A Self-Assembling Peptide Scaffold Functionalized for Use with Neural Stem Cells

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ABSTRACT

The performance of a biological scaffold formed by the self-assembling peptide RADA16 is comparable to the most commonly used synthetic materials employed in the culture of neural stem cells. Furthermore, improvements in the performance of RADA16 have recently been made by appending the self-assembling peptide sequence with various functional motifs from naturally occurring proteins.

The focus of this work is to further analyze the performance of these functionalized self-assembling peptide scaffolds when used for the culture of neural stem cells, and to characterize these newly developed materials for comparison with RADA16. The effect of the functional motifs on the structure of the peptide scaffold was evaluated with circular dichroism and scanning electron microscopy, and the mechanical properties of the peptide scaffolds were examined through rheological analysis. The functionalized peptides were found to have lower percentages of beta-sheet structure as well as reduced storage moduli in comparison with RADA16. SEM images confirmed the ability of the functionalized peptides to form three-dimensional nanofiber scaffolds capable of encompassing neural stem cells. Three-dimensional cell culture techniques were used to evaluate the ability of the functionalized peptide scaffolds to promote neural stem cell proliferation, and a scaffold formed by the combination of different functionalized peptides was found to increase the proliferation of neural stem cells in comparison to non-functionalized RADA16.

Thesis Supervisor: Shuguang Zhang
Title: Associate Director of the Center for Biomedical Engineering

Acknowledgements

It would be impossible for me to thank Dr. Shuguang Zhang enough for the opportunity he gave me to study and work in his lab at the Center for Biomedical Engineering. He is an excellent teacher and has truly created an environment conducive to innovation, collaboration and learning at his lab. I would also like to thank Dr. Fabrizio Gelain for being an excellent teacher who was always more than willing to share his expertise in cell culture and biomaterial analysis. Also, Prof. Krystyn Van Vliet played an essential role in the realization of this work, she introduced me to Dr. Zhang and was an exceptional source of guidance towards the completion of this thesis and my degree. I would also like to thank Kathy Farrell for all of her assistance and guidance during my time here.

As noted in certain areas of this thesis, parts of the material presented are from work that was done in the Zhang lab before and during my time there by Fabrizio Gelain, and I would like to say again how thankful I am to him for allowing me to take part in his work and for helping me to find my own part of this area to explore.

1. Introduction

A general description of self-assembly is the spontaneous association of numerous individual entities into a coherent organization and well-defined structure, maximizing the structural benefit of the individual entity without external instruction. This type of self-assembly is ubiquitous in nature, from schools of fish in the sea to oil droplets in water (1-2). More specifically, molecular self-assembly has been defined as the spontaneous organization of molecules under conditions of thermodynamic equilibrium into structurally well-defined and rather stable arrangements through a number of non-covalent interactions (3-5). It is this more specific definition that provides a description of the basic fundamentals behind the self-assembling process of the peptides focused upon in this work.

The following section includes a discussion of the discovery, development and application of the general class of material formed by self-assembling peptides similar to those studied here. Also included in the background section is an explanation of the inherent advantages of using this class of biomaterial for cell culture studies and tissue regeneration applications.

A detailed description of the specific functionalized peptides studied here can be found in the materials section. The results section is divided into two categories, describing separately the details of the characterization of these functionalized peptides and the evaluation of their use as substrates for the culture of neural stem cells, while the specific protocols used in these analyses are detailed in the methods section. A section

has also been dedicated to conclusions drawn from this work and possible avenues for future investigations and applications of this class of functionalized biomaterial.

2. Background

2.1 Self-Assembling Peptides

A class of biological materials made from self-assembling peptides has the potential to significantly advance the field of tissue regeneration. This class of material overcomes several of the shortcomings inherent in many of the traditional material systems employed in tissue engineering. (1,6-10)

Scaffolds formed by the type of peptides studied in this work are produced when a solution of the self-assembling peptide is exposed to physiological media or salt solution. Upon assembly, a hydrogel consisting of greater than 99% water (peptide content 1-10 mg/ml) is produced (11-13).

This general class of self-assembling peptide consists of repeating units of positively charged lysine or arginine followed by negatively charged aspartate and glutamate. These peptides are composed of repeating sequences of alternating hydrophilic and hydrophobic amino acids. Thus, the beta-sheets have distinct polar and non-polar surfaces (11-14). The first molecule of this class, EAK16-II, a 16 amino acid peptide, was found as a segment in the yeast protein zuotin, which was originally characterized by binding to left-handed Z-DNA (15). A number of additional self-assembling peptides, including RADA16 in which arginine and aspartate residues substitute the lysine and glutamate in EAK16, have been designed and characterized (16-18).

Several peptide scaffolds have been shown to support the attachment of a variety of mammalian cells, including neural cells, chondrocytes, human aortic endothelial cells, liver progenitor cells, neural cells and osteoblasts (19-28).

2.2 Advantages of Self-Assembling Peptide Scaffolds for Tissue Regeneration

Attempts have been made to culture cells in 3-D using synthetic polymers and their copolymers. However, many processed synthetic polymers consist of microfibers ~10–50 micrometers in diameter, which are similar in size to most cells (~5–20 micrometers in diameter). Thus, cells attached to microfibers are still in a two-dimensional environment with a curvature dependent on the diameter of the microfibers (29). In addition, because of their micro-scale sizes, the mechanical strength of these polymeric fibers often prevents material structural adaptations from the forces exerted by cells during their adhesion, migration and maturation processes. Furthermore, the pores (~10-200 micrometers) between the microfibers are often ~1,000–10,000 times larger than the size of biomolecules, which consequently can quickly diffuse away. Thus, although these microfibers provide an artificial extracellular environment, they are very different from the natural nanoscale ECM. For a true ECM-like 3-D environment, a scaffold's fibers and pores must be substantially smaller than the cells (29). Scaffolds formed by the self-assembling peptides studied in this work are composed of a network of nanofibers (~10-20nm in diameter) exhibiting pore sizes of 5-200nm (19-21) which surround seeded cells in a manner similar to the natural extracellular matrix.

Many synthetic scaffolds used in tissue engineering release harmful degradation products. However, the self-assembling peptide scaffolds studied in this work are formed

by, and upon degradation break down into, L-amino acids that occur naturally in the body (30). RADA16 has been shown not to elicit noticeable immune response or inflammatory reactions in animals (31).

