ($n = 1$) pups received an injection of cells with no GCCAG scaffold. This group served as a control to validate that the cell preparation itself did not cause any host reaction. A second control group, HI ($n = 1$) and sham ($n = 1$), was transplanted with material containing no cells to validate that the material alone did not cause a local host reaction. A total of 11 pups HI ($n = 8$) and sham ($n = 3$), received GCCAG scaffolds with MASCs. Seven days post-transplant, the pups were deeply anesthetized and perfused with 4% PFA. The brains were then collected for immunohistochemistry.

2.4. Immunostaining and Unbiased Stereology

For detection and characterization of the grafted GFP cells, tissue sections were processed for immunofluorescence with polyclonal antibodies against GFP (1:1000, Invitrogen) together with either mouse anti- β -III tubulin (1:1000, Promega) or with mouse anti-GFAP (1:10, Shandon). Sections were incubated overnight at 4°C in primary antibodies diluted in PBSt containing 10% FBS. They then were rinsed twice with PBS and incubated at room temperature for 2 h with PBSt containing fluorescence-labeled secondary antibodies, before being washed, mounted, cover-slipped and evaluated with epifluorescence microscopy or spinning-disk confocal microscopy.

A Zeiss axiophot equipped with a Microfire CCD camera (Optronics) was used for counting cells using unbiased stereology. Real-time images were analyzed using the Stereologer 2000 version 2.1 (Stereology Resource Center). The numbers of viable appearing GFP expressing cells were counted in a 100 μ m area around and including the injection track. Each GFP positive cell was then evaluated for the expression of GFAP or β -III tubulin. Mean coefficient of error (CE) values were 0.14 and 0.33 for GFP and GFAP probes, respectively.

3. Results

Synthesis of GCCAG scaffolds *via* our method was successful (Fig. 1); Fig. 2A shows the gross morphology of 2/2.6/0.5 GCCAG materials in the raw gel state (left) through a conditioned scaffold (right), ready for culture or implantation. The apparent swelling/de-swelling of the materials shown in Fig. 2A is representative, i.e., a raw gel block (left) swells significantly during cross-linking, and de-swells upon media conditioning. GCCAG scaffold average capillary diameter was found to be 31 ± 5 μ m and the average capillary density was 102 ± 33 caps/mm²; these data correspond to average 2/2.6/0.5 GCCAG volumetric surface

area of $10 \pm 3 \text{ mm}^2/\text{mm}^3$ (excludes the outer surface area of the gel block). The histogram (Fig. 2B) shows that the distribution of capillary diameters is skewed toward larger capillary diameters.

Optical microscopy and SEM-EDS straightforwardly provided data revealing 2/2.6/0.5 GCCAG scaffold microstructure and elemental composition (Fig. 3). The phase contrast micrographs (Fig. 3A, B) show an array of essentially round, evenly distributed regular capillaries running in parallel through any given volume of material. Although subtle, careful inspection revealed a curious periodic wave propagating longitudinally down the lengths of many capillaries peculiar to these new materials (Fig. 3B). The SEM images (Fig. 3C, D) are in agreement with the optical microscopy findings; an upside-down L-like fracture in the material illustrates both the patent and 3-D nature of GCCAG scaffolds (Fig. 3D, black arrows). In conjunction with SEM imaging, EDS was used to probe the elemental composition of conditioned 2/2.6/0.5 GCCAG. Detection of copper was of particular interest since small copper ion concentrations are generally believed to affect cells. The EDS spectrum (Fig. 3E) shows elements common to the 2/2.6/0.5 GCCAG and/or the conditioning media were detected (counts/keV); a copper K_{α} peak was not observed ($= 8.0481 \text{ keV}$, dashed line).

