

Three-dimensional growth and function of neural tissue in degradable polyethylene glycol hydrogels

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Received 7 September 2005; accepted 6 November 2005
Available online 28 November 2005

Abstract

Graft survival and integration are major factors that limit the efficacy of cell therapies for the treatment of disease and injury in the central nervous system. Efforts to improve cell survival and integration have focused in part on the development of biocompatible scaffolds that support neural cell growth and function. Here we photoencapsulate neural cells within degradable hydrogels and use confocal microscopy to non-invasively monitor these key cell functions over time. By directly imaging fluorescently labeled cells we show that neural cells cultured within three-dimensional polymer networks create their own cellular microenvironment to survive, proliferate and differentiate and form neurons and glia that are electrophysiologically responsive to neurotransmitter. By changing the degradation rate of the polymer network, the time-scale over which neural cells extend processes throughout the hydrogel could be tuned on a time-scale that ranged from 1–3 weeks. These studies were carried out in the absence of serum and extracellular matrix molecules that can be immunogenic and identify degradable PEG hydrogels as suitable synthetic cell carriers for neural transplantation.

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Keywords: Neural regeneration; Stem cells; Tissue engineering; Hydrogels

1. Introduction

Polymer scaffolds play a central role in three-dimensional cell culture and as delivery vehicles for cell-based therapies in regenerative medicine. For example, neural cells seeded throughout implantable PLGA scaffolds extend processes and migrate out of the scaffold to innervate host tissue; host cells and processes innervate the scaffold [1]. Injectable matrices based on natural or synthetic materials that gel in response to ionic strength or temperature are being developed for neural cell delivery and may be particularly useful for treatment of deep tissue structures. Materials that are being explored for this purpose include alginate [2], fibrin [3], agarose [4–7], collagen [8], methylcellulose [9] and peptide-based hydrogels [10].

In gel-based materials, cells are assembled throughout a network of cross-linked polymer chains. The distance

between cross-links in the polymer chains is defined as the mesh size of the hydrogel. During tissue regeneration, cells and growing processes must penetrate through this space. Processes emerge from encapsulated cells in alginate, fibrin, agarose, collagen, and methylcellulose based hydrogels within 24 h following encapsulation, unless the average mesh size is too narrow and growth is inhibited [5]. A similar time-scale for process growth is observed in peptide-based hydrogels, when growth promoting peptide sequences are present within the gel network [10]. These findings indicate that two factors, the mesh size and the chemistry of the surrounding polymer network are both important factors that influence process growth through gel-based materials. Regardless of the scaffold platform, intimate control of the scaffold architecture and cell environment on many scales are critical.

Here, we assemble cells throughout a polymer hydrogel in which the mesh size of the network changes with time. Neural precursor cells were assembled throughout three-dimensional PEG hydrogels that contain hydrolytically degradable lactide units. Hydrogels were formed by

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photopolymerizing methacrylate groups covalently linked to degradable PEG macromers. Because of the robustness of the gel properties and the processing platform, gels with spatial dimensions that are suitable for injection into tissue can be created (Fig. 1a). On average 3 lactide units were grafted to each side of a core PEG (4600 MW) molecule. The degradation behavior of hydrogels formulated with this macromer was monitored over time by measuring the mechanical strength (compressive modulus) and by calculating the average mesh size of the hydrogel (from swelling ratio data and the Flory Rehner equation [11]) (Fig. 1b). Immediately following encapsulation the network of polymer chains is highly cross-linked. As degradation proceeds ester bonds within the PLA block are hydrolyzed, the hydrogel swells imbibing more water, compressive modulus decreases and the overall mesh size of the network increases until the network completely dissolves.

Neural cells assembled throughout the hydrogel were isolated from the developing rat forebrain at 14 days of gestation. Results from the literature indicate that this cell population contains a mixture of post-mitotic neurons and proliferative precursor cells. In a recent study, direct counting of positively labeled cells in acutely dissociated cell cultures indicates that $53 \pm 4\%$ of the cell population is positive for beta-tubulin-III an immunocytochemical marker for post-mitotic neurons; the remainder of the cell population ($57 \pm 3\%$) is positive for nestin, an intermediate filament protein found in precursor cells [12]. Nestin was not observed in beta-tubulin positive cells indicating that these antibodies label distinct, non-overlapping cell populations [12]. In addition, BRDU labeling experiments were used to show that all nestin positive cells are BRDU positive, indicating that precursor cells isolated from this tissue are actively proliferative [12]. These results are in agreement with quantitative data obtained by FACS analysis in a separate study [13].

The choice to study the growth and development of neural cells isolated from the E14 developing forebrain in degradable PEG hydrogels is based on the demonstrated potential of this cell population in cell therapies for the treatment of degenerative disorders of the central nervous system [14–19]. Neural cells isolated from developing E14 forebrain tissue self-renew or differentiate to form neurons or glia when exposed to extracellular matrix molecules and specific growth factors [20–22]. Successful survival and differentiation of donor cells has been observed when freshly isolated neural precursor cells are transplanted into the adult CNS suggesting that a sufficient chemical microenvironment persists in the adult extracellular space to support and maintain the survival and development of neural precursor cells [19,23].

In vitro expansion of neural precursor cells prior to transplantation offers the possibility of an unlimited source of donor cells—a factor that may significantly increase the availability and application of transplants for neural regeneration. Several studies have explored the in vitro expansion and differentiation of neural precursor cells on two-dimensional extracellular matrix coated surfaces [24–29]. For use in transplantation therapy, however, these cells must be dislodged from the two-dimensional substrate by mechanical or chemical means—a process that unnecessarily exposes donor cells to shear forces and stress.