Several types of naturally derived animal products, such as collagen-based biological scaffolds, their derivatives and bio-compatible co-polymers can be used as scaffolds for cell attachment (31). While animal derived scaffolds often provide an environment more similar to the natural ECM than synthetic scaffolds, one potential problem with all animal-derived biomaterials is that they can potentially carry dangerous pathogens (30). Although capable of forming an environment much more similar to natural ECM than most synthetic materials, the self-assembling peptide scaffolds chosen for this study are not biologically derived and contain only known constituents which are completely defined and can be modified with ease (1,7,8,10,32-34).

2.3 RADA16

The specific self-assembling peptide RADA16 repeats the sequence RADA four times. The molecule and self-assembling process are represented below (Fig. 1).

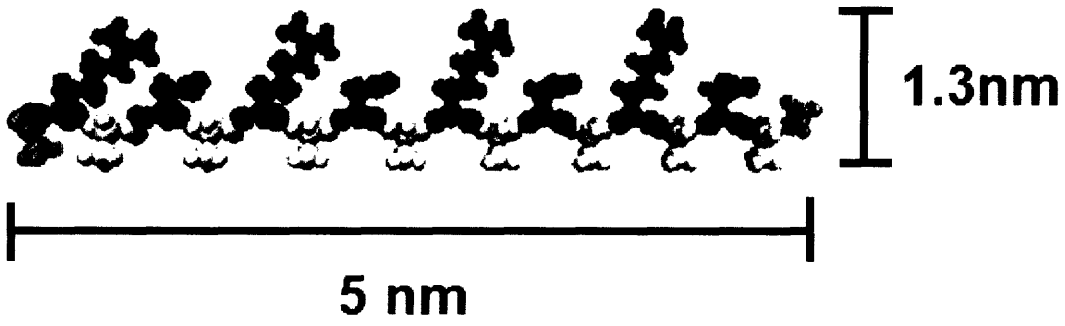


Figure 1. Molecular Model of RADA16 peptide. RADA16 is a self-assembling 16-residue peptide with an alternating sequence of basic arginine (blue), hydrophobic alanine (white) and aspartic acid (red). These peptides self-assemble once exposed to physiological pH solutions or salt solutions. The peptide is capable of self-association via hydrophobic interactions formed by the alanines as well as polar interactions between the positively charged arginines and negatively charged aspartates. (Photo courtesy of Fabrizio Gelain)

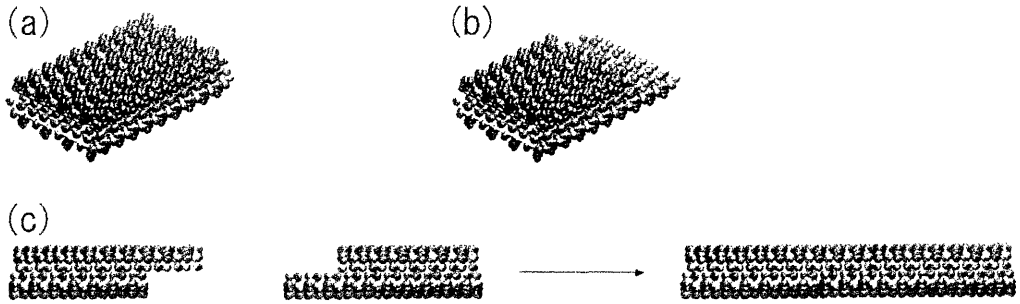


Figure 2. Proposed molecular model of RADA16 assembly. The self-assembling peptides form stable β-sheets in water through intermolecular hydrogen bonds along the peptide backbones. The β-sheets have two distinctive regions, one hydrophobic with an array of alanines (shown in green) and the other hydrophilic with negatively charged aspartic acids and positively charged arginines. (Photo courtesy of H. Yokoi)

The RADA16 peptide forms β-sheet secondary structures as the alanines pack together through hydrophobic interactions and form the interior of the structure. The exterior side

of the structure is formed by positively charged arginines and negatively charged aspartic acids as they pack together through intermolecular hydrogen bonds (14). Beta-sheet fragments can form various assemblies similar to restriction digested DNA fragments, such as blunt ends (figure 2a) and semi-protruding ends (figure 2b), which can readily reassemble (figure 2c) (35-36).

3. Methods

3.1 Peptide Solutions

All peptide sequences used were obtained from SynPep Corporation (www.synpep.com) and dissolved in distilled sterile water (Gibco) at a final concentration of 1 % (v/w) (10 mg/ml) and sonicated for 30 min (aquasonic, model 50 T, VWR).

3.2 Circular Dichroism Spectroscopy

Circular Dichroism Spectroscopy (CD) was used to determine the secondary structure of the self-assembling peptides in solution. Solutions for analysis were made by diluting 1% (w/v) stock peptide solution to a concentration of 20 μm using Milli-Q water. CD Spectra were taken from 190nm to 260nm at wavelength intervals of 1nm. CD signals were measured in millidegrees and subsequently converted to mean residue ellipticity. The measurements were performed with an AVIV 202 CD Spectrometer.

3.3 Rheometry

The mechanical properties of the assembled hydrogels were studied with a TA Instruments AR2000 rheometer. Storage moduli were measured at frequencies from 1 to 10 rad/sec using a 20mm, .5° stainless steel cone with a truncation gap of 9μm while the strain was held constant at 1%. Each measurement was performed with 35μl of gel assembled with 70μl of PBS solution after allowing 1 hour for assembly.

3.4 Scanning Electron Microscopy

After seeding neural stem cells onto the peptide scaffolds in basal medium, samples destined for SEM imaging were cultured for 7 days. The peptide matrices were removed from culture medium and dehydrated with a Tousimis Sam-Dri 1000 critical point dryer. After drying, the samples were coated with a gold-palladium target and examined using a JOEL JSM 6060 SEM at 2000-20000X magnification, 6kV acceleration voltage, 29-32 spot size, and 12 mm electronic working distance.

