Peptide-modified alginate surfaces as a growth permissive substrate for neurite outgrowth

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Abstract: Different strategies are being investigated for treatment of spinal cord injuries, one of the most promising being application of neurotrophic factors, which have been shown to prevent neuronal death and stimulate regeneration of injured axons. *Ex vivo* gene therapy has emerged as the leading delivery method at the site of the injury, and we have shown previously that encapsulating genetically engineered fibroblasts in an immunoprotective alginate capsule can permit implantation of the factor-secreting cells without need for immunosuppression. This strategy could be greatly enhanced by providing the sprouting neurons with a permissive substrate upon which to attach and grow. We report here studies on the modification of an alginate gel surface by either coating it with laminin or by covalent attachment of YIGSR peptide. Using NB2a neuroblastoma cells, we found that native alginate elicited minimal cell attachment (~1.5%); however, YIGSR-alginate conjugate elicited a five-fold increase in numbers of cells attached using peptide ratios of 0.5 and 1 mg/g alginate, ranging from 9.5% of the cells at the lower ratio, to about 44% at the higher. Only a further 19% increase was obtained at an increased peptide density of 2 mg/g alginate (~63% over control). Laminin-coated gels showed ~60% cell attachment. However, laminin coating did not stimulate differentiation and neurite growth, whereas both numbers and lengths of outgrowths increased with increasing peptide density on peptide-modified alginate. We demonstrate here the ability of the peptide-modified alginate gels to allow adhesion of NB2a neuroblastoma cells and to promote neurite outgrowth from these cells when attached to the peptide-modified alginate surface. Also, we show that the adhesion of NB2a neuroblastoma cells and neurite outgrowth from the attached cells is a function of the peptide density on the gel surface. © 2004 Wiley Periodicals, Inc. J Biomed Mater Res 71A: 191–200, 2004

Key words: spinal cord repair; alginate gel; YIGSR peptide modification; growth-permissive

INTRODUCTION

Alginate is a naturally occurring linear polysaccharide extracted from brown seaweed. It is composed of 1–4 linked α-L-guluronic (G) and β-D-mannuronic (M) acid residues. Alginate can form hydrogels by reaction with divalent cations such as Ca^{2+}, Ba^{2+}, Sr^{2+}, and more, with the exception of Mg^{2+}. Trivalent cations such as Al^{3+} and Fe^{3+} have also been used. The preparation of these hydrogels simply involves dropping a sodium alginate solution into a solution that provides the crosslinking cations, and can be performed under very mild aqueous conditions and with nontoxic reactants. This makes it a very attractive choice as a matrix for the encapsulation of biologicals, including drug-containing liposomes and cells. Liposomes encapsulated in alginate have been studied for protein delivery,[1–3] and several different cell lines including pancreatic islets[4] and genetically engineered fibroblasts[5,6] have been encapsulated in alginate for therapeutic applications. In recent years, alginate has been investigated for use as a scaffold in tissue engineering.[7] Covalently crosslinked freeze dried alginate hydrogels have been investigated for their effectiveness in wound healing,[8,9] and as bridges for resected peripheral nerves[10,11] and the transected spinal cord.[12,13] Alginate hydrogels with covalently coupled peptides have been studied as synthetic extracellular materials[14,15] and as a tissue bulking agent.[16]

Approximately 11,000 people suffer from spinal

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cord injuries every year in the United States alone. These injuries result in a need for lifelong care and rehabilitation, and the estimated costs for a quadriplegic patient's lifetime has been estimated to exceed $1 million.[17] The devastating effects of spinal cord injuries are due to the death of neurons that cannot be replaced, the inability of the surviving neurons to regenerate their axons, and an inhospitable environment produced by the injury. Several different strategies are being investigated for treatment of spinal cord injuries, one of the most promising ones being application of neurotrophic factors that have been shown to prevent death of neurons and stimulate the regeneration of injured axons.[18] Systemic administration is not effective because of the inability of the neurotrophic factors to cross the blood–brain barrier. *Ex vivo* gene therapy has emerged as the leading strategy for delivering these factors effectively at the site of the injury.[19] We have previously shown that fibroblasts that are genetically engineered to produce brain-derived neurotrophic factor (BDNF) and encapsulated in unmodified alginate in a capsule system continued to express BDNF and also induced sprouting of neurons at the site of the injury without need of immune suppression.[5] *In vivo*, these capsules were demonstrated to significantly improve recovery in a rat spinal cord injury model (unpublished results). However, a strategy of delivering therapeutic products from encapsulated cells could be greatly enhanced by also providing the sprouting neurons with a permissive substrate upon which to grow. Cell adhesion plays a critical role in several cellular functions including migration, proliferation, differentiation and apoptosis.[14] Because cells do not interact naturally with the alginate used in our studies, we investigated *in vitro* the possibility of creating a growth permissive surface for the BDNF-delivering microcapsules in an effort to produce a prototype that would enhance the regeneration of the axons in the injured spinal cord. In these proof-of-concept studies, the fibroblast cells used in the *ex vivo* gene therapy studies were omitted for clarity. The system will eventually contain both modified alginate and BDNF-secreting cells.