Alternatively, expansion and differentiation of neural precursor cells within a transplantable three-dimensional degradable matrix eliminates the need to remove cells from a substrate prior to grafting. Three-dimensional collagen gels are being considered for this purpose [30,31]. When cultured in three-dimensional collagen gels neural precursor cells survive, proliferate, and differentiate to form beta-tubulin-III positive neurons. While collagen is a suitable substrate for in vitro neural precursor cell growth, however, the in vivo utility of this matrix is unclear. The

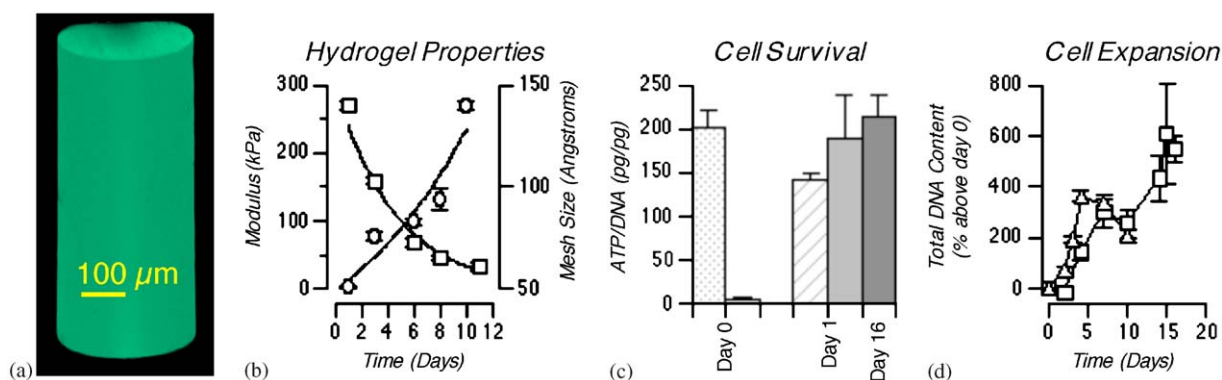


Fig. 1. Neural cell survival and expansion in degradable PEG hydrogel culture. (a) Gels with dimension suitable for injection through a needle can be prepared by photopolymerization. PEGPLA hydrogel loaded with fluorescein. (b) Temporal change in compressive modulus (squares) and calculated mesh size (circles) of the PEGPLA hydrogel as the network degrades. (c) Neural cell survival in hydrogel culture (solid bars) relative to monolayer culture (hatched bars). Quantitative comparison of cell survival was made by normalizing total ATP content to total DNA content. As a reference normalized ATP content in a population of cells that is greater than 95% (dotted bar, day 0) or less than 5% (solid bar, day 0) viable is provided. (d) Total DNA content as an indicator of cell expansion in hydrogel culture (squares) and monolayer culture (triangles). Data in (b–d) are expressed as the mean \pm SEM for greater than (b, 3–4) (c–d, 5) samples derived from 3 different experiments.

mechanical properties and degradation rate of protein-based scaffolds are difficult to control.

In this work, we evaluate the utility of a degradable synthetic PEG hydrogel as a three-dimensional environment for neural precursor cell expansion and differentiation in culture. Neural precursor cells were photoencapsulated throughout degradable PEG-based hydrogels. The polymer network is synthetic and is modified with no chemical cues that instruct neural precursor cells to morphologically or biochemically differentiate. Neural precursor cells were cultured within hydrogels in serum-free medium supplemented with a single mitogen, bFGF-2, to allow the cells to grow. This approach enabled us to identify the extent to which neural cells regenerate their own three-dimensional tissue-like microenvironment that supports precursor cell survival, proliferation, and differentiation and to evaluate how this tissue-like environment evolves as the physical properties of the three-dimensional polymer network change with time. In addition to end-point assays, confocal microscopy was used to non-invasively visualize the spatial pattern of neural tissue development and function in fragile three-dimensional degradable hydrogels ~1–2 mm thick. A high degree of (1) cell survival, (2) proliferation and differentiation to form morphologically and biochemically identifiable neurons and glial cells, and (3) electrophysiological response to neurotransmitter were taken as positive indicators of successful neural tissue formation. PEG hydrogels are non-immunogenic and are well tolerated in many tissues of the body, including the central nervous system. The results from this work suggest the potential of PEG hydrogels as cell carriers for transplantation into the CNS.

2. Materials and methods

2.1. Hydrogel preparation and characterization

Using previously developed procedures, poly(lactic acid) (PLA) and poly(glycolic acid) (PGA) were grafted from linear PEG chains (MW 4600) to form triblock copolymers [32]. Macromers were end-capped with methacrylate functionalities, purified via dialysis, and characterized with ^1H NMR. Hydrogels were prepared with a 10 wt% PEG-macromer in sterile culture medium containing cells and 0.05 wt% photoinitiator (Darocur 2959). Gels were formed by exposing the solution to UV light ($\sim 4\text{ mW/cm}^2$) for 10 min. Hydrogel discs prepared from PEG-PLA-DM macromers were weighed immediately after polymerization and were then placed in culture medium at 37 °C. At specific time intervals 3–4 discs were removed from the medium and the discs were weighed again to determine the swollen mass of the hydrogel (m_s). The discs were lyophilized to determine the dry, polymer mass (m_p). The volume swelling ratio, Q and the mesh size of the hydrogel were calculated as described elsewhere [11]. The compressive modulus was measured using a Materials Testing System (Synergie 100).