3.5 Cell Seeding and Culture

Cell seeding was performed two days after the last mechanical dissociation in order to seed the maximum percentage of neural stem cells. To form the three-dimensional cell seeded scaffolds, approximately 10^5 cells in 10 µl were mixed with 40 µl of 1% gel solution and then transferred to individual wells of 24-well multiplates. Each cell/peptide mixture was then assembled with 200 µl/well of basal medium. The cells were cultured for three days with basal medium supplemented with βFGF (10ng/ml). After 3 days, medium containing Leukemia Inhibitory Factor (LIF, Chemicon) (20 ng/ml) and Brain Derived Neurotrophic Factor (BDNF, Peprotech) (20 ng/ml) was used as a medium replacement.

4. Materials

Through the work of Shuguang Zhang and Fabrizio Gelain, peptide scaffolds were tailor-made to incorporate known functional motifs from extracellular matrix proteins. This section has been taken from the descriptions of these materials written by Fabrizio Gelain (21). In order to incorporate functional motifs into the self-assembling peptide sequence, a simple strategy was utilized for an easily reproducible method with potential for widespread use. Functional motifs were directly linked to self-assembling peptide sequences through automated solid phase peptide synthesis. This method is a conventional and well-known synthetic technique, and depending on the length of the sequences, peptide samples of very high purity can be produced at a reasonable cost (37-38).

A schematic representation of the general process is illustrated below. Blue lines represent self-assembling peptide sequences, and the red, green, purple, yellow and brown lines represent various functional peptide motifs. Once electrostatic repulsions are screened by electrolytes, the molecules assemble into nanofibers by hydrogen bond formation and through hydrophobic interactions caused by the unfavorable contact among hydrophobic segments and water molecules. These nanofibers bundle to form 3D networks and produce a gel-like material. Thus, the nanofibers that form around cells in 3D present the functional epitopes at an artificially high density relative to a natural extracellular matrix. Although it is not expected that all of the epitopes will participate in receptor binding, this type of scaffold should be a good vehicle for intense signal presentation to cells in 3D (39).

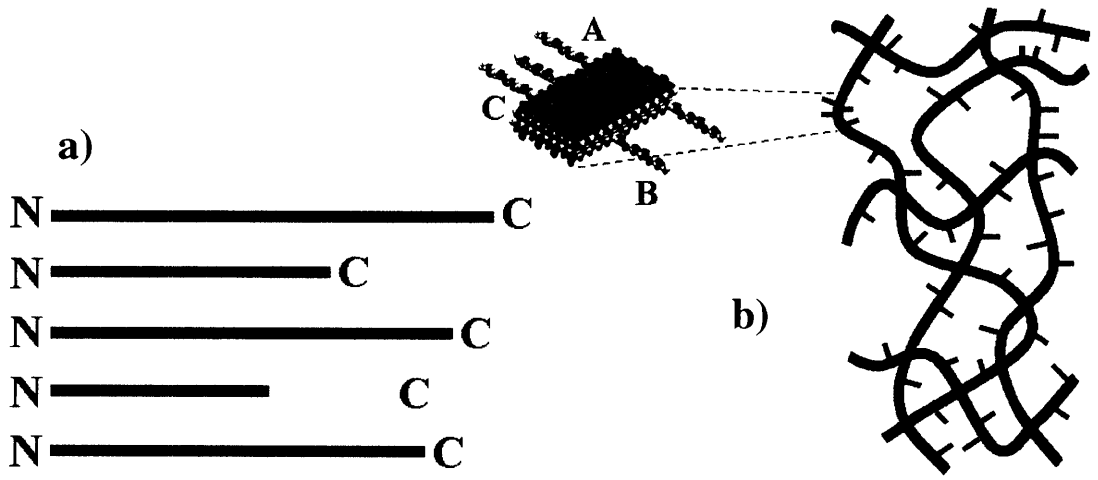


Fig. 3 - (a) Direct extension of the functional motifs from the self-assembling peptides. The blue lines represent the self-assembling portion of the peptide sequence and the red, green, purple, yellow and brown lines represent various functional motifs. (b) Schematic representation of a self-assembling peptide scaffold attached with various functional motifs. Either single or multiple functional motifs, labeled as **A, B & C**, can be mixed together. The density of these motifs can be easily adjusted by simply mixing them in various ratios, 1:1-1,000,000 or more, prior to the assembling step. (Photo courtesy of Shuguang Zhang)

One possible problem that can be envisioned is that by extending the self-assembling peptides with functional motifs that do not contribute to the self-assembly process, a disruption in the structure of the assembled peptide network may occur. This disruption is examined in the section of this work dedicated to the characterization of these functionalized scaffolds.

The self assembling peptide RADA16-I was functionalized with fifteen different motifs from various types of collagen (40-42), laminin (42), fibronectin, osteopontin (43), osteogenic peptides (44), bone marrow homing (BMH) (45) and regulatory peptides (46). However, only the four peptides of greatest interest were studied in this work.

In the following figure, all of the sequences discussed above are represented with a corresponding number for to simplify the subsequent discussion. The RADA16 portions of the sequences are represented with the alternating basic (blue), hydrophobic (white) and acid (red) residues necessary to guarantee the self-assembling property of the functionalized peptides. Two neutral hydrophobic spacers (glycine) are positioned between RADA16 and each functional motif. Neutral polar residues are drawn in green.

