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Long-term three-dimensional neural tissue cultures in functionalized self-assembling peptide hydrogels, Matrigel and Collagen I

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ABSTRACT

Designer peptides with self-assembling properties form nanofibers which are further organized to form a hydrogel consisting of up to 99.5% water. We present here the encapsulation of neural stem cells into peptide nanofiber hydrogel scaffolds. This results in three-dimensional (3-D) neural tissue cultures in which neural stem cells differentiate into progenitor neural cells, neurons, astrocytes and oligodendrocytes when cultured in serum-free medium. Cell survival studies showed that neural cells in peptide hydrogels thrive for at least 5 months. In contrast, neural stem cells encapsulated in Collagen I were poorly differentiated and did not migrate significantly, thus forming clusters. We show that for culture periods of 1–2 weeks, neural stem cells proliferate and differentiate better in Matrigel. However, in long-term studies, the population of cells in Matrigel decreases whereas better cell survival rates are observed in neural tissue cultures in peptide hydrogels. Peptide functionalization with cell adhesion and cell differentiation motifs show superior cell survival and differentiation properties compared to those observed upon culturing neural cells in non-modified peptide hydrogels. These designed 3-D engineered tissue culturing systems have a potential use as tissue surrogates for tissue regeneration. The well-defined chemical and physical properties of the peptide nanofiber hydrogels and the use of serum-free medium allow for more realistic biological studies of neural cells in a biomimetic 3-D environment.

1. Introduction

Neurons in living organisms reside in three-dimensional (3-D) architectures; however, most neural cell studies are performed currently in 2-D cultures. While the simple Petri dish has been an indispensable tool for in vitro cell biology studies for over 100 years, it is less than ideal for studying mammalian cell biology for several reasons: (i) a significant portion of the cell surface is attached to the plastic material of the plate instead of interacting with other cells and molecules as in the tissue environment; (ii) diffusion phenomena of cell nutrients and cell waste products in 2-D and 3-D tissue cultures are dramatically different; (iii) many cell types, including neurons, have altered metabolism and gene expression patterns in 2-D cultures compared to 3-D cultures [1,2]; (iv) the morphology of neural cells growing in a 2-D environment differs from that of neural cells growing in 3-D (e.g. in 2-D cultures an increase in spreading is observed rather than expanding in three dimensions as in the brain tissue) [3].

To meet the need for a realistic tissue culture system, bioengineers and polymer scientists have developed new materials to create a 3-D environment, primarily focusing on synthetic polymers and animal-derived materials. In past decades, many synthetic polymers tested for tissue engineering applications consisted of microfibers ~10–50 μm in diameter. Since the microfibers are the same size or larger compared to mammalian cells, the latter adhere on top of the polymer fiber, resulting in a 2-D environment [4,5]. New polymers, including those fabricated using electrospun technologies, address this incompatibility and consist of nanofibers. However, many polymer matrices contain toxic polymerization initiators such as free radicals and toxic chemicals which are required for the synthesis of the polymer or are produced during polymer degradation.

Some animal-derived biomaterials, such as collagen, laminin and Matrigel™, are of the right scale and meet most of the criteria for an ideal 3-D tissue culture. However, due to their origin (i.e. they are isolated from living tissues) their use for medical applications in humans is often questioned. Matrigel is isolated from the mouse EHS sarcoma, a cancerous tissue. Cell studies in Matrigel are often not reproducible because the matrix composition varies from lot to lot and the biochemical pathways observed may be due to unknown cell signaling factors present in the matrix.

Self-assembling peptides, made from natural amino acids, form nanofiber scaffold hydrogels which surround cells in a manner

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similar to the extracellular matrix. At physiological conditions, short self-assembling peptides with alternating hydrophobic and hydrophilic amino acids undergo spontaneous self-assembly into nanofibers, 6–10 nm in diameter as determined by atomic force microscopy (AFM), transmission electron microscopy (TEM) and scanning electron microscopy (SEM) analyses [6–8]. These fibers are further organized to form a hydrogel scaffold containing water >99%, with 5–200 nm pores [9]. These designer self-assembling peptides can be chemically synthesized and tailor-made to incorporate specific ligands from the extracellular matrix including ligands for cell receptors. Peptide hydrogels have been characterized and tested in a number of biomedical applications including bone and cartilage reconstruction, heart tissue regeneration, angiogenesis, and drug delivery [9–13]. Preliminary studies showed that self-assembling peptides support neuronal cell attachment, extensive neurite outgrowth, and promote active and functional synapse formations when neural cells were plated on the surface of pre-formed peptide hydrogel matrices [14]. Here we report that adult mouse neural stem cells can be encapsulated inside 3-D matrices of self-assembling peptide hydrogels for up to 5 months in serum-free medium providing a platform to study neural cell proliferation and differentiation.