MASCs were successfully cultured on 2/2.6/0.5 GCCAG scaffolds for 2 weeks. The *in vitro* MASC-GCCAG scaffold culture results are summarized in Fig. 4. Capillaries containing cells appeared consistently filled with cords [40] of cells possessing healthy nuclei at all time points (Fig. 4A, E, I); GFP positive cells appear attached and their processes spread along the capillaries at all time points as well (Fig. 4B, F, J). No positive cellular staining within the scaffold was detected for β -III tubulin at 5, 9 or 14 days in culture (Fig. 4C, G, K); the red signals in the images are assumed staining artifacts caused by debris. Again, the 'wavy' nature of many scaffold capillaries is highlighted by the shape of the MASC resident within scaffold capillaries. To check for neurogenic potential of scaffold-cultured MASCs, we harvested MASCs from the scaffold and re-plated them after 12 days in culture; serum was withdrawn from the culture media to spur neurogenic differentiation.

Immunofluorescent staining of these harvested cells revealed that MASCs are clearly attached and spread, possessing healthy nuclei and clusters of β -III-tubulin-positive cells are readily identifiable, indicating the neurogenic potential of the cultured MASCs (Fig. 4M, N). As expected, GCCAG scaffolds maintained their capillary microstructures and showed no obvious indications under optical microscopy (e.g., holes, fraying, erosion) of degradation after multiple weeks in *in vitro* culture. The scaffold should be degraded *in vivo* as GCCAG is composed of alginate polysaccharides and gelatin proteins cross-linked together *via* peptide bonds, thus rendering it susceptible to degradation from endogenous proteases.

All the hypoxic-ischemic injured neonatal rat pups and controls survived the microinjections (approx. $1 \mu\text{L}$) of GCCAG scaffold materials and MASCs. Figure 5 shows the state of cell-gel combos at the brain injection site after a 1-week survival period. Faintly autofluorescent material observed within the injection sites was designated as remnant GCCAG. Grafted GFP⁺ (green) cells judged viable were found within the injected GCCAG scaffold materials and in the tissue surrounding the injection sites (Fig. 5C, D). Some GFP⁻ cells, evidenced by the presence of DAPI-stained nuclei (blue), were also observed within injected GCCAG material, indicating the possibility of integration between grafted and host cells. Most GFP⁺ donor cells adopted morphologies that resemble mature astrocytes and presented extended processes (Fig. 5C). Small numbers of grafted cells apparently migrated from the injection sites and incorporated into the surrounding host tissues (arrow Fig. 5D); some of these cells were both GFP and GFAP positive (inset Fig. 5D). A reactive gliosis/astrocytosis, manifested by an increase in GFAP staining was noted in the areas surrounding the injection sites but did not differ in appearance from control injections using cells without

materials (Fig. 5A) or materials alone (Fig. 5B). Unbiased stereologic analysis revealed that the average total number of transplanted GFP⁺ MASC had a broad range and on average $29 \pm 13\%$ of these transplanted cells were GFAP⁺ at the one week time point (Fig. 5E); β -III tubulin⁺ transplanted cells were not detected.

4. Discussion

Our focus has been on deriving hydrogel scaffolds from copper–capillary alginate gels for stem/progenitor cell-based tissue engineering [30, 35], and we suspected that the incorporation of gelatin into CCAG scaffolds would enhance cell attachment and spreading. Previously, only studies adding gelatin to the calcium–capillary alginate gel system have been conducted, and this work was focused on producing capillary-structured bioceramics for bone regeneration [34, 41]. A 1% addition of gelatin is described in these prior studies; however, we found that adding this amount of gelatin to the copper system produced erratic, unacceptable distortions in the gel microstructure. Based on the theory of CAG self-assembly, we postulated that the distortions likely resulted from the significant increase in viscosity (even when warmed) of the alginate solution after gelatin addition. This idea seems consistent with previous findings where it was noted that addition of gelatin correlated with less uniform CAG microstructures [34]. We, therefore, heated (cooked) the gelatin in base to decrease the average chain length (and consequently M_w) before it was blended with the alginate solution yielding a significant reduction in overall solution viscosity. After stewing, a 10% (w/v) gelatin solution formed only a weak jelly upon cooling to RT and diluted solutions were easily pourable with apparently low viscosities. A native 10% (w/v) solution formed a firm gel and essentially was non-flowable once cooled to RT. Solidification of the oligo-gelatin solution did occur upon refrigeration at 4°C (soft gel).