2.2. Cell culture

Embryonic day 14 forebrain was dissected from rats and was dissociated enzymatically. Single cells were cultured on poly ornithine-coated coverslips in basal culture medium containing 10 ng/ml bFGF and

N2 (GIBCO) or in three-dimensional hydrogel culture at a density of 1×10^7 cells/ml. Culture medium was exchanged every 2–3 days to maintain appropriate growth factor levels.

2.3. Direct imaging of neural cells in degradable PEG hydrogel culture

Cell viability within hydrogels was assessed with a fluorescence-based membrane integrity assay (LIVE/DEAD kit, Molecular Probes). To facilitate dye penetration into the interior regions of the hydrogel, on the day of imaging hydrogel cultures were vibratome sectioned to produce 400 μm thick sections of gels. Cells within each 400 μm section of hydrogel were incubated in PBS containing 10 μm calcein-AM and 2 μm ethidium bromide for 30 min and were directly visualized by confocal microscopy. During imaging hydrogel slices were incubated within a Petri dish containing artificial cerebrospinal fluid. Hydrogel slices were immobilized in the dish by placing two stainless steel rod-shaped weights on top of the gel slice. Hydrogels were discarded immediately following imaging.

All images were captured using a Zeiss LSM Pascal equipped with an Axioplan upright microscope. Images were captured with a 10 \times water immersion objective (Zeiss Achromplan 10 \times , numerical aperture 0.3, working distance 3.1 mm) or a 40 \times water immersion objective (Zeiss Achromplan 40 \times , numerical aperture 0.8, working distance 3.1 mm). Aggregate diameter was calculated by tracing the perimeter of aggregates in images obtained using confocal microscopy in the Zeiss LSM Image Examiner software program. Aggregate size data was obtained from at least 50 aggregates derived from 3 separate experiments.

Calcium imaging experiments were performed with confocal microscopy. Cells encapsulated within a degradable hydrogel at 14–16 days of degradation were loaded with the calcium indicator Fluo-3 (Molecular Probes) by incubation in PBS containing 10 μm Fluo-3 for 30 min. At this late stage of degradation, positively labeled Fluo-3 cells were visible throughout the entire width of the 1–2 mm thick hydrogel when observed with the 10 \times water immersion objective. The neurotransmitter GABA was applied exogenously in artificial cerebral spinal fluid bathing the hydrogel. Images of encapsulated cells were captured with confocal microscopy over the course of 10 min. Images were captured at a rate of 1 image per second. The Fluo-3 dye was excited with a 488 nm argon laser and emitted light was captured with a 520 nm long pass filter. Calcium imaging experiments were performed on greater than 50 cells assembled throughout micro-tissues in 3 separate experiments.

2.4. Homogenization of PEG hydrogel cultures

Each hydrogel culture was rinsed with cold PBS and was transferred to a 15 ml centrifuge tube containing cell lysis buffer. Each hydrogel was homogenized for 1 min at 40% power on ice. This procedure disrupts the polymer network and lyses cell membranes. The resulting homogenate is a liquid mixture composed of intracellular proteins, nucleic acids and extracellular proteins that are released into the hydrogel environment. Protein and nucleic acid levels in supernatants were quantified using specific biochemical assays as described below. The supernatant was stored at $-80\text{ }^\circ\text{C}$ until the day on which assays were performed.

2.5. Measurement of total DNA and ATP content in hydrogel culture supernatant

Total DNA content was quantified using the PicoGreen assay (Molecular Probes). ATP levels were quantified with the CellGlo assay (Promega). Data are presented as mean \pm standard error of the mean (S.E.M.). Statistical significance was determined using Student's t test, with $p < 0.05$ considered significant. Data was obtained from 3 separate experiments involving 5 hydrogel cultures per time point.

2.6. Western blot analysis on cell culture supernatants

Hydrogel culture supernatants prepared by sonication were run on a 10 wt% Tris-HCl gel. Monoclonal antibodies were used to probe levels of glial fibrillary acidic protein and beta-tubulin-III on immunoblots of supernatants derived from day 1 of hydrogel culture and day 16 of culture. Beta-actin was utilized as a loading control. Primary antibodies were purchased from Chemicon. Secondary antibodies and detection reagents and protocols were performed according to BIORAD Opti-4CN kit recommendations.

2.7. Immunocytochemistry on cryosections of hydrogel culture

After 16 days of culture, cells were fixed in 4% paraformaldehyde. Sections of hydrogel cultures, 40 μm thick were cut with a cryostat. Using standard immunocytochemical techniques, hydrogel sections were processed for staining with antibodies against nestin (progenitor cell), beta-tubulin (neuron), glial fibrillary acidic protein (glial cell), fibronectin, and synaptophysin (SIGMA). Unless otherwise noted all antibodies were purchased from Chemicon. Secondary antibodies conjugated to alexafluor 633 were purchased from Molecular Probes. Sytox Green was utilized as a nuclear counterstain (Molecular Probes). Quantitation of specific cell types present in cultures was completed for greater than 50 micro-tissues in cyro-sections of hydrogel culture derived from 3 separate experiments.