2. Materials and methods

2.1. Self-assembling peptide nanofiber hydrogels

The CH_3CO-(Arg-Ala-Asp-Ala)$_4$-$CONH_2$ (ac-(RADA)$_4$-$CONH_2$) 1% w/v peptide solution in water was purchased by BD Biosciences (Bedford, MA). Functionalized peptides (Fig. 1) were designed, tested for stability using computational tools, and custom-made by MIT Biopolymers Lab and CPC Scientific (San Jose, CA). The functionalized peptides, which were obtained in powder form, were dissolved in distilled water to a final concentration of 1% w/v and sonicated for 20 min to disassemble peptide aggregates prior to use. Sol–gel transition occurs upon interaction of the peptide monomers in water with an electrolyte solution such as phosphate-buffered saline (PBS) or cell growth media. Experiments were performed in tissue culture treated 96- or 384-well plates (BD Labware, NJ). SEM imaging was performed using a JEOL JSM-6060 (Tokyo, Japan). Peptide hydrogel preparation for SEM imaging involved slow exchange of water with ethanol (10% increment steps of increasing ethanol to water solutions for 15 min at each condition). Once the hydrogel was in 100% ethanol, the ethanol was replaced with liquid CO_2 using a CO_2 critical point dryer (Tousimis, Samdri 780A, Rockville, MD). Upon CO_2 removal, peptide hydrogels were sputter-coated with a 10 nm gold layer (Denton Vacuum, Moorestown, NJ) and SEM imaged. This method allows for preservation of the structure of the hydrogel without compromising the nanofiber structural characteristics and avoiding nanofiber collapse resulting from hydrogel dehydration.

2.2. Mechanical properties of the hydrogels

Rheological tests of the peptide hydrogels, Matrigel and Collagen I hydrogel were performed using an AR1000 rheometer (TA Instruments, Inc., New Castle, DE). All hydrogels were formed on the surface of the rheometer and storage (G′) and loss (G″) moduli were measured using a 8 mm plate. Data were obtained at room temperature at constant angular frequency 6.3 rad s^{-1} and at constant oscillatory torque in the frequency range 1.0–49.9 rad s^{-1} (n = 3).

2.3. Neural stem cells

Adult mouse neural stem cells were isolated from the subventricular zone of an 8 week old CD-1 albino mouse striatum and expanded [15]. Cell proliferation was performed in serum-free basal medium (Neurocult®, StemCell Technologies, Inc., Canada) supplemented with human basic fibroblast growth factor (FGF-2) and human epidermal growth factor (EGF) (Peprotech, Inc., NJ) [15,16]. In this medium, cells self-renew extensively to form neurospheres and can be propagated for at least 80 passages without detectable genetic or phenotypic changes. Incubation of adult mouse neural stem cells (37 °C, 5% CO_2) in 150 cm^2 flasks resulted in proliferation and formation of characteristic, non-adhering stem cell neurospheres. To passage, the neurospheres were mechanically dissociated by vigorous pipetting. During this process differentiated cells in the neurosphere rapidly perished but neural stem cells survived, giving rise to new neurospheres.

2.4. Neural cell tissue cultures

For the 3-D neural tissue cultures, 5 μl of freshly passaged neural stem cells suspended in serum-free, basal neural cell culture medium (with added 10 ng ml^{-1} human FGF-2 to increase neural stem cell differentiation to neural progenitors but without EGF) were mixed with 45 μl of the peptide, Matrigel or Collagen I solutions in the wells of a 96-well plate as described below. The final cell concentration in the hydrogel tissue culture was 5×10^5 cells ml^{-1}, whereas the concentration of the peptide, Matrigel and Collagen I were 0.9, 0.9 and 0.15% w/v, respectively.

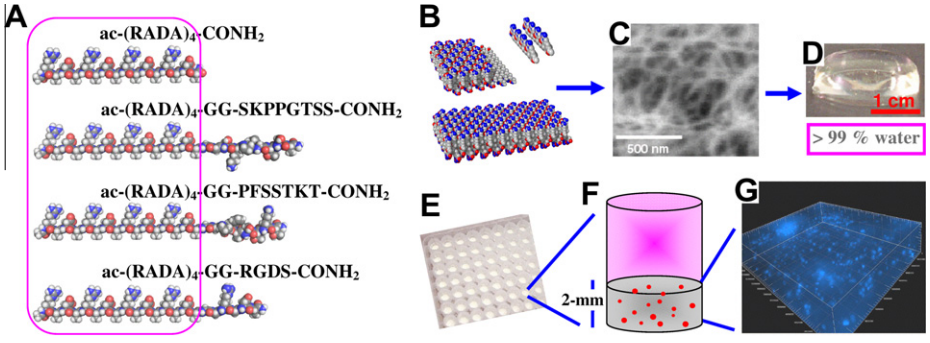

Fig. 1. (A) Molecular models of the self-assembling peptide ac-(RADA)$_4$-$CONH_2$ and of the modified self-assembling peptides carrying a di-glycine linker and the functional motifs SKPPGTSS, PFSSTKT and RGDS. Color code: carbon, grey; oxygen, red; nitrogen, blue; hydrogen, white. (B) Molecular model of the self-assembling peptide and of the nanofiber formed upon assembly of the peptide monomers. (C) SEM picture of the peptide nanofibers inside the hydrogel. (D) Picture of the peptide hydrogel. (E, F) 96-well plate and graphical representation of a plate well in which the cells (red spheres) are encapsulated inside the peptide hydrogel (grey shaded volume at the bottom of the well); the well is filled with cell growth medium (red shaded volume). (G) Microscopy image volume representation of neural cells encapsulated in the peptide hydrogel; series of images were taken at different z-planes of cells cultured in the 3-D environment of a 2 mm thick peptide hydrogel (cell nuclei were stained blue with DAPI).