Various reports using hydrogels to enhance neuronal growth have appeared in the literature.[20–22] For example, Marchand et al.[20] showed spinal axons grow into a collagen matrix implanted between the stumps of a transected spinal cord, but rapid denaturation required the collagen hydrogel to be crosslinked, and in fact, the resulting improved mechanical properties both modified the normal scarring process, and favored axonal regeneration. In another study,[21] peptide (RGD) and aminosugar-modified hydrogels of N-(2-hydroxypropyl) methacrylamide (HPMA) showed increased adhesion properties with host neural tissue, were vascularized, and were infiltrated by host nonneuronal cells compared to unmodified (control) hydrogels that showed no cellular infiltration or axonal growth. Axonal regeneration has also been shown in Schwann cell-seeded matrige-guidance chanels.[22] Further, studies have appeared that show that the deformable nature of the substrate is also important, for example, glia did not survive on deformable substrates even though the chemical environment was permissive for their growth, while neurons grown on softer substrates formed more than three times as many branches as those grown on stiffer gels.[23] Other strategies have employed guidance channels fabricated from biodegradable polymers with elastomeric properties, for example, the reported use of poly[glycolide-*co*-(ε-caprolactone)]-diol and crystallizable blocks of poly[(*R*)-3-hydroxybutyric acid-*co*-(*R*)-3-hydroxyvaleric acid]-diol (PHB).[24]

Laminin is an extracellular matrix protein found in basement membranes and is both a structural component as well as a biologically active one. The laminin molecule (MW ~850,000) is a cross-shaped structure with three short arms and one long arm. Laminin consists of three distinct chains called A, B1, and B2. Several studies have shown that laminin enhances axonal growth *in vitro*.[25,26] Several domains and amino acid sequences from laminin that are believed to promote neurite outgrowth have been isolated such as the heparin binding domain,[27] P1543 peptide,[28] YIGSR peptide,[29] IKVAV peptide,[29] and the p20 peptide.[30] *In vitro* studies using these domains and peptides involved attaching them on glass coverslips via aminosilanes,[31] or on gold-coated films of fluoropolymers such as PCTFE.[32] The oligopeptide domain CDPGYIGSR covalently linked to an agarose gel has been shown to be a successful substrate for 3D neurite outgrowth from dorsal root ganglia (DRG) *in vitro* and in a transected rat dorsal root model *in vivo*.[33] Subsequently, studies have been reported on the safety and effects on nerve regeneration of these peptides *in vivo* by Itoh et al.,[34] who describe the use of collagen fibers coated with the YIGSR peptide to demonstrate their ability to promote peripheral nerve regeneration *in vivo*.

We studied the modification of the gel surface by either coating it with laminin or by covalent attachment of YIGSR peptide to the carboxylic acid groups on the alginate. We demonstrate here the ability of the peptide-modified alginate gels to allow adhesion of NB2a neuroblastoma cells and to promote neurite outgrowth from these cells when attached to the peptide-modified alginate surface. Also, we show that the adhesion of NB2a neuroblastoma cells and neurite outgrowth from the attached cells is a function of the peptide density on the gel surface. The model cell line was chosen because it has been demonstrated that NB2a cells treated with RA and db cAMP are a model

system for the study of distinct stages of differentiation; because the cells respond to laminin (and therefore will have receptors) and because of the familiarity of use of this robust line in our group.[35–37]

MATERIALS AND METHODS

Materials

Manugel® DMB, a high G-content alginate was a gift from ISP Alginates, (San Diego, CA). YIGSR peptide was obtained from American Peptide Company Inc. (Sunnyvale, CA). Morpholinoethanesulfonic acid sodium salt (MES), 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) and N-hydroxysulfosuccinimide (sulfo-NHS), dibutyrylcyclic AMP (db cAMP) were from Sigma Chemical Company (St. Louis, MO). Natural mouse laminin and serum replacement were purchased from Gibco BRL (Rockville, MD). Dulbecco's Modified Eagle's Medium (DMEM), Fetal Bovine Serum (FBS), L-glutamine, trypsin, and Hank's Buffered Salt Solution (HBSS) were obtained from Fisher Scientific (Fair Lawn, NJ). All other chemicals were from Sigma Chemicals, and were reagent grade. Six-well culture plates were from Falcon, Becton Dickinson Labware (Franklin Lakes, NJ).