3. Results

3.1. Cell survival in degradable peg hydrogels

ATP content normalized with respect to total DNA content was utilized as a quantitative indicator of cell viability in hydrogel culture over time. As a reference monolayer cultured cells that are 97% viable (by trypan blue exclusion) contain 1486 ± 121 ng of ATP and 7386 ± 678 pg of DNA. When expressed as a ratio, a 97% viable culture contains 201 ± 20 pg of ATP per pg of DNA (Fig. 1c, dotted bar day 0). In contrast, a population of non-viable cells (induced to die by a 10 min exposure to saponin) contains 1 ± 0.4 pg of ATP and 8230 ± 230 pg of DNA. When expressed as a ratio, a nonviable culture contains 6 ± 3 pg of ATP per pg of DNA (Fig. 1c, solid bar day 0). A similar analysis was performed on hydrogel cultures. Total ATP and DNA content were quantified on day 1 and day 16 of gel culture (Fig. 1c). Twenty-four hours after encapsulation, neural cells in hydrogel culture contain 190 ± 50 pg of ATP per pg of DNA (90% viability, light gray bar). This level of cell viability is significantly greater than that observed in monolayer culture (143 ± 8 pg/pg, $p < 0.05$, hatched bar). High levels of cell viability were maintained after 16 days of gel culture, 215 ± 25 pg/pg (100% viability, dark gray bar).

Imaging results obtained by confocal microscopy are consistent with our ATP viability assay. Dead cells were visualized in culture by confocal microscopy during the first 3 days of culture (Fig. 2a). Few dead cells were imaged throughout the hydrogel after 16 days of culture (Fig. 3). The ratio of ATP/DNA indicates that every cell present in the hydrogel culture at day 16 is viable. Those cells that died during the initial stage of culture were likely removed by phagocytic glial cells. Alternately, a fraction of non-

viable cells may be released from the hydrogel at late stages of degradation. A low number of free-floating cells are observed in the culture medium at late stages of degradation.

3.2. Cell expansion in degradable peg hydrogels

Over the course of 16 days of culture, neural tissue is formed in the hydrogel as cells divide. Cell division and neural tissue formation within a polymer network may occur more slowly, because cellular resources must be directed towards creating additional space for newly generated cells. To investigate this possibility we compared the rate of cell growth in monolayer culture where cells are not bounded by a polymer network to that in hydrogel culture (Fig. 1d). For both culture systems total DNA content increased with time. During the first week of the experiment, total DNA content in gel culture was not statistically different from that in monolayer culture (one-tail student *t*-test, $p > 0.05$). These findings indicate that cells do proliferate in gel culture and that the force of the polymer network on an encapsulated cell does not significantly impede the rate of neural cell expansion.

3.3. Tissue morphology throughout degradable PEG hydrogels

Confocal microscopy was used to directly visualize neural tissue formation throughout degradable PEG hydrogels. The spatial pattern of tissue formation in the hydrogel changed with time. Initially, on day 0 of culture, single cells were distributed throughout the hydrogel. Over the course of 3 days of culture, both single cells and small clusters of cells (20 ± 2 μm in diameter) were visible throughout the hydrogel (Fig. 2a). Cell cluster size increased with time (Fig. 2b,c). By day 7 cells were predominantly associated together in multi-cellular aggregates 30 ± 2 μm in diameter (Fig. 2b), or micro-tissues. The mechanism by which micro-tissues are formed in hydrogel culture involves cell proliferation and not cell migration as the mesh size of the polymer network is significantly less than the diameter of a cell body. Indeed, immunocytochemical labeling of nuclear incorporated BRDU confirmed that proliferating cells are present in micro-tissues after 2 weeks of culture (Fig. 2d).

During the first week of culture cells within micro-tissues extended long processes that penetrated through the tissue interior; some processes wrapped around the exterior surface of the micro-tissue. The first evidence of neural tissue formation throughout the hydrogel environment was observed between day 10 and day 12 of culture. At this stage of degradation, processes wrapped around the micro-tissue and growth occurred in a shell $\sim 17 \pm 4$ μm thick concentric to the micro-tissue center (Fig. 3a,b). During the next 24–48 h of culture, hydrolysis of the network proceeded at a rapid rate and the mesh size of the gel increased by a factor of 3 (Fig. 1b). By day 13 of culture,