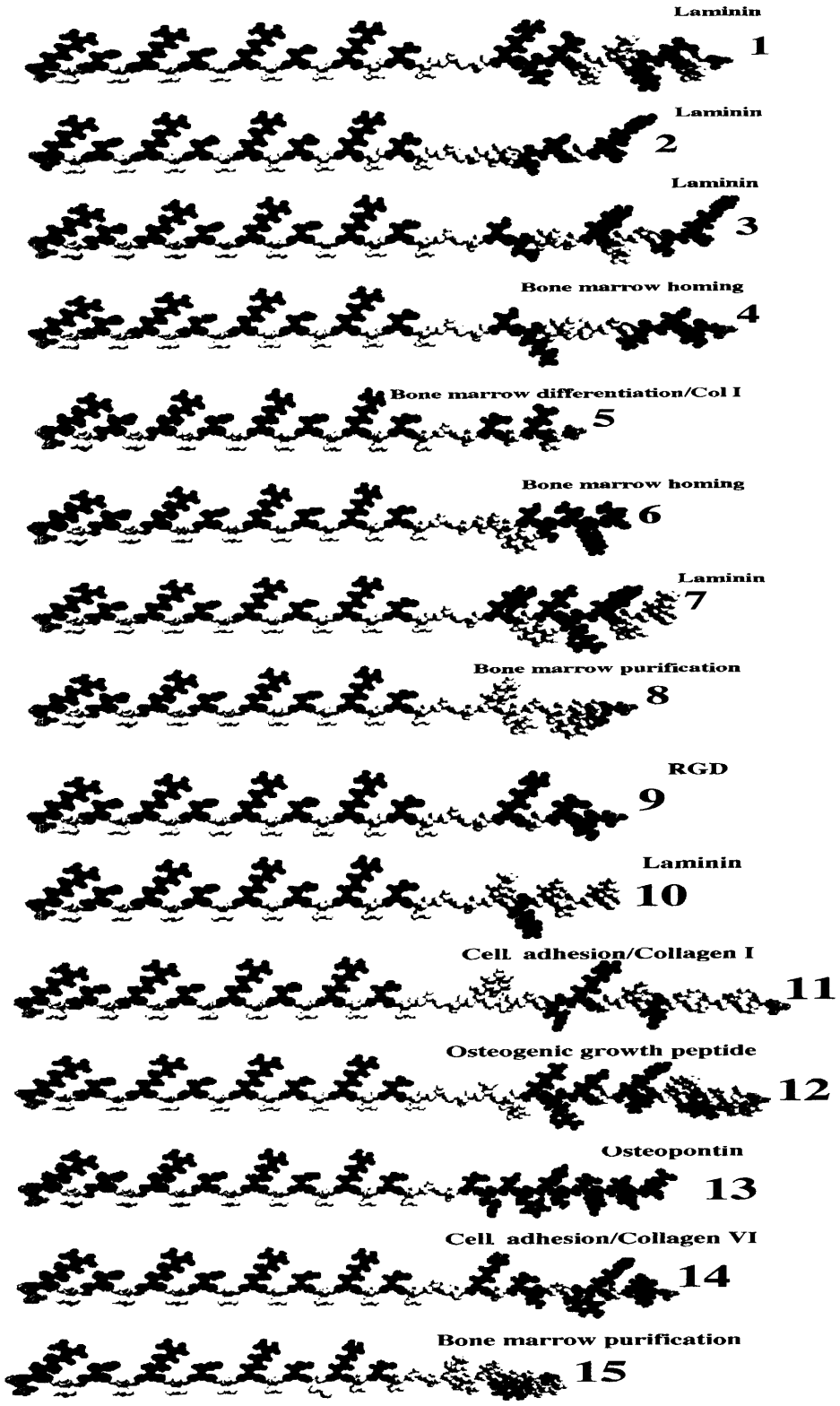

Laminin
1
Laminin
2
Laminin
3
Bone marrow homing
4
Bone marrow differentiation/Col I
5
Bone marrow homing
6
Laminin
7
Bone marrow purification
8
RGD
9
Laminin
10
Cell adhesion/Collagen I
11
Osteogenic growth peptide
12
Osteopontin
13
Cell adhesion/Collagen VI
14
Bone marrow purification
15

Fig. 4 – Direct extension of RADA16 peptide sequence with 15 different functional motifs. The alternating basic (blue), hydrophobic (white) and acid (red) residues represent the RADA16 portion of the sequences. Two neutral glycine residues are placed between the RADA16 sequence and the functional motifs as spacers to guarantee flexibility of the functional sequences for improved accessibility to cell membrane receptors. Neutral polar amino acids are marked in green. (Photo courtesy of Fabrizio Gelain)

Through previous analysis in the Zhang laboratory, these peptides showed various chemical and physical properties due to the different molecular structure of each functional motif. Gelain showed that "Peptides 1, 7 and 15 were found to be insoluble in water at room temperature, and remained insoluble even after exposing the peptide suspension to changes in pH (1-12) and temperature (from 4° to 70°C), and were therefore not tested. Peptides 12 and 13 were not capable of self-assembly at concentrations from 1% to 4%(w/v), so they were excluded from any further investigation. The mechanical strengths of the assembled peptides were also noticeably different: relatively weak gels were formed by peptides 4, 6, 11 and 14; gels with a stiffness comparable to RADA16 were formed by peptides 2, 3, 5, 9 and 10; and an extremely viscous solution before assembly and the stiffest gel after assembly resulted from peptide 8. By increasing or decreasing the concentration of peptide solutions, it is possible to vary the mechanical strength of the assembled gels." (21)

A previous study (21) conducted by Fabrizio Gelain tested the ability of the above functionalized peptides to promote neural stem cell proliferation and differentiation found that peptides 4 and 6, the two peptides incorporating bone marrow homing motifs, showed the largest increases in cell proliferation and differentiation when compared to RADA16. For this reason, peptides 4 and 6 are focused upon in the characterization portion of this work. Peptide 8 was also studied due to its increased mechanical strength and potential for combination with the weaker peptides 4 and 6. Results are shown below (Fig. 5)

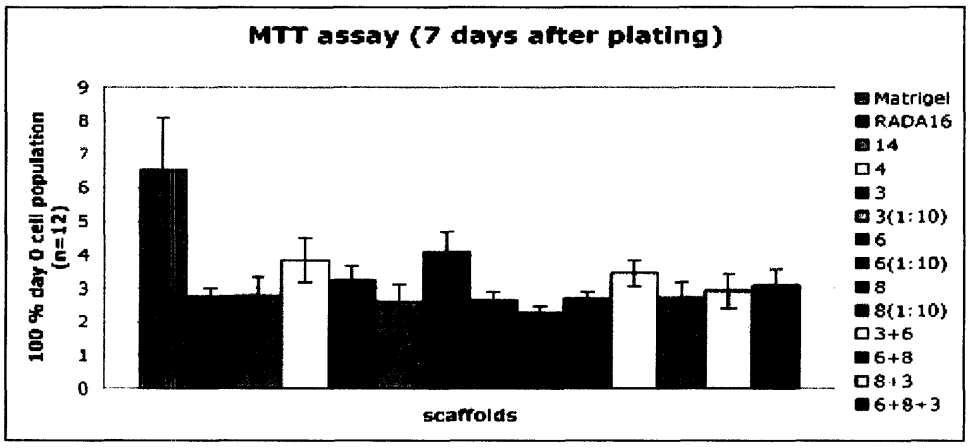


Fig. 5. Increase of NSC proliferation 7 days after plating. Results are expressed as percentage of the initial population seeded. Peptides number 4 and 6 show the most significant improvement over RADA16 after 7 days of culture. (Photo courtesy of Fabrizio Gelain)