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When the peptide solution in water is mixed with the cell suspension containing 150 mM NaCl, amino acids and other electrolytes, gelation begins, resulting in encapsulation of neural stem cells inside the nanofiber hydrogel (Fig. 2F, G). The aquatic peptide solution is acidic with pH 3–4. To avoid instant death of the neural stem cells during gelation we adjusted the pH of the basal medium to pH ≈ 9 using 0.1 M NaOH. Peptide self-assembly strongly depends not only on the addition of electrolyte but also on pH. An increase of the pH of the peptide solution to a value of pH > 5 prior to cell encapsulation would result in immediate and irreversible gelation and peptide aggregation, preventing encapsulation of the neural stem cells. Neural stem cell viability is not significantly affected when they are suspended in a growth medium of pH 9 for the short period of time (a few seconds) required for mixing the cell suspension with the peptide solution. Then we added 200 μl of serum-free, basal medium supplemented with FGF-2 (preconditioned to 37 °C), pH ≈ 9, to neutralize the acidic hydrogel environment. The basal medium contains phenol red as pH indicator. Therefore, a color change of the medium from red to yellow indicates that the pH is still acidic and the medium was replenished. When the pH of the system was neutral, we added standard serum-free, basal pH ≈ 7.4 medium supplemented with FGF-2. Three days after encapsulation, the medium was replaced with FGF-2-free basal medium supplemented with 20 ng ml^{-1} leukemia inhibitory factor (LIF) (Chemicon, CA) and 20 ng ml^{-1} human brain derived neurotrophic factor (BDNF) (Peprotech, Inc., NJ) to promote maturation of the neural cells and glial formation. The medium was changed every 12 h during the first 2 days, and then every 3 days. The hydrogel-encapsulated neural cells were incubated at 37 °C, 5% CO_2 at all times and efforts were made to minimize the time required for medium changes. Cells were cultured for up to 5 months.

Tissue cultures of neural cells were also performed in Matrigel™ (growth factor reduced from mouse EHS sarcoma, BD Biosciences) and Collagen I (rat tail, type I, BD Biosciences). Matrigel and Collagen I hydrogel formation occurs upon exposure of the materials to 37 °C. Therefore, neural stem cells were mixed and encapsulated inside the Matrigel or Collagen I solutions before the materials were placed in the incubator to induce gelation. In tissue cultures using Collagen I, the neural stem cells were suspended in a solution containing phenol red, deionized water, NaOH and Collagen I solution.

2.5. Cell viability and differentiation in 3-D matrices

The LIVE/DEAD kit (Molecular Probes Inc., Eugene, OR) was used to assess cell viability. The cells were incubated for 30 min to ensure that the reagents had diffused through the matrix and properly interacted with the encapsulated cells. After incubation, cells were washed five times with PBS for 30 min to remove unbound dyes and imaged immediately using a fluorescent microscope (Nikon TE300). The percentage of live and dead cells was determined by counting 200-300 cells at five different, non-overlapping focal planes of the 3-D neural tissue cultures in 5–8 different tissue culture samples. Variation in survival of the cell population in the different matrices was evaluated by Student's *t*-test.

Neural stem cell differentiation in 3-D tissue cultures was studied in self-assembling peptide hydrogels, Matrigel and Collagen I. Tissue cultures were fixed with 4% formaldehyde in water, incubated with blocking solution (10% goat serum in PBS containing 0.3% Triton-X100) for 1 h at room temperature, and then incubated at 4 °C with primary antibodies diluted in blocking solution. Neuronal and glial differentiation was determined by single and double immunostaining with lineage-specific antibodies: anti-nestin (Chemicon, Temecula, CA) for progenitor cells, rabbit neuronal anti-neuronal class III β-tubulin (Tuj1) (Covance, Berkeley, CA)

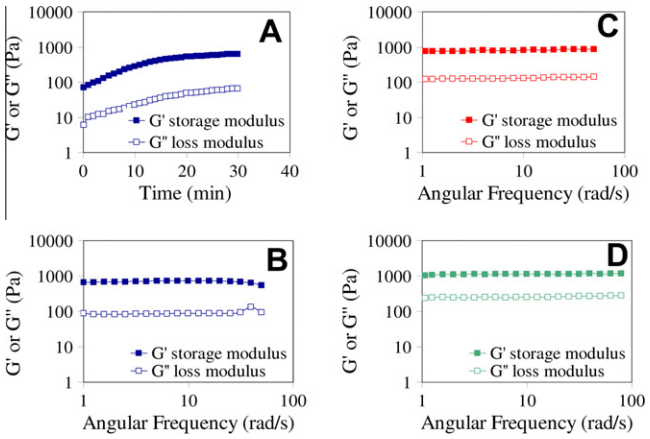

Fig. 2. Mechanical properties of (A, B) self-assembling peptide hydrogels that were used for 3-D neural cell tissue cultures. Storage (G′) and loss (G″) moduli of hydrogels consisting of 0.9% w/v peptides were monitored (A) at constant angular frequency 6.283 rad s^{-1} as a function of time and (B) at frequency sweep conditions where preformed peptide hydrogels were examined. Mechanical properties of (C) 0.9% w/v Matrigel and (D) 0.15% w/v Collagen I were determined on preformed hydrogels because both Matrigel and Collagen I materials gel when exposed 37 °C. Each experiment represents the average (n = 3) and the standard deviation is less than 10%.

for early differentiating neurons committed to be mature not recognizing β-tubulin found in glial cells, mouse anti- glial fibrillary acidic protein (anti-GFAP) (Chemicon) for astrocytes, and anti-galactocerebroside (anti-GalC) (Chemicon) for oligodendrocytes. Species-specific fluorophore-conjugated secondary antibodies used were ALEXA 488 goat anti-mouse (Molecular Probes) and CY3 AffiniPure F(ab′)2 fragment goat anti-rabbit (Jackson ImmunoResearch, West Grove, PA). Cell nuclei were stained with DAPI (Molecular Probes). Negative controls for immunostaining were also performed. Fluorescent and phase-contrast images of the neural tissue cultures were acquired by a Nikon TE300 microscope; confocal images were collected using a Olympus FluoView FV1000 confocal laser scanning microscope (Olympus, Japan) and 3-D visualization analysis was performed using Imaris (Bitplane, MN). For each hydrogel system, quantitative analyses of the differentiated cells were performed using an inverted Nikon TE300 microscope by counting 200–300 cells from randomly selected non-overlapping fields, from different focal planes, and from 5–8 different tissue culture samples from different wells.