Reduction of the gelatin M_w allowed for production of defined, high gelatin-content gels with regular capillary microstructures (Figs 1B and 3). The calculated average GCCAG volumetric surface area (excluding the outer faces of the gel block) is lower than that of the OCCAG scaffolds we synthesized in our first studies [30] due to the difference in observed capillary densities. This difference is an apparent consequence of the oligomeric gelatin addition to the alginate solution. The origin of the ‘wavy’ clusters of capillaries present in the optical micrographs (Fig. 2A, B) is unknown; vibration was considered as a possible culprit, but gels grown on a suspension had similar features. It is possible that the torus-shaped fluid cells responsible for mapping the microstructure onto the growing gel during assembly [42] travel a spiral path rather than a linear one through the gel. The SEM–EDS data (Fig. 2D, E) confirm the GCCAG capillaries are patent and that conditioning the scaffolds in a volume of media prior to culture serves to extract copper from the material. The 3-D cell culture results clearly show that MASCs can attach, spread, grow, be maintained undifferentiated, and retain their multipotency in GCCAG scaffold culture. Though not the focus of this study, a cursory inspection of the nuclear orientation ratios of the scaffold-cultured MASC (Fig. 3A, E, I) clearly shows that cells were oriented in line with the tubular microstructure of the GCCAG scaffolds.

To demonstrate that GCCAG scaffolds can serve as an injectable stem-cell delivery system, we performed cortical microinjections of GCCAG mixed/loaded with MASCs into neonatal rats modeling HIE. We have previously demonstrated that MASCs migrate to the region of cortical injury and differentiate into neuronal phenotypes once in the injured cortex, but ultimately tend to line the walls of the porencephalic cyst and not fill the actual cavity [1]. The *in vivo* results of the present study (Fig. 5) clearly show that MASCs can be delivered to the brain with GCCAG material *via* microinjection. Grafted/transplanted MASCs and possible host cells populated the scaffold material (Fig. 5C, D), and the reactive gliotic response to GCCAG was nominal (Fig. 5A, B). The average total number of transplanted

MASCs will likely need to be increased and optimized to maximize the ultimate therapeutic potential of GCCAG–MASC combinations; the absence of detected β -III tubulin⁺ transplanted cells in this pilot *in vivo* study could be related to the relatively low numbers of transplanted MASCs.

Our group has developed a new injectable biomaterial tissue scaffold (GCCAG) to facilitate the repair of brain lesions caused by hypoxic–ischemic injury. We hypothesized that this novel biomaterial, copper–capillary alginate gels incorporated with oligomeric gelatin, could potentially provide a transplantable environment in which stem cells could attach, survive, integrate into the host brain tissue and ultimately fill the cystic cavity. We have initially tested that hypothesis *in vitro* in this work and have demonstrated that MASCs can be cultured and maintained for at least 2 weeks within GCCAG scaffolds. We have further demonstrated that GCCAG–MASC combinations can be successfully transplanted *via* injection into the brain of neonatal rats in our rat model of HI; the hydrogel functioned as a scaffold for grafted MASCs and a possible integration site for grafted cells and host tissues *in vivo*. The surviving cells were an apparent GFAP⁺ glial population, and even though future studies should focus on optimizing the biomaterial support system for directing cell fate of such grafted cells toward neuronal lineages as needed for cell replacement therapies, the grafting of precursor cells like MASCs that were shown here to generate astrocytes may also have therapeutic utility since these cells also provide important homeostatic factors to reactive and healing brain tissue. GCCAG scaffolds are, therefore, a promising new injectable biomaterial system for use in tissue engineering.

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Figure 1.