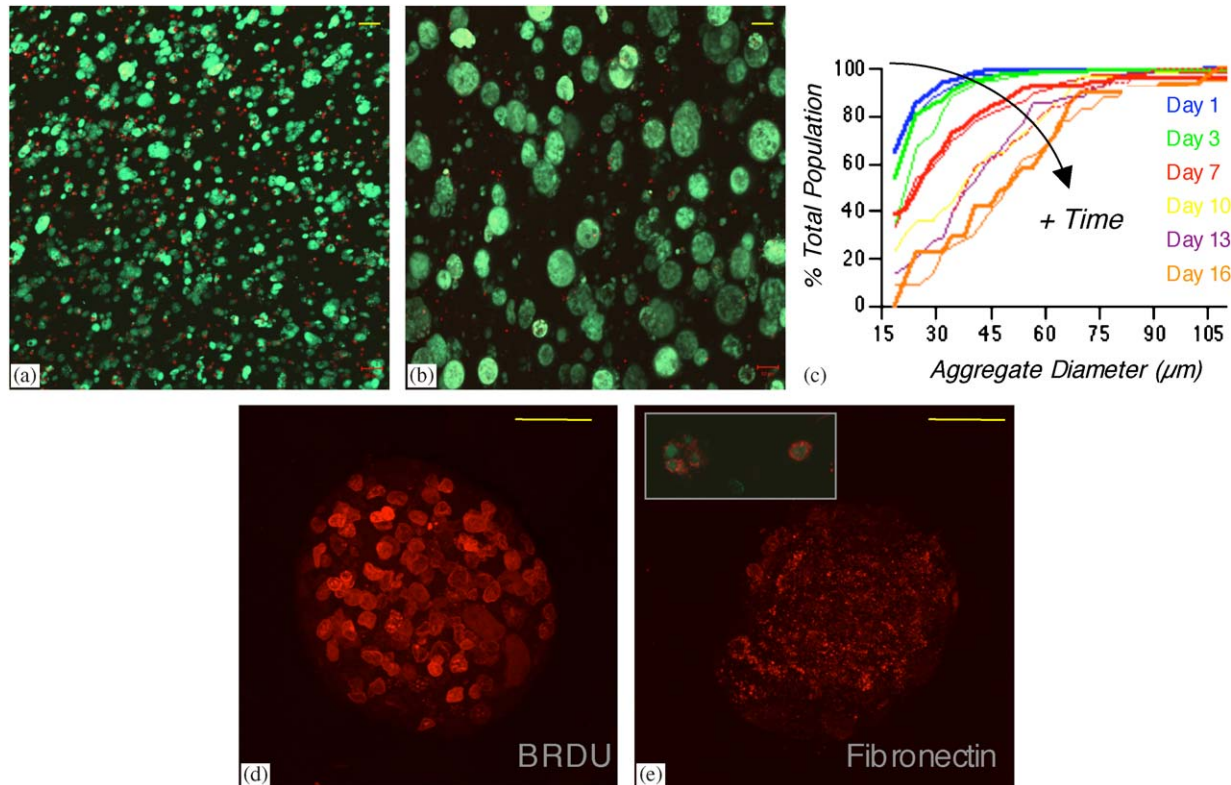


Fig. 2. Distinct micro-tissues form in hydrogel culture during week 1. Neural cells in degradable hydrogels were fluorescently labeled with a dye that fluoresces green upon de-sterification in living cells (calcein-AM) and a dye that fluoresces red when bound to DNA in dead membrane compromised cells. Living and dead cells were directly visualized using confocal microscopy. (a) By day 3 of culture single cells and small clusters of cells are distributed throughout the hydrogel. (b) Over the course of 7 days multicellular aggregates or micro-tissues are visible throughout the culture. (c) Micro-tissue diameter continued to increase over the course of time in culture (d) Positive immunolabeling for BRDU (red) indicates that micro-tissues are formed as cells proliferate. (e) Cells create an environment that is enriched in fibronectin (positive immunolabeling in red, nuclei green). Images in (a) and (b) represent a projection of 50–60 optical sections 10 μm thick throughout the interior region of the hydrogel. Images in (d) and (e) represent a projection of 5 optical sections 2 μm thick through a single micro-tissue. Aggregate size data was obtained from greater than 50 aggregates derived from 3 separate experiments. Scale bar represents 50 μm .

micro-tissues spatially integrated throughout the hydrogel network. At this time point a dense plexus of processes emerged radially from micro-tissues to penetrate and grow through the hydrogel environment (Fig. 3c,d). On average these processes extended $52 \pm 20 \mu\text{m}$ into the hydrogel. In some cases processes emerging from one micro-tissue connected to another one. Cells began to migrate away from the micro-tissue center. Processes and cells continued to penetrate through the hydrogel until the gel was completely hydrolyzed.

During development of the central nervous system receptors on the surface of a neurite bind to matrix molecules present in the extracellular space. Through these contacts traction forces are generated within the developing neuron that push and pull the neurite forward. The extracellular matrix in the developing central nervous system contains several molecules that support neurite extension including laminin, fibronectin, and collagen IV [24–29]. The extracellular environment surrounding a cell in a synthetic hydrogel is dramatically different. Immediately following encapsulation cells are surrounded by synthetic polymer that is non-adhesive. In hydrogel culture

cells define their own chemical microenvironment by producing extracellular matrix molecules. We observed positive labeling for fibronectin in hydrogel culture after 16 days. However, labeling for laminin and collagen IV, two prominent components of the ECM in developing neural tissue was not observed.

These findings identify two distinct spatial patterns of neural tissue formation in degradable PEG hydrogels: (1) formation of distributed micro-tissues, and (2) production of fibronectin and integration and connection of micro-tissues as processes extend throughout the gel environment.

3.4. Temporal control of neural tissue formation

The time-scale over which these spatial patterns of neural tissue develop depended on the degradation rate of the hydrogel. Processes emerged to penetrate through the hydrogel environment after 2 weeks in culture. This time-scale was decreased to 1 week by incorporating a fast-degrading macromer into the network (PEG 2.5-glycolide) or extended to 3 weeks by incorporating a more slowly degrading macromer (PEG 2-lactide) into the network