3. Results and discussion

3.1. Designer peptide sequences

Peptide sequences inspired by extracellular matrix and stimuli-inducing proteins/peptides have generated attention as a potential means to provide biological cues for synthetic biomaterials. We used molecular modeling and information from the literature to design functionalized peptides that exhibit optimal properties for culturing neural cells (Fig. 1A). The peptide sequence SKPPGTSS which was added to the ac-$(RADA)_4$- self-assembling peptide unit appears in (i) the neuronal apoptosis inhibitory protein family which inhibit neural cell apoptosis [17]; (ii) the nitric oxide synthase which catalyzes the production of nitric oxide, i.e. an important cellular signaling molecule with a vital role in the development of the nervous system and mediating neurotransmission [18]; (iii) the neurogenic locus notch homolog protein 2 (NOTCH-2) which regulates interactions between adjacent neural and stem cells [19,20]; (iv) protocadherin family proteins which mediate cell adhesion and also act as signaling molecules [21]; (v) collagen XI which is found in the mammalian brain [22].

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In another designer self-assembling peptide the functional motif PFSSTKT was attached to the self-assembling sequence. This modification contains an amino acid sequence that appears in (i) multiple copies of cAMP-specific phosphodiesterase isoforms which are enzymes that regulate cellular responses to extracellular signals, such as hormones and neurotransmitters, and thereby play a role in signal transduction [23]; (ii) several mucins whose production is regulated by neural cells [24,25]; (iii) many serine protease inhibitors (serpins) which are beneficial for tissue cell cultures by increasing the lifetime of growth factors that otherwise would be quickly eliminated from the growth medium by proteases; (iv) semaphorins which act as axonal growth cone guidance molecules and are therefore important in the development of the neural system [26].

Another designed self-assembling peptide has the Arg-Gly-Asp (RGD) sequence. RGD-based domains are present in many glycoproteins and in fibronectin, mediate cell adhesion through the integrin family of receptors, and were shown to improve sprouting of hippocampal mouse neurons [27,28].

3.2. Mechanical properties of the hydrogels and cell behavior

Tissue cells respond to the mechanical properties of their microenvironment and alter their properties depending on stiffness and tissue elasticity. In many tissue culture studies, diverse biomaterials are tested and compared without discussing the effect of matrix stiffness on cell proliferation, migration and tissue formation. Rheological measurements of the peptide hydrogels, Matrigel and Collagen I were performed to determine biomechanical properties and to address this common source of error. We recorded the storage and loss moduli G′ and G″ of cell-free hydrogel matrices at constant oscillatory torque (from 1 to 60 rad s^{-1}, Fig. 2B, D). Furthermore, we studied the self-assembling peptide gelation kinetics as a function of time at constant angular frequency and we observed that G′ and G″ values increased until reaching a plateau at 730 Pa after ~20 min (Fig. 2A). The presence of 30 mol.% of functionalized self-assembling peptides mixed with the ac-$(RADA)_4$-$CONH_2$ peptide did not significantly alter the mechanical properties of the final hydrogel. Preliminary studies showed that at this ratio of functionalized to ac-$(RADA)_4$-$CONH_2$ peptide, embedded neural stem cells were exposed to a sufficient number of stimuli groups from the floppy, non-assembling side chains of the self-assembling ac-$(RADA)_4$ unit. The stiffness of peptide nanofiber hydrogels can be adjusted by altering the concentration of the peptide water solution prior to addition of the neural cell growth medium which induces the self-assembly of the peptides.

Using rheometry, the mechanical properties of Matrigel and Collagen I were also determined (Fig. 2C, D). To our knowledge, the mechanical properties of Matrigel measured using rheometry have not been reported previously. In the case of Collagen I (Fig. 2D), the G′ and G″ values that we recorded are in agreement with those found in the literature for Collagen I hydrogels of similar concentration, conditions, and instrumentation used in the present study [29,30].

We observed that the Matrigel and Collagen I concentrations used in our experiments result in hydrogels with rheological properties resembling those of the peptide hydrogels (Fig. 2B). Therefore, differences in cell behavior in different matrices could not be due to differences in the mechanical properties of the hydrogels.

3.3. Adult neural stem cells

We used neural stem cells from the subventricular zone of an adult mouse. These cells should be distinguished from the so-called ‘‘progenitor stem cells’’ which are transiently dividing cells that produce only restricted phenotypes and have limited proliferating capacity or from other genetically immortalized cells which have been modified to proliferate in a stem cell-like manner [15]. A property of stem cells is the generation of identical progeny through unlimited numbers of cell divisions whilst they respond to physiological demands and environmental conditions by producing daughter cells that are committed to differentiate. To stimulate extensive self-renewal of the neural stem cell population, we added human FGF-2, human EGF and insulin in the stem cell propagating medium. We found that continuous provision of both FGF-2 and EGF was necessary to sustain non-adherent neurosphere formation of neural stem cells. To maintain a pure neural stem cell population in every passage, the neurospheres were mechanically dissociated by vigorous pipetting which induced cell death of differentiated cells. We found that cell viability was ~55% after passaging as determined by Trypan Blue exclusion experiments. Serial subculture of these cells over a period of 3 years did not result in measurable loss of pluripotency upon removal of the FGF-2 and EGF growth factors.