5. 3-D Culture

Many factors exist that are likely to contribute to the superior performance of Matrigel in Figure 5. One obvious difference between Matrigel and the functionalized peptides is a large difference in the mechanical stiffness of the scaffolds formed by these materials. With this in mind, mixtures of the relatively stiff peptide 8 with the relatively weak peptides 6 and 4 were formulated in an attempt to create a scaffold with mechanical properties as similar to Matrigel as possible while still incorporating the most beneficial motifs. In addition, by incorporating two functional motifs into the same scaffold, the potential for interactions between distinct sets of cell integrins and the scaffold should increase. It has previously been shown that mixtures of multiple functionalized peptides are still capable of self-assembling into scaffolds (23). The results below show that mixtures of peptides 4 and 6 with petide 8 increase cell proliferation in comparison with RADA16 alone and RADA16 mixed with peptides 4 and 6. The following mixtures were composed of equal amounts of each peptide at a total peptide concentration of 1% (w/v).

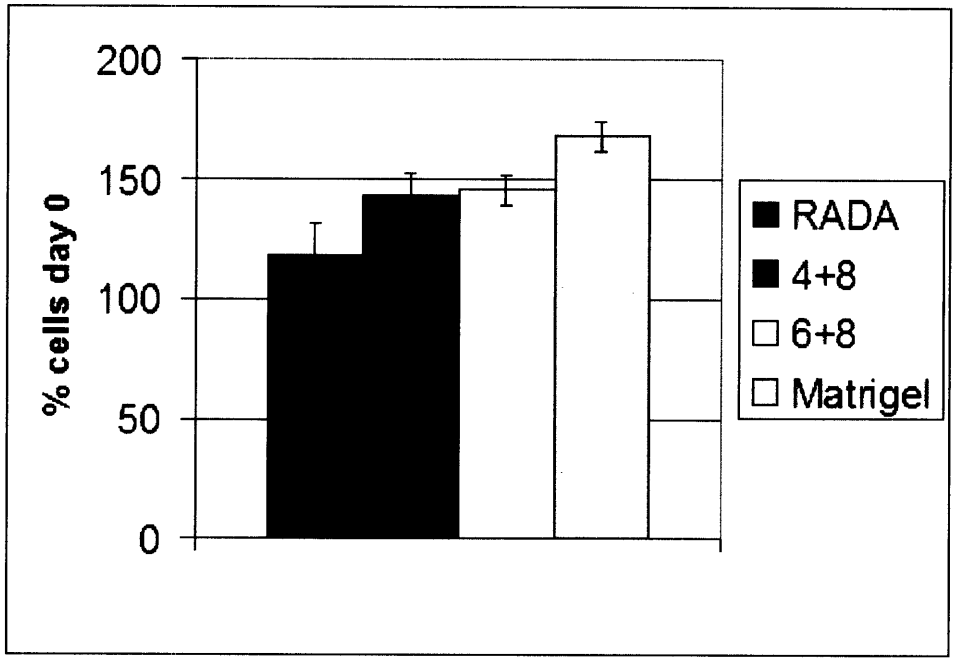


Figure 6 Effect of mixing Peptides 4 and 6 with either RADA16 or Peptide 8. It was found that mixing peptides 4 and 6 with peptide 8 led to increased cell proliferation when compared with mixtures of RADA16 alone. Matrigel was used as a positive control, and mixtures of the functionalized peptides showed increased strength imparted by peptide number 8.

6. Circular Dichroism

CD spectra were obtained for each peptide sequence to establish the presence of β-sheet secondary structure. A typical spectrum for β-sheet structures with a minimum mole residue ellipticity (deg cm^2/decimole) at 217 nm and a maximum at 195 nm was observed for the peptides studied (Fig. 7a). The addition of the functional peptide sequences reduces the β-sheet content, as shown by a decrease in the intensity of mole residue ellipticity at 217 nm. Using the estimation method of Greenfield and Fasman (47), the percentages of β-sheet and random coil content were calculated. These calculations assume that there are no other secondary structures such as α-helices present.

	Mean Molar Ellipticity per Residue at 217nm	% β-Sheet	% Random Coil
RADA	-10296.88	64.77	35.23
#4	-6788.46	49.52	50.48
#8	-6136.25	46.68	53.32
#6	-6010.40	46.13	53.87
β-sheet Standard	-18400.00	100.00	0.00
Random Coil Standard	4400.00	0.00	100.00

Table 1 Calculated percentages of peptide secondary structure based on comparison of spectra minima at 217nm. Reference standards taken from Greenfield and Fasman (47).