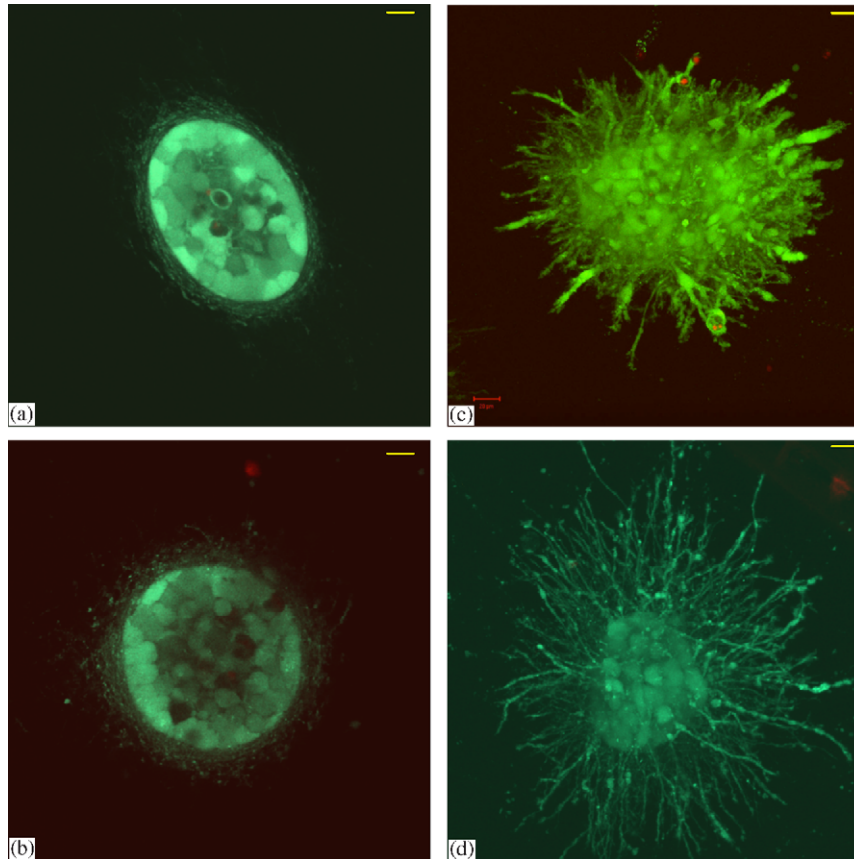


Fig. 3. Spatial integration of micro-tissue during week 2 of culture. Neural cells in degradable hydrogels were fluorescently labeled with calcein-AM (green, live) or ethidium bromide (red, dead). Images in (a) and (b) represent a projection of 5 optical sections $2\ \mu\text{m}$ thick throughout a single micro-tissue on day 10 (a) and day 12 (b) of culture. At this stage of degradation, neurites wrap around the micro-tissue and growth occurs in a shell $\sim 17 \pm 4\ \mu\text{m}$ thick concentric to the micro-tissue center. Images in (c) and (d) represent a projection of 50–60 optical sections $1\ \mu\text{m}$ thick throughout a single micro-tissue on day 14 (c) and day 16 (d) of culture. At this time point a dense plexus of processes emerged radially from aggregates to penetrate and grow through the hydrogel environment. Scale bar represents $20\ \mu\text{m}$.

(data not shown). These findings identify the degradation rate of the polymer network and the concomitant change in mesh size as important material properties that can be tuned to temporally control the spatial pattern of neural tissue formation throughout three-dimensional materials.

3.5. Generation of three cell types in hydrogel culture

Neural precursor cells isolated from the developing forebrain are multipotent and can self-renew or differentiate to form neurons or glia. The differentiation pathway is determined by a balance of intrinsic and extrinsic cues in the surrounding extracellular microenvironment. Here we evaluate the effect of culture in a three-dimensional synthetic gel on cell composition by immunostaining cryo-sections of hydrogel culture and counting positively labeled post-mitotic neurons and glial cells (Fig. 4a–e). At day 0, 66% of the population of neural cells isolated from the developing forebrain was positive for the neuronal protein beta-tubulin-III. No GFAP expression was detected in culture at day 0. Initially 4×10^5 cells were encapsulated within each hydrogel. Based on the fraction

of cells that are beta-tubulin positive on day 0, this represents 2.6×10^5 neurons (Fig. 4e). On day 16 of culture, 2.6×10^6 cells are present in hydrogels. Of this total population 35% are beta-tubulin positive (Fig. 4b) and 38% are GFAP positive (Fig. 4a) corresponding to 9.0×10^5 neurons and 9.8×10^5 glial cells (Fig. 4e). A total of 7.3×10^5 neurons are generated (a 3.5-fold expansion) in hydrogel culture.

A western blot analysis is a semi-quantitative technique that was used as a secondary indicator of changes in cell culture composition in hydrogel culture over time. Beta-actin was utilized as a loading control. Our western blot results indicate that GFAP positive cells are not present until day 16 of culture (Fig. 4f). The banding pattern for beta-tubulin suggests that the fraction of cells that are beta-tubulin positive decreases slightly over the course of 16 days of culture (Fig. 4f). This semi-quantitative analysis is consistent with our direct measurement of cell composition based on cell counting.

Positive localization of synaptophysin in micro-tissues suggests that these neurons are capable of forming synapses (Fig. 4d). We also observed positive MAP-2