Freshly dissociated neural stem cells were regularly examined for differentiation by immunostaining. We did not observe a statistically significant population of differentiated neurons or glial cells inside the neurospheres. These neural stem cells generate different neuronal sub-types and did not show preferential differentiation into astrocytes or neurons as previously observed for other neural stem cell lines. Therefore, they represent an ideal system for in vitro studies of biological phenomena of the brain and neural tissue regeneration.

3.4. Cell survival in 3-D neural tissue cultures

The self-assembling peptide nanofiber hydrogel scaffolds are transparent, and therefore suitable for microscopic analyses. Direct observations of neural cells in 3-D hydrogel matrices were performed in situ inside the wells of 96- or 384-well plates to facilitate high throughput analyses. Earlier work in our group, focused on cell plating on the surface of preformed peptide hydrogels resulting in a 3-D-like tissue culture. While the cells are deposited on the ‘‘hills’’ and ‘‘valleys’’ of the hydrogel formed during gelation, the cells are still on the surface rather than within the gel. In this work, cells are embedded within the hydrogel and the study was performed in a true 3-D environment. We observed neural and glial cells dispersed homogeneously inside the peptide hydrogel scaffolds, forming 2 mm thick neural tissue cultures inside the well of a 96- or 384-well plate (Fig. 1F, G). We performed live/dead assay analyses of the 3-D tissue cultures at 1, 2, 4 and 8 weeks and at 5 months. At all time points, we observed that tissue cultures in functionalized peptide hydrogels contain more than 50% viable cells (Fig. 3). The highest percentage of living cells, ~69% after 3 months, was observed in the peptide nanofiber hydrogel containing the SKPPGTSS functional motif, which suggests that this amino acid sequence may be important to support cell viability. It is possible that the mechanism involves inhibition of neural cell apoptosis because the SKPPGTSS motif is important in the neuronal apoptosis inhibitory protein family [17]. However, more work will be required to confirm this hypothesis.

At 3 months, a higher rate of cell survival was also observed for neural tissue cultures in peptide nanofiber hydrogels carrying the PFSSTKT (56%) and the RGDS (58%) motifs compared to tissue cultures in Matrigel (37%) and Collagen I (25%) (Fig. 3).

To confirm that increased cell viability was a direct result of adding the specific sequence motifs, SKPPGTSS, PFSSTKT and RGDS, for the functionalization of the self-assembling peptide, we performed negative control experiments using self-assembling peptides carrying sequences of scrambled or mutated motifs. Encapsulation of neural stem cells and subsequent tissue cultures

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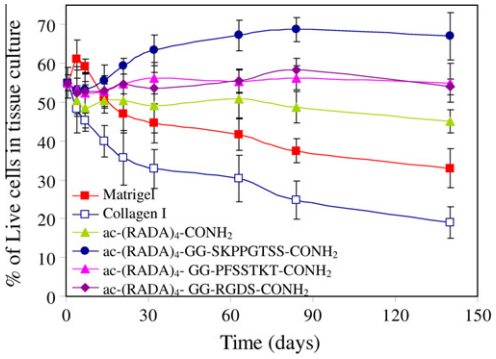

Fig. 3. Cell survival as a function of time. Live/dead assay of 3-D neural cell tissue cultures in modified peptides show increased cell survival compared to the unmodified ac-$(RADA)_4$-$CONH_2$ hydrogel, Matrigel and Collagen I.

in ac-$(RADA)_4$-GG-STFTKSP-$CONH_2$, ac-$(RADA)_4$-GG-FSSPTTK-$CONH_2$ and ac-$(RADA)_4$-GG-PDSGR-$CONH_2$ peptide hydrogels showed that cell viability was similar or less compared to that of the non-functionalized ac-$(RADA)_4$-$CONH_2$ peptide. This result suggests that the beneficial effect of the SKPPGTSS, PFSSTKT and RGDS sequences on neural cell growth, migration, adhesion and differentiation is specific to the functionalized group.

Tissue cultures of neural cells in Matrigel showed increased living cell population during the first week post-encapsulation of undifferentiated neural stem cells. However, following the initial increase of the cell population, the number of living cells in the tissue culture in Matrigel consistently decreased over time. One and two months post-encapsulation the per cent viability of living cells in Matrigel were 16% and 31% less, respectively, compared to freshly encapsulated living cells (Fig. 3). Neural stem cells embedded in Matrigel proliferate and differentiate uncontrollably even in the absence of added growth factors. Therefore, it is likely that the initial increase in per cent living cells in Matrigel (Fig. 3) is due to growth factors and cytokines present in Matrigel, resulting in increased numbers of the undifferentiated and differentiated cells. However, the initial increase in cell viability is lost over time. It is likely that these growth factors and cytokines are "consumed" by the neural cells, degraded over time, by proteases for example, or simply removed from the Matrigel hydrogel because our protocol involves addition of fresh growth medium (more frequently during the first week and then every 3 days).