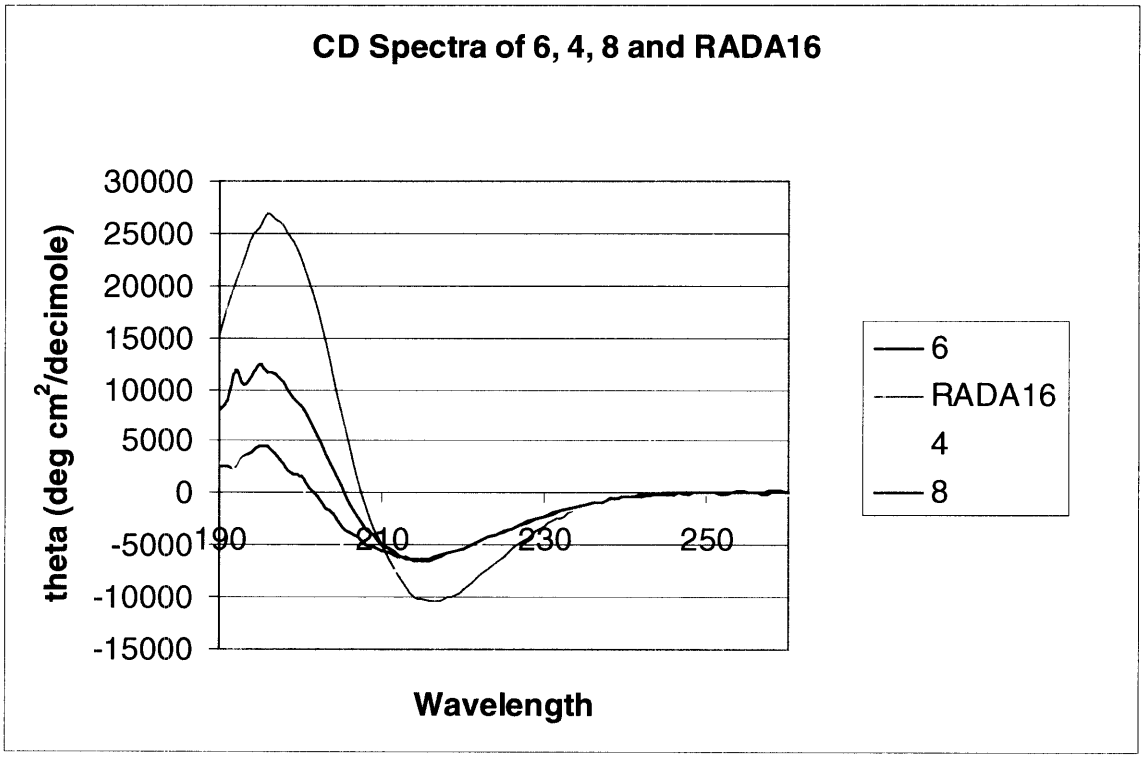


Figure 7. (A) Circular Dichroism Spectra of Peptides 6, 4, 8 and RADA16. The spectra of functionalized peptides show characteristic β-sheet structures, but it can be seen that extending the RADA16 sequence with functional motifs causes a decrease in total β-sheet formation.

7. SEM Imaging of Cells Cultured on 3D Scaffold

Upon incorporation of functional motifs to the RADA16 peptide, one concern was that the appended portion of the peptides could inhibit nanofiber formation. To address this concern, scanning electron microscopy (SEM) was used to study the nanofiber structure of the functionalized peptide scaffolds. The scaffolds of the functionalized peptides after self-assembly in PBS and cell culture medium were found to form structures with nanofibers and nanopores similar to those previously reported for RADA16 (19-21).

To examine cell-scaffold interactions, SEM was used to image the cell-seeded scaffolds after seven days in culture. It was found that cells were embedded in the functionalized nanofiber scaffolds (Figure 8), and that the scaffolds appear to wrap cell bodies in a three dimensional manner similar to the natural ECM environment. As can be seen from the images, intimate interactions are formed between the cells and the surrounding scaffold. Such interaction may not be likely on typical 2-D coated surfaces commonly used in cell culture. Images showing specific cell processes that grew during culture show that the processes also appear to be wrapped by the nanofibers, suggesting that cells may have the ability to remodel the surrounding peptide nanofibers.

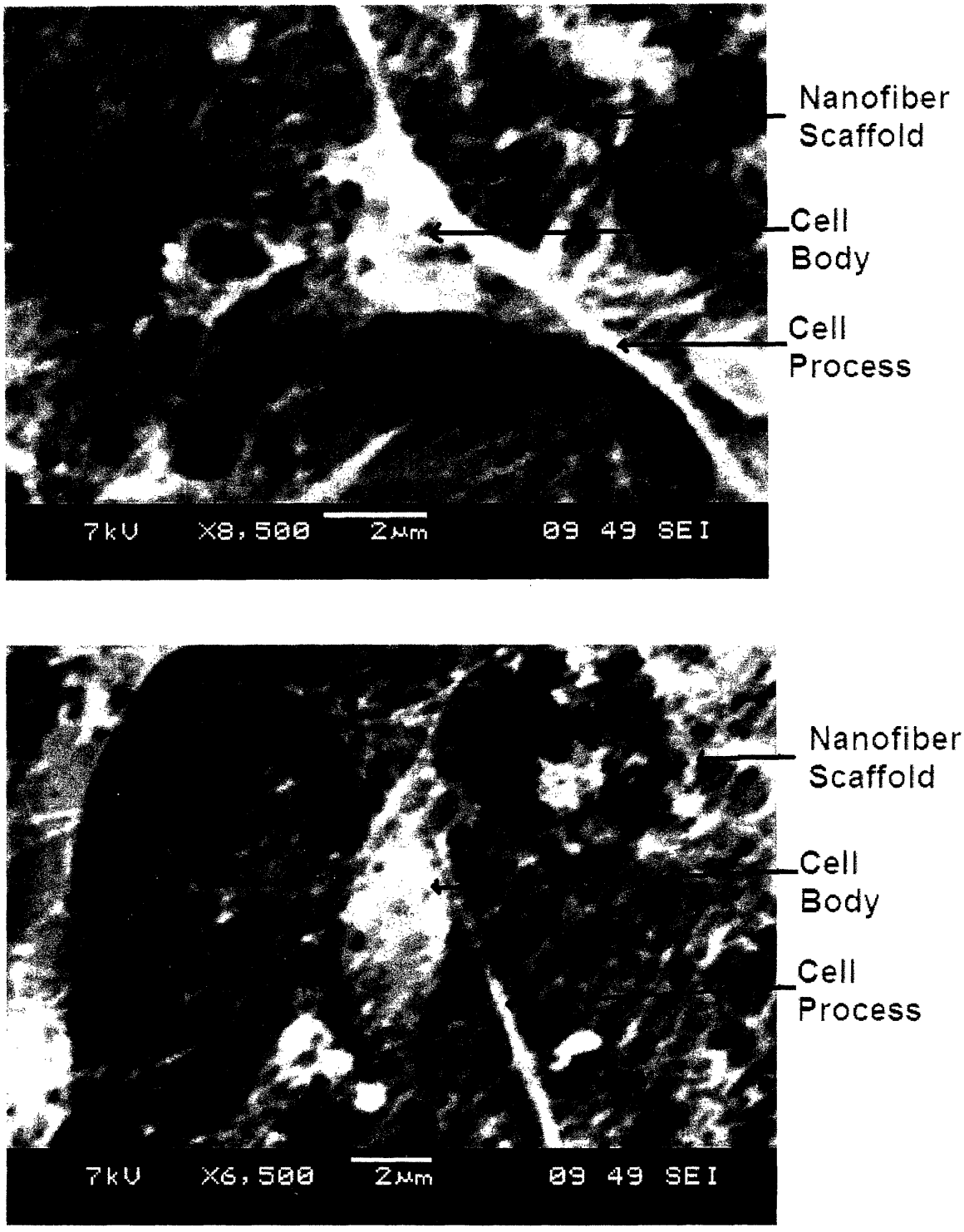
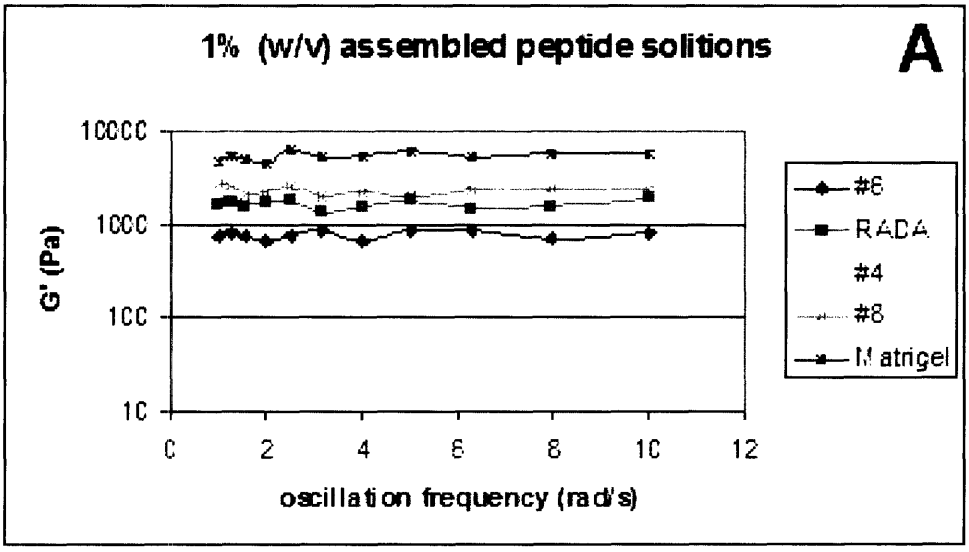