Preparation of YIGSR–alginate conjugate

YIGSR peptide was covalently attached to the alginate using an adaptation of aqueous carbodiimide chemistry,[38] resulting in the formation of an amide bond between the carboxylic acid groups of the alginate and the amine terminus of the peptide. Alginate was dissolved in MES buffer (0.1 *M* MES, 0.3 *M* NaCl, pH 6.5) to obtain a 1% (w/v) solution. EDC was added to activate the carboxylic acid groups of the alginate. The amount of EDC added was such that 5% of the carboxylic acid groups of the alginate are activated (50 mg EDC/g alginate). This was followed by the addition of sulfo-NHS in the molar ratio 1:2 to EDC (28 mg sulfo-NHS/g alginate). The solution was stirred for 15 min to allow the activation of the carboxylic acid groups, following which the appropriate amount of YIGSR peptide was added. The conjugation reaction was allowed to proceed for 24 h at room temperature under gentle stirring. The reaction mixture was then dialyzed for 4 days against about 20 liters of deionized water to remove buffer salts, reaction byproducts, and unreacted peptide using Spectra/Por dialysis tubing (MWCO 3500). The purified YIGSR-alginate conjugate solution was transferred to 50 mL polypropylene tubes, and lyophilized. The final fibrous product was then stored in airtight tubes at −20°C for future use.

Preparation of YIGSR–alginate discs

Alginate gels were prepared in the form of discs (to provide flat surfaces) using a $CaSO_4$ slurry method as described by Rowley et al.,[14] with some modifications. A 1% (w/v) solution of was prepared in deionized water containing 0.2% (w/v) sodium metaphosphate. The solution was sterilized by filtration through a 0.45-μm syringe filter. The sterilized alginate solution (5 mL) was transferred to a 50 mL conical tube and 100 μL of $CaSO_4$ slurry (0.4 g/mL in deionized water) was added. The contents of the tube were shaken vigorously to ensure complete mixing of the alginate and the $CaSO_4$ slurry. The mixture was then poured into three wells of a six-well plate and allowed to gel for 2 h, after which they were ready for use. Alternatively, the mixture was poured into a 50-mm crystallization dish and allowed to set. Following complete gelation in about 2 h, small discs (about 5 mm diameter × 2 mm thick) were punched out of the gels using the wider end of a sterile pasteur pipette and the small discs were used for further experiments.

Culture of NB2a neuroblastoma

NB2a neuroblastoma cells were a gift from Dr. Thomas Shea at the University of Massachusetts, Lowell Campus. NB2a were cultured in 10-cm culture dishes in DMEM containing 10% FBS and 2 m*M* L-glutamine. The cells were passaged at about 75% confluency.

Adhesion of NB2a on YIGSR–alginate

The adhesion of NB2a cells on YIGSR-alginate gels prepared in the wells of a six-well plate was studied with varying amounts of peptide used in the preparation of the conjugate. NB2a cells were harvested at approximately 75% confluency using 0.25% trypsin, counted using a haemocytometer and resuspended in serum-free medium containing DMEM + 10% serum replacement + 2 m*M* L-glutamine. The gels were then seeded with 100,000 cells in 5 mL of serum-free medium and placed in an incubator at 37°C and 5% CO_2. After 24 h, the gels were washed with (HBSS) to remove any unattached cells. The gels were transferred to 15-mL conical tubes and treated with 3 mL of 0.25% trypsin for 10 min to detach the cells from the alginate surface. Standard growth medium (7 mL) was added and the medium containing the cells was transferred to counting vials followed by dilution to 20 mL with HBSS. The cells were counted using a Coulter Multisizer II (Coulter Electronics Ltd., England) with a 100-μm orifice and a manometer volume of 100 μL.

Alternatively, three small discs, prepared as described above were placed in each well of a six-well plate and seeded with 100,000 cells in 5 mL of serum-free medium. After 24 h, the discs were washed with HBSS, placed in counting vials, and treated with 1 mL of 0.25% trypsin for 10 min followed by a 30-min treatment with 10 mL of 10% (w/v) EDTA to dissolve the alginate discs. The cells were then counted using the Coulter Multisizer.