Our results show decreased living cell population in neural tissue cultures 2 weeks post-encapsulation in Matrigel. The long-term experiments reveal the real effect of the Matrigel matrix on neural cells. It is apparent that Matrigel, free of the initial chemical stimuli, does not offer an advantage towards maintaining a thriving neural cell population.

3.5. Neural stem cell differentiation in 3-D tissue cultures

Prior to encapsulation in the hydrogels, neural stem cells were propagated, dissociated and passaged as neurospheres suspended in growth medium containing EGF/FGF-2. Neural stem cell differentiation was studied following encapsulation of 5×10^5 neural stem cells ml^{-1} inside ac-$(RADA)_4$-$CONH_2$, functionalized self-assembling peptides, Matrigel and Collagen I hydrogels. Neuronal and glial differentiation was assessed by single and double immunostaining with lineage-specific antibodies: anti-nestin for progenitor cells, neuronal class III anti-β-tubulin (anti-Tuj1) for neuronal committed cells, anti-GFAP for astrocytes and anti-GalC for oligodendrocytes (Fig. 4). Tuj1 is an immunocytochemical marker for post-mitotic neurons while nestin is an intermediate filament protein found in precursor cells. Nestin was not observed in Tuj1(+) cells, suggesting that these antibodies label distinct cell populations. Also, in double-staining experiments, nestin and GFAP labeled distinct neural stem cell-derived cells and no co-localization was observed.

Three days post-encapsulation the growth medium contained EGF/FGF-2 to sustain the neural stem cell population inside the hydrogels. During this period, neural stem cell proliferation and partial differentiation occurred. Immunostaining showed that the majority of the encapsulated cells expressed the marker nestin, approximately 5% were immunoreactive for the neuronal-specific marker Tuj1, and less than 1% expressed the astroglial and oligodendrocyte markers GFAP and GalC. Subsequently, the growth medium was EGF/FGF-2-depleted, but leukemia inhibitory factor (LIF) and the neurotrophin BDNF were added to promote the maturation of the neuronal and glia cells [31].

In our 3-D neural tissue culture studies, we did not observe preferential differentiation of the encapsulated neural stem cells into astrocytes or neurons as previously described in studies performed in 2-D conditions by plating the same neural stem cell line on the surface of laminin, collagen or polymers [32]. This result highlights the advantage of 3-D neural tissue cultures and suggests that our approach allows the confidence to study biochemical processes of the brain in vitro. Our results show that neuronal subtypes including astrocytes and oligodendrocytes survived in 3-D peptide nanofiber hydrogels significantly longer and better compared to tissue cultures in Matrigel (Fig. 5). Tuj1(+) cells appeared quickly, suggesting that establishment of neuronal lineage occurs earlier in the life of the differentiated cells compared to the appearance of astrocytic and oligodendrocytic phenotypes which occurs later. With continued tissue culture in the presence of LIF and BDNF more cells acquire neuronal identity as shown by Tuj1 immunoreactivity and cytological profiling while notable differentiation into GFAP(+) astrocytic and GalC(+) profiles occurs 2 weeks post-encapsulation (Fig. 5).

In the functionalized peptide nanofiber hydrogels, a significant amount of encapsulated neural stem cells enter neuronal lineage, i.e. approximately 25% Tuj1 immunoreactivity at 2 weeks, which increased to 30% at 4 and 6 weeks post-encapsulation (Fig. 5). These Tuj1(+) cells were initially monopolar or bipolar, but later developed multiple extensions and have average cell body diameter of 15 μm. One week post-encapsulation approximately 62% of the neuron Tuj1(+) cells were monopolar, ~23% bipolar and 15% multipolar. Examination of the neuronal processes 6 weeks post-encapsulation revealed that monopolar cells account for 19% of the total Tuj1(+) cell population, whereas bipolar are 38%, and multipolar are 44%. None of the cells co-expressed Tuj1 and GFAP. Among the different functionalized peptide hydrogels that we tested, the highest number of Tuj1(+) cells was observed in hydrogels carrying the SKPPGTSS sequence. At 6 and 12 weeks post-encapsulation the number of Tuj1(+) cells were approximately 20% and 30% higher, respectively, compared to those counted in PFSSTKT and RGDS peptide hydrogels.

Representative images of 3-D neural tissue cultures in peptide nanofiber hydrogels are shown in Fig. 4. Depending on the functionalization of the self-assembling peptide, quantitative immunocytochemistry analyses showed that after 3 months in tissue culture, neural stem cells encapsulated in peptide hydrogels had differentiated into approximately ~27% neurons, ~25% astrocytes and ~26% oligodendrocytes (Fig. 5). Contrary to other 3-D hydrogel or polymeric matrices that did not allow for neural stem cell differentiation towards astrocytes [33], we observed that neural tissue cultures in peptide hydrogels contained multipolar GFAP(+) cells. Peptide nanofiber hydrogels do not inhibit the formation of astrocytes, and therefore they represent a more realistic tissue culture system to study neural cell biology and tissue engineering.