Figure 8 SEM images of neural stem cells embedded in the 3-D scaffold of peptide 6 (1% v/w). Arrows point to cell bodies and processes and the surrounding scaffold.

8. Rheology

Rheology was used as a method for comparison of the relative mechanical stiffness of the assembled peptides. The storage moduli (G') were measured at low frequencies where the loss moduli (G'') were too low to be measured. Therefore, the average G' over the frequency range of 1 to 10 radians/sec is used to compare the relative stiffness of the scaffolds. This information is useful in describing the type of mechanical environment that seeded cells are presented with and can serve as a macroscopic measure of the extent of interactions formed between assembled peptides.

The functionalized peptides were generally found to exhibit reduced storage moduli when compared to RADA16 (G'=1630Pa), however, peptide number 8 (G'=2320Pa) showed an increase in stiffness. This is perhaps due to the relatively high degree of hydrophobicity of the added motif (illustrated in Fig. 3). The peptides of greatest interest, numbers 4 (G'=762Pa) and 6 (G'=53.5Pa), show reduced storage moduli in comparison with RADA16. All of the assembled peptides exhibit relatively low storage moduli in comparison with Matrigel (G'=5408Pa), which was used as a positive control in the cell culture studies.



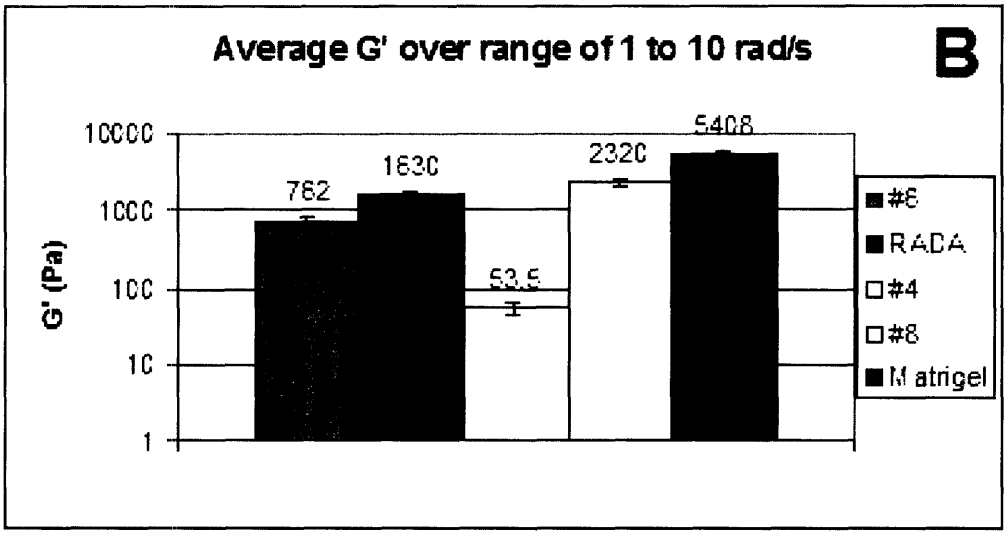
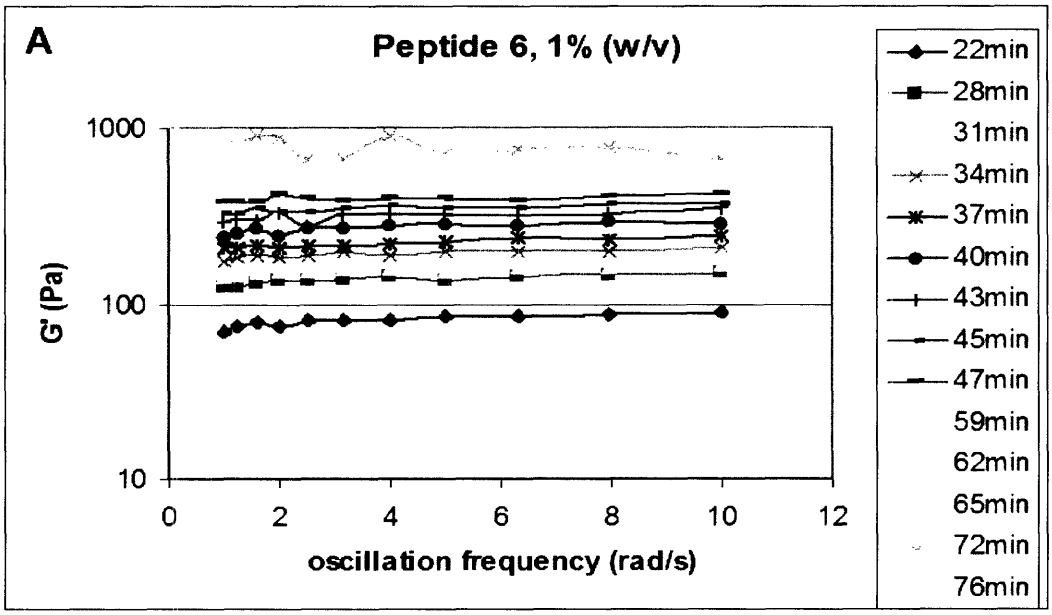