2/2.6/0.5 GCCAG synthesis and experimental scheme. A 10% (w/v) solution of gelatin was heated at 80°C for 72 h with 1% (v/v) 1 M NaOH. This oligomeric-gelatin solution was then mixed with a 4% (w/v) solution of sodium alginate to yield a 2% (w/v) sodium alginate/ 2.6% (w/v) oligomeric-gelatin solution; an alginate-coated Petri dish was then filled with this combined solution and submerged in a tank of 0.5 M CuSO₄ solution for 36–48 h. After gelation (self-assembly) the gel was carefully separated, sectioned into 5-mm-thick strips and washed in 4°C water overnight (2×). The strips were then cut parallel to the capillary long axis into 3-mm-thick rectangular blocks; the top and bottom of the blocks were then cut off perpendicular to the capillary long axis to yield a gel block with approximate length (parallel to the capillary long axis), width and thickness dimensions of 5 × 5 × 3 mm³, respectively. GCCAG blocks were cross-linked using EDC Sulfo-NHS chemistry at a mole ratio of 1:2:2 alginate/EDC/Sulfo-NHS in PBS at 4°C for 21 h. GCCAG scaffolds were then sterilized in 70:30 ethanol, conditioned in media used for MASC culture or transplantation into the brains of neonatal rats modeling HI injury. This figure is published in colour in the online edition of this journal, that can be accessed via <http://www.brill.nl/jbs>

Figure 2. 2/2.6/0.5 GCCAG stages and morphology. (A) Representative materials yielded at each major step of GCCAG scaffold synthesis. (B) Representative histogram showing the distribution of capillary diameters present in conditioned 2/2.6/0.5 GCCAG scaffolds. Scale bar = 5 mm. This figure is published in colour in the online edition of this journal, that can be accessed via <http://www.brill.nl/jbs>

Figure 3. Representative micrographs and EDS spectrum of a conditioned 2/2.6/0.5 GCCAG scaffold. (A, B) Phase-contrast images perpendicular (A) and parallel (B) to the capillary long axis (oriented horizontally); note the 'wavy' character of the capillaries (inset). (C, D) SEM images perpendicular and tilted with respect to the capillary long axis; note the patent, continuous capillary microstructure revealed by a convenient split (middle and upper right, black arrows) in the sample. (E) EDS spectrum of a conditioned scaffold; a peak for copper was not observed (dashed lined indicates the position of Cu K α). Scale bar = 200 μ m for all images.

Figure 4.

Representative micrographs of MASC cultured within and harvested from 2/2.6/0.5 GCCAG scaffolds at select time points. β -III is red, GFP is green and nuclei are blue for all appropriate images. (A–L) Complementary fluorescence images/merges after 5 (A–D), 9 (E–H) and 14 days (I–L), respectively, in scaffold culture. (M, N) Complementary phase contrast (M) and fluorescence (N) images of cells harvested (HARV) after 12 days in scaffold culture, replated (overnight, complete medium) and then subject to 48 h serum withdrawal (WD). Scale bar = 30 μ m for A–L and 100 μ m for M and N. This figure is published in colour in the online edition of this journal, that can be accessed via <http://www.brill.nl/jbs>

Figure 5. Representative fluorescence micrographs of MASC–GCCAG scaffold transplant and control sites in the brains of neonatal rats modeling hypoxic ischemic encephalopathy (HIE) one week post injection. (A) Cell-only and (B) scaffold-only transplants; note the nominal GFAP response to the materials. Scale bar = 100 μ m. (C) Grafted cells are shown resident in the injection site; the donor cells adopted morphologies that resemble mature astrocytes by presenting extended processes, some into the surrounding host tissues. GFP is green, GFAP is red and DAPI is blue, scale bar = 50 μ m. (D) Another example of the injection site filled with a mixture of GFP⁺ cells and the GCCAG scaffolding materials; a small number of the grafted cells left the injection site and incorporated into the surrounding host tissues (arrow). Some of these cells show signs of differentiation by double labeled with astrocyte marker GFAP (inset). GFP is green, GFAP is red and DAPI is blue, scale bar = 50 μ m. (E) Bar graph showing the average total number transplanted GFP⁺ MASC, the average total number of these cells that are also GFAP⁺ and the average % total of the GFP⁺ transplanted

cells that are also GFAP⁺; top scale is cell number, bottom is percentage. This figure is published in colour in the online edition of this journal, that can be accessed via <http://www.brill.nl/jbs>