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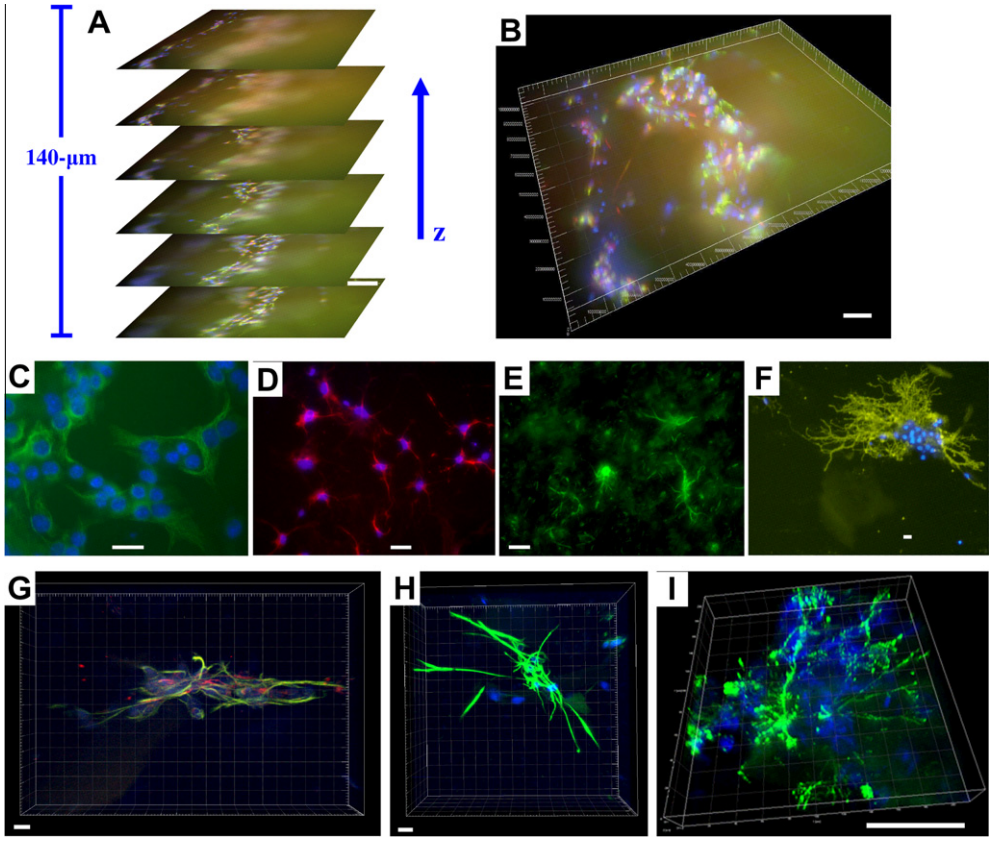

Fig. 4. Neural cells in 3-D peptide nanofiber scaffold containing the ac-$(RADA)_4$-GG-SKPPGTSS-$CONH_2$ peptide. Neural stem cells were encapsulated in a 2 mm thick peptide hydrogel, proliferated and differentiated therein. (A, B) Images of 2 week tissue culture in which nestin(+) neural progenitors, cells (green) and Tuj1(+) neurons (red) appear at different z-planes; image (B) was generated from image (A) using an image analysis software. (C, G) Neural progenitors were stained with anti-nestin (green), (D, G) neurons were stained with anti-Tuj1 (red), (E, H) astrocytes were stained with anti-GFAP (green), and (F, I) oligodendrocytes were stained with anti-GalC (green). Cells nuclei were stained with DAPI (blue). Images were collected using an inverted fluorescence microscope (A–F) and a confocal microscope (G–I). Scale bar is 200 μm in images (A, B) and 20 μm in images (C–I).

The amino acid sequence of the peptides used to form the nanofiber hydrogels defines the microenvironment surrounding the encapsulated cells and appeared to affect the neural stem cell fate. We observed that there was a delay in the differentiation rate of astrocytes in the non-functionalized peptide nanofiber hydrogels compared to the functionalized peptide nanofiber hydrogels. We found that 1 and 2 weeks post-encapsulation, the percentage of GFAP(+) cells were between ~15% and ~26% in the functionalized peptide hydrogels and between ~12% and ~21% in the non-modified peptide hydrogel, respectively. Furthermore, we observed better survival rates and higher numbers of astrocytes in the functionalized peptide hydrogels compared to the non-functionalized ac-$(RADA)_4$-$CONH_2$ peptide hydrogel (Fig. 5). Between 4 and 12 weeks post-encapsulation peptide hydrogels carrying the SKPPGTSS sequence contained approximately 25% more glial GFAP(+) and GalC (+) cells compared to PFSSTKT and RGDS peptide hydrogels.

Our results also show that peptide hydrogels containing the RGDS sequence, which facilitates cell adhesion to the nanofibers, did not result in significantly improved cell viability or promoted neural stem cell differentiation in 5 month neural tissue cultures. This suggests that cell adhesion, although an important trait of a biomaterial, is perhaps not the most important factor to consider for the development of an ideal neural tissue system.

Furthermore, it appears that the SKPPGTSS sequence which is found in proteins that inhibit apoptosis promotes neural cell viability and differentiation. Further work will be required to add evidence to this notion.

Contrary to the trend observed in peptide nanofiber hydrogel tissue cultures, in Matrigel, we observed increased differentiation of neural and glial cells from the first week post-encapsulation (Fig. 5). Furthermore, the decrease in the number of progenitor cells inside Matrigel was faster compared to tissue cultures performed in the peptide hydrogels. It is likely that growth factors and cytokines impurities that are present in Matrigel have a significant effect in the rate of cell differentiation and neural stem cells enter the differentiation path faster. However, biological phenomena studies must be performed in an inert material that is free of impurities. Neural stem cells embedded in Matrigel differentiated significantly even in the presence of EGF/FGF-2 growth factors that normally maintain an undifferentiated neural stem cell population.