Differentiation of NB2a on YIGSR–alginate

NB2a cells were also studied for their ability to differentiate on the modified alginate surfaces. After 24 h of seeding

with NB2a cells, the gels were washed with HBSS to remove any unattached cells and 5 mL of differentiation medium (DMEM + 0.1% FBS + 10 μM db cAMP) was added to each well. The differentiation medium was changed every day to replenish the db cAMP supply. The differentiation of the cells was monitored visually by observation under an inverted microscope and images were recorded using an Olympus DP-11 digital camera.

In all the adhesion and differentiation experiments, unmodified alginate gels served as the negative control and alginate gels coated with a 25 μg/mL solution of laminin for 1 h served as the positive control.

Image analysis

The digital images were analyzed using Scion Image 4.02 (Scion Corporation, Frederick, MD). The lengths of neurites developed were measured for 10 randomly selected cells for each peptide density after 7 days of differentiation. Data were analyzed using one-way ANOVA with a significance level of 0.05. Analysis was performed using Microcal™ Origin™ software by Microcal Software Inc. (Northampton, MA).

RESULTS

We studied the adhesion and differentiation of NB2a cells on alginate surfaces that were coated with laminin or on gels that were prepared with alginate with YIGSR peptide conjugated to it. Laminin coating was used as a positive control, but is considered to be a less ideal modification than a covalently attached peptide. The eventual use will involve encapsulation of genetically engineered cells that will excrete growth factors, and the large molecular weight laminin is likely to inhibit diffusion of such substances out of the alginate. The NB2a mouse neuroblastoma cell line was chosen because it is a well accepted and convenient model of differentiation and can differentiate in response to various factors such as retinoic acid and db cAMP.[35] Upon differentiation, these cells adopt a neuron-like phenotype with cessation of proliferation and development of axon-like neurites. Similar to studies using primary neurons, laminin also promotes neurite outgrowth from these cells.[36,37]

Adhesion of NB2a cells on modified alginate surfaces

We evaluated the effect of surface density of the conjugated YIGSR peptide on cell adhesion, because varying the amount of peptide added during the conjugation reaction would be expected to change the number of peptide molecules per alginate molecule and present different surface densities of the peptide on the surface of the gel. Initial studies (results not shown) determined that seeding the cells for times longer than 24 h did not give an appreciable increase in cell attachment, and therefore, all subsequent studies were carried out only to 24 h.

YIGSR peptide was conjugated to the alginate gel at ratios of 0.5 mg/g alginate, 1 mg/g alginate, and 2 mg/g alginate. The peptide ratios were so chosen depending on the minimum peptide density required for cell adhesion. Rowley et al.[14] suggested that a ratio of 1 mg GRGDS peptide/g alginate was about 2.5 orders of magnitude higher than that required for cell adhesion. Figure 1 shows the variation of NB2a adhesion on YIGSR-alginate gel discs prepared in the wells of the plate *in situ*. In every experiment the different gels were seeded with 100,000 cells. As expected, because alginate by itself does not possess bioadhesive properties, a very small number of cells (~1.5%) attached to the unmodified alginate gels. These could be cells that had settled down on the gel surface and did not get removed during the washing of the gel. However, we see an increase in cell attachment when cells are seeded on gels of the YIGSR–alginate conjugate. There is a fivefold increase in the number of cells attached between input peptide ratios of 0.5 mg/g alginate and 1 mg/g alginate [Figs. 4(a) and 5(a), respectively]. While about 9.5% of the cells attached on gels made with the lowest peptide ratio, about 44% of the cells attached on the YIGSR–alginate gels with input peptide ratio of 1 mg/g alginate. This, we believe is a result of the increased peptide density on the surface of the gel. However, while there is a further increase in the number of cells attached (~63% over control) with an input peptide density of 2 mg/g alginate, the increase is not incremental with peptide density [Fig. 6(a)]. The pronounced increase in cell attachment between peptide density of 0.5 mg/g alginate and 1 mg/g alginate might be the result of a critical minimum ligand density required for good cell attachment. Alginate gels that were coated with laminin showed about 60% cell attachment.