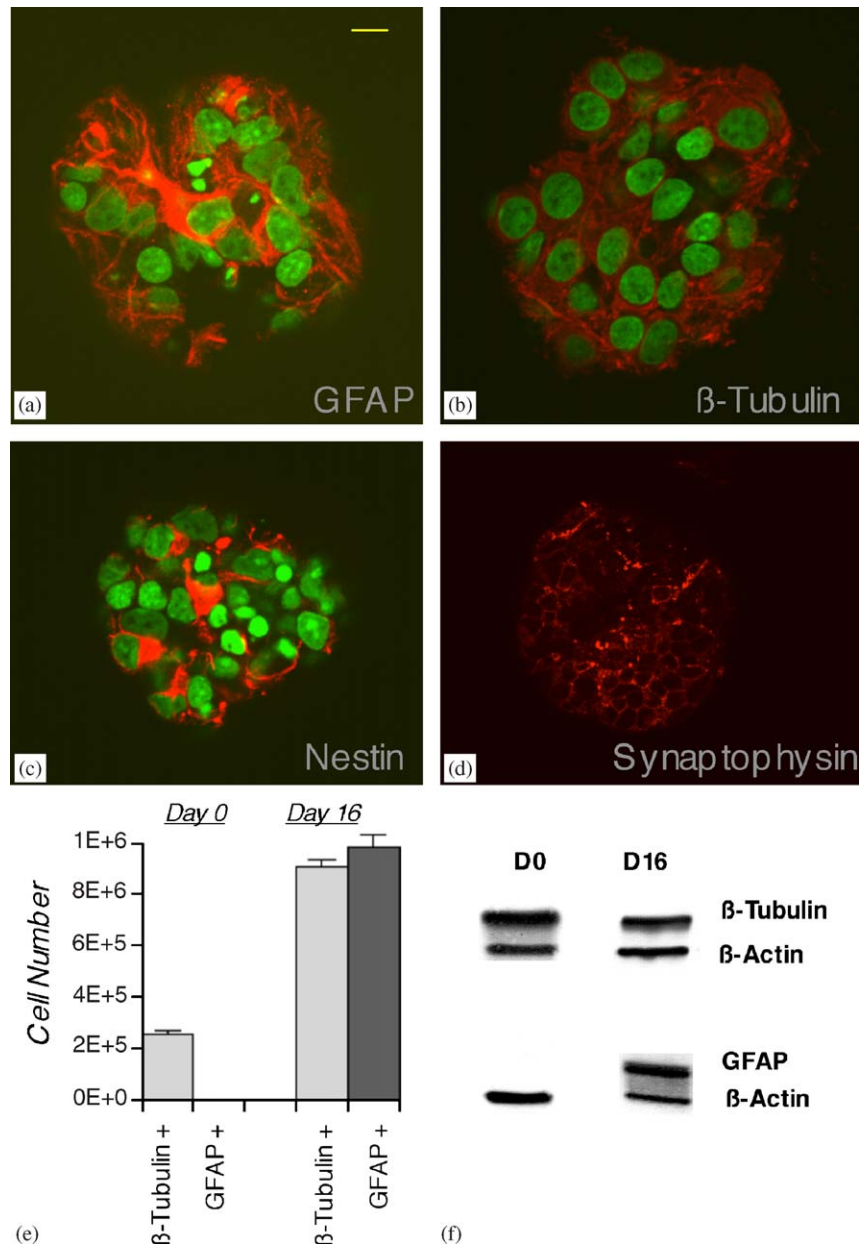


Fig. 4. Cell differentiation in micro-tissues. Differentiation of NPCs in a synthetic three-dimensional polymer network in FGF-2 supplemented media with no serum or extracellular matrix molecules. (a–d) Immunofluorescence analysis using antibodies against glial fibrillary acidic protein (astrocytes), beta-tubulin (neurons), nestin (precursors), and synaptophysin (synapses). (e) Quantification of positive labeling for cell-specific proteins on day 0 and 16 of culture. Data shown are mean \pm SEM for greater than 50 micro-tissues from 3 separate experiments. Images represent a projection of 5 optical sections 2 μ m thick throughout different micro-tissue interiors. Nuclei in (c–e) were counterstained with Sytox Green. Scale bar 10 μ m. (f) Western blot analysis of beta-tubulin, GFAP, and beta-actin expression in freshly isolated NPC (D0) or after 16 days (D16) of hydrogel culture.

labeling in gel cultures, indicating that neurons in hydrogel culture are maturing to form dendrites (data not shown).

3.6. Cell function in degradable PEG hydrogels

Cells within micro-tissues are functional: they are responsive to neurotransmitter. Within seconds following exposure to the neurotransmitter GABA, calcium transients are observed in cells on the exterior and interior of micro-tissues and in cell processes (Fig. 5). All cells in the

micro-tissues displayed an increase in intracellular calcium levels. The time-scale of this increase varied (Fig. 5c). Fast-rising global calcium spikes were observed and slower calcium transients or waves were observed. Calcium transients that vary in spike frequency and duration suggest that multiple cell phenotypes are present throughout the hydrogel culture—a finding consistent with immunocytochemical data. Due to the depolarized equilibrium potential for chloride in neural precursor cells, GABA activation triggers a depolarization response [33].

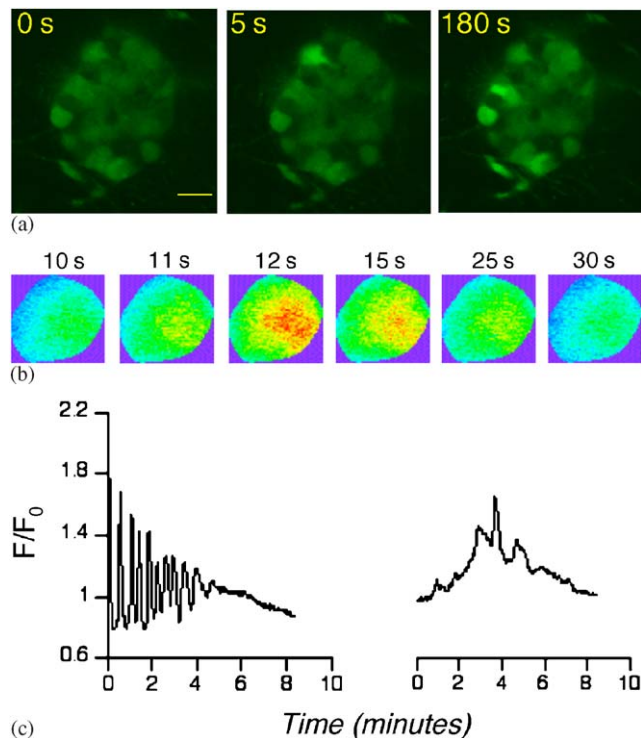


Fig. 5. GABA-evoked Calcium Transients in Micro-tissues. (a) Optical section $2\mu\text{m}$ thick through the interior of a micro-tissue loaded with the fluorescent calcium indicator dye Fluo-3 at different time points following exogenous addition of GABA. An increase in intracellular Ca^{2+} level is present in multiple cells and processes throughout the microtissue interior. (b) Pseudocolor images of a single cell within a micro-tissue at multiple time points. Blue represent background level, red is the highest level of intracellular Ca^{2+} . (c) All cells examined exhibited an increase in Ca^{2+} levels, two types of transients were apparent, fast-rising spikes (left) and slower sustained elevations (right). Data were obtained from greater than 50 cells assembled throughout micro-tissues in 3 separate experiments. Scale bar $15\mu\text{m}$.