Tissue cultures in Collagen I gel were characterized by low cell migration, cell clustering and limited neural stem cell differentiation. This may be due to the biological incompatibility between neural cells and Collagen I, a substance not found in mammalian brain tissue. Traditionally, Collagen I has been and is still used widely in neural tissue culture studies because of the lack of suitable materials. Our results provide more evidence that Collagen I is not suitable for neural tissue culture studies. Neural cells (especially differentiated neurons and immortalized neural cells) survive in Collagen I in the presence of a cocktail of growth factors. However, such studies are performed for short periods of times (i.e. up to 2 weeks) and they are characterized by low cell migration and differentiation. We believe that the peptide hydrogel may be a good material for long-term neural tissue cultures to study neural cell biology.

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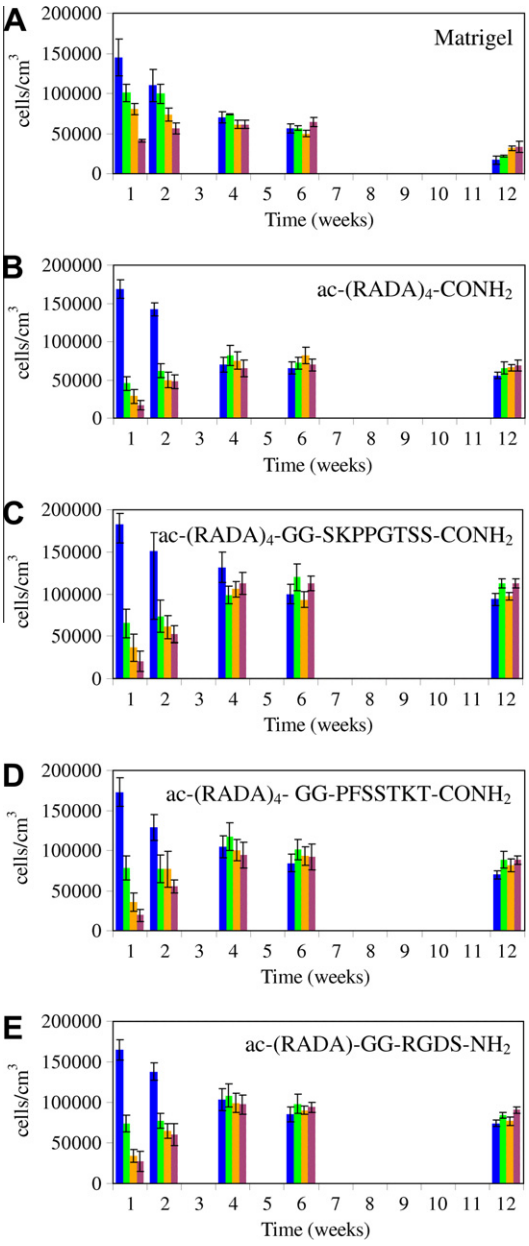

Fig. 5. Neural cell differentiation as function of time in tissue cultures performed in self-assembling peptide hydrogels and Matrigel. Color code: neural progenitor cells (blue), neurons (green), astrocytes (orange), and oligodendrocytes (brown).

4. Conclusions

3-D tissue cultures in self-assembling peptide nanofiber hydrogels represent a more realistic system compared to traditional 2-D studies and 3-D models that employ animal derived materials such as Matrigel and Collagen I. Our results show that during the first 2 weeks of culture, neural stem cells in Matrigel appear to proliferate, differentiate and form processes better than in peptide nanofiber hydrogels. However, this effect of Matrigel is limited to the initial growth period and the situation is reversed over time. When neural tissue cultures were investigated for longer periods of time, we observed better cell survival rates in peptide nanofiber hydrogels compared to Matrigel and Collagen I, suggesting that the beneficial effect of Matrigel on tissue cultures is mainly due to non-quantified growth factors and cytokines that are present in the material. It is apparent that when these chemical stimuli are removed, the material itself is not suitable to support a viable neural tissue culture. This is yet another indication of the important role of the matrix environment in inducing neural stem cells into diverse differentiation pathways.

We showed that neural stem cells can be encapsulated successfully in hydrogel matrices of self-assembling peptide nanofibers and present marked differentiation into projection neurons, astrocytes and oligodendrocytes. Our observations prompted inquiry into the functionality and mechanism of interaction between the self-assembling peptide functionalization motifs and the neural cell types in the tissue cultures. The biocompatible and non-immunogenic peptide hydrogel scaffold provided a suitable material for stem cells to proliferate and differentiate. This results in a system that allows for realistic studies of neural cell biology, including examining synapse activity in the presence of various factors such as drugs and growth factors, neural development under stress factors, and regenerative medicine to repair the insults of age, disease or injuries to the brain tissue. The peptide hydrogel biomimetic system using human cells may substitute or append to animal studies which are expensive, raise ethical concerns and often lead to conflicting data because the biology of neural cells of genetically modified animals may be different from that of the wild-type animals. The simplicity of the system and the ease of microscopic analysis of neural tissue cultures provide a fast and efficient method for in vitro investigation of neural cell biology in a biomimetic 3-D environment.

Acknowledgement

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Appendix A. Figures with essential color discrimination

Certain figures in this article, particularly Figs. 1–5, are difficult to interpret in black and white. The full color images can be found in the on-line version, at http://dx.doi.org/10.1016/j.actbio.2012.09.010.

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