During the dissociation of the NB2a cells from the alginate hydrogels, we observed some breakdown of the alginate gel along its edges into small particles, and this particulate matter could interfere with the counting of cells giving false readings. To determine whether the trend observed in NB2a cell attachment with varying input peptide density as shown in Figure 1 was accurate, we used an alternative method in which the cells were seeded on small discs of the alginate gel and following attachment and treatment with trypsin to dissociate the cells, the gels were dissolved by treatment with 10% (w/v) EDTA. After complete dissolution of the gels in about 30 min, the cells were counted. The results are shown in Figure 2. The trend observed was similar to Figure 1. There was an increase in cell attachment between peptide ratios

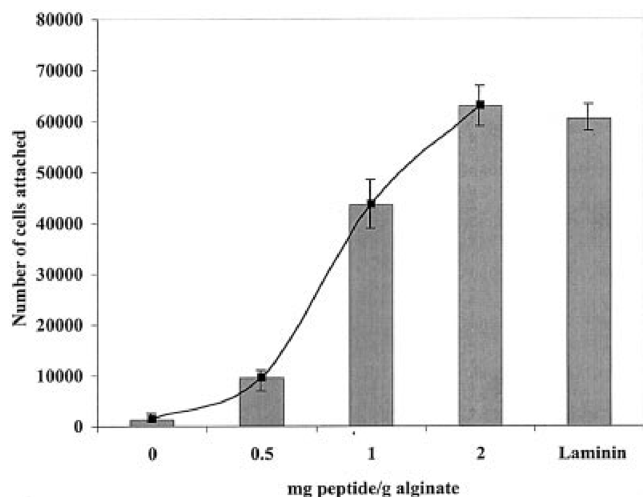


Figure 1. Adhesion of NB2a on YIGSR–alginate surfaces with varying peptide densities. NB2a cells attached on YIGSR–alginate gels after seeding for 24 h were counted using a Coulter Multisizer. Unmodified alginate gels were used as the negative controls while alginate gels coated with laminin served as the positive control. There is a fivefold increase in cell attachment on gels of YIGSR–alginate compared to unmodified alginate gels. Cell attachment increases dramatically between peptide–alginate ratios of 0.5 mg/g and 1 mg/g. However, the sequential increase is not as marked with gels of YIGSR–alginate with a peptide–alginate ratio of 2 mg/g. Each data point represents the average of three different samples, with each sample being counted thrice.

of 0.5 mg/g alginate and 1 mg/g alginate, but the increase was not as dramatic with an input peptide ratio of 2 mg/g alginate.

These observations show that the covalent grafting of YIGSR peptide on the alginate backbone imparts cell adhesion properties to alginate hydrogels. The YIGSR peptide has been identified as one of the major sites of cell adhesion in laminin, and cell adhesion studies have demonstrated that the specific binding of cells to the YIGSR peptide is mediated by a nonintegrin binding protein.[39] This nonintegrin binding protein with a molecular weight of 68 kDa has been characterized in several cell types including skeletal muscle cells and neuronal cell lines.[39,40] Because cell adhesion is a requirement for differentiation, YIGSR-alginate hydrogels could be used in the spinal cord to allow attachment of regenerating neurons, which in turn, would encourage differentiation of those neurons. These hydrogels could be prepared in different geometries including tubes and bundles to promote directional neurite outgrowth consistent with the orientation of the tube.

Differentiation of NB2a cells on modified alginate surfaces

The ability of the YIGSR–alginate surface to promote neurite outgrowth in the attached NB2a cells was studied by treating the cells with 10 μM db cAMP for 7 days, which has been shown to induce differentiation of neuroblastoma cells via activation of protein kinase A.[41]

Differentiation was studied on alginate gels with different amounts of YIGSR peptide conjugated to it and compared with differentiation on alginate gels coated with laminin, from which the YIGSR peptide is derived. Although a large number of cells attached on the laminin-coated alginate gels, NB2a cells seeded on these did not show marked neurite outgrowth after 7 days, as seen in Figure 3. However, very extensive neurite outgrowth was observed when NB2a cells were seeded on laminin-coated tissue culture plates and treated with differentiation medium (data not shown). This shows that while laminin does promote neurite outgrowth from NB2a cells, the coating of laminin on alginate gels causes it to lose this activity to a great extent, possibly through masking of the sites promoting neurite outgrowth or steric hindrance. This was one of the incentives to find an alternative method of creating a growth permissive surface. Figures 4 through 6 present micrographs tracking the cell attachment to modified alginate as the concentration of conjugated peptide is increased. Figure 4 shows the differentiation of NB2a cells on YIGSR–alginate with 0.5 mg peptide/g alginate. In Figure 4(a) the cells are shown after seeding (day 0 of differentiation), and in Figure 4(b) after differentiation for 7 days. It can be