As cells mature, GABA-receptor activation serves an inhibitory function within neuronal networks.

4. Discussion

Here we demonstrate that developing neural precursor cells tolerate photoencapsulation and culture in degradable PEG hydrogels with minimal cell death. A low level of cell loss was observed during the first 24 h of culture (10% of the initial population). This low level of cell loss may be caused by exposure to free radicals during the photopolymerization procedure; however, studies have also shown an increase in apoptotic signaling following removal of neural precursor cells from their natural developmental microenvironment [34]. This form of apoptosis (termed “anoikis”) can occur within 24 h of removal and may contribute to the cell loss that is observed immediately following encapsulation. But the overall high level of cell viability observed after 16 days of culture indicates that the synthetic, serum-free polymer hydrogel environment is sufficient to support neural precursor cell survival. In fact our results indicate that culture in synthetic three dimen-

sional hydrogels may provide a more suitable environment for expansion of neural precursor cells as survival in gel culture was found to be significantly greater than that in monolayer culture 24 h post-isolation.

When cultured in degradable hydrogels, the spatial pattern of tissue formation throughout the gel varied with time in culture. During the first week of culture precursor cells and neurons with long processes assembled together to create small micro-tissues. At this early stage of degradation process growth throughout the hydrogel was not observed presumably because the overall mesh size of the hydrogel is too low (50\AA) to permit extracellular matrix molecule diffusion or process growth into the gel environment.

After 2 weeks of culture, processes emerged from cells in micro-tissue cores to penetrate and grow through the hydrogel environment. At this time point three types of cells are present in hydrogels: glia, neurons, and precursor cells. Positive staining for the extracellular matrix protein fibronectin is also present (Fig. 2e). Process growth throughout the gel environment most likely involves a combination of adhesive interactions between cell-surface molecules and cell-secreted extracellular matrix molecules, as both types of interactions promote process growth during development of the CNS [35] and are available to developing processes in gels.

Micro-tissues can be considered building blocks for the creation of functional neural circuits both in vitro, and ultimately, upon transplantation in vivo. They are composed of a mixed population of cells, neurons and glia that are electrophysiologically responsive to neurotransmitter. As the gel degrades, the building blocks begin to connect to one another. The time-scale over which connection occurs is tunable by controlling the degradation rate of the polymer network. While cellular aggregates have been formed using a variety of techniques [36,37], no methods have been developed to study the development of connections between tissue micro-tissues. In this regard, spatially distributed micro-tissues may be useful to better understand factors that influence morphogenesis of neural tissue structures and, as the gel degrades, factors that influence the development of connections between neural tissues.

Neural precursor cells isolated from developing rat brain have been shown to survive and proliferate within other three-dimensional environments. For example in collagen gel cultures by day 5 of growth, cells extend long processes throughout the gel interior [31]. Process growth throughout the gel interior occurs on an earlier time-scale than that observed in this study most likely because the porosity of the collagen gel is higher and cells are presented with a scaffold to which they can adhere and elongate across. We see a similar elongation of processes on day 5 of PEG hydrogel culture. However, this process growth is restricted to micro-tissue interiors until the gel has significantly degraded and process growth proceeds throughout the gel environment (day 14–16). Interestingly, immunocytochemical results indicate positive staining for neurons and glial

cells in both PEG hydrogels and collagen gel cultures. The relative fraction of each cell type may differ in the two environments. The relative fraction of beta-tubulin-III positive neurons and glial cells was not reported in the collagen gel study.

Several types of cells have been successfully cultured in PEG hydrogels including chondrocytes and osteoblasts [38–40]. Chondrocytes secrete extracellular matrix molecules that diffuse throughout the gel network as it degrades. The extent to which secreted ECM molecules distribute throughout the gel depends on the pore size of the network. A more homogeneous distribution of ECM can be achieved by incorporating degradable macromers into the network. Osteoblasts form mineralized bone throughout the gel network. The spatial pattern of bone formation is also dependent on the pore size of the network. In this study, a significant amount of ECM deposition throughout the gel was not observed at any time point during culture. However, process growth throughout the gel environment was dependent on the cross-linking density of the polymer network. In addition, micro-tissue formation was not observed in gel cultures with other cell types. The formation of micro-tissues is only observed with neural precursor cells and is similar to the behavior of these cells in suspension culture [41,42].

5. Conclusions

These studies were carried out in cell culture in the absence of serum and extracellular matrix molecules and identify degradable PEG hydrogels as suitable synthetic cell carriers that may ultimately provide temporal control over donor and host cell interactions in the central nervous system. Assembly and transplantation of cells throughout a hydrogel that is initially impermeable to surrounding cells and growing processes will afford donor cells protection from cell-contact mediated inflammatory signals in the adult extracellular space immediately following transplantation. At later time points, as the gel degrades and the overall mesh size of the gel increases, the network can serve as a scaffold to support, and one day direct, regeneration within the host environment until the material is ultimately resorbed by the tissue.

Acknowledgments

This work was supported by HHMI and the Packard Foundation.

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