Figure 9 Comparison of the Storage moduli of the functionalized scaffolds with RADA16 and Matrigel. Peptides 6 and 4 formed gels with lower storage moduli than RADA16 while Peptide 8 was found to have a higher storage modulus than RADA16. All peptide scaffolds were found to have a lower storage moduli than Matrigel.

The time required for the self-assembly process to occur was also examined through rheological analysis. Peptide 6 was found to have a fairly linear increase in storage modulus with time for approximately one hour, at which point the G' values stabilized at 762 Pa.



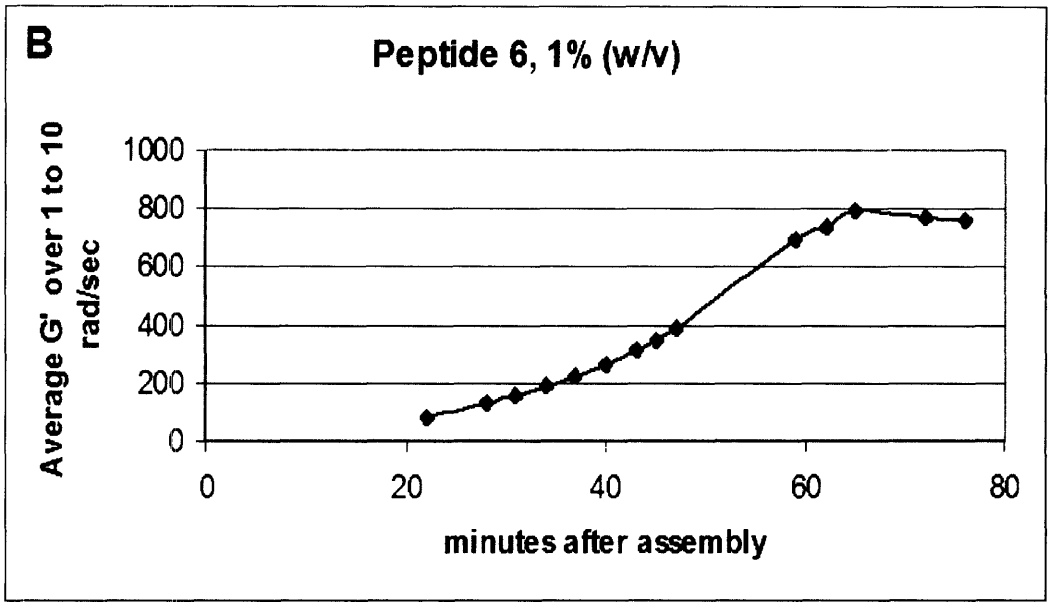


Figure 10 Kinetics of the self-assembling process. The storage modulus of a gel formed by peptide 6 was measured at varying time intervals after exposure to assembling solution. Approximately one hour of assembly time was required for the storage modulus to stabilize.

9. Future Considerations

Several other options exist that may be capable of improving the performance of this class of biomaterial. Based on the improvements in scaffold performance seen by appending functional motifs to self-assembling peptides, it should also be possible to improve performance by directly linking growth factors and other cell-signaling agents to the RADA16 sequence. Self-assembling peptides directly linked to growth factors could be fabricated with recombinant DNA technology. Elevated and sustained levels of growth factors such as LIF (Leukimia Inhibitory Factor), which has been shown to promote neuronal and glial cell population maturation in NSC progeny (48), could be presented to cells by directly linking complete proteins to the self-assembling sequence.

Only two of the many possible combinations of the functional peptides have been tested, and many more functional motifs exist for the synthesis of additional functionalized peptides. Also, the influence that the mechanical properties of the scaffolds have on NSC proliferation and differentiation has yet to be determined. In all likelihood, in order to simulate the natural ECM environment as precisely as possible, the optimal combination of functionalized peptides will include a very large number of functional motifs.

One of the ultimate applications for this class of functionalized material could be for the repair of spinal cord injury. With this application in mind, the capability of aligning the scaffold nanofibers in order to direct nuerite growth could be very useful, and the possibility of this represents another area for feasible future investigations.

Part of the results of this work may be used in an effort to create a self-assembling peptide that better approximates the ability of matrigel to support cells. The large difference in mechanical properties seen between the functionalized peptides and matrigel should account at least in part for the differences in cell performance seen on these materials. As was seen with peptide 8, by incorporating large hydrophobic regions into the sequences, mechanical properties can be enhanced. This causes a decrease in the peptide solubility necessary for their application, but finding the optimal degree of hydrophobicity to incorporate without creating insoluble peptides could be useful. Unfortunately, the production price of these peptides rises sharply with increases in length. It may be possible to keep length down by reducing the self-assembling sequence from four repeats of RADA to just two repeats, but again optimization for solubility purposes will be important.

10. Conclusions

Self-assembling peptides form a novel class of biomaterial with applications for tissue regeneration. By incorporating functional motifs from natural ECM proteins into the self-assembling peptide sequence, it is possible to improve the survival and proliferation of cells seeded in this class of biomaterial. In particular, neural stem cells exhibit increased proliferation when seeded onto a scaffold of RADA16 that had been functionalized with bone marrow homing peptide motifs. While the addition of these motifs does decrease the percentage of β-sheet secondary structure, the ability of the functionalized peptides to form a nanofiber scaffold is not lost. The incorporated motifs were found to significantly alter the mechanical stiffness of the scaffold, but through combinations of different peptide mixtures and modification of peptide concentration, the difference in mechanical properties can be corrected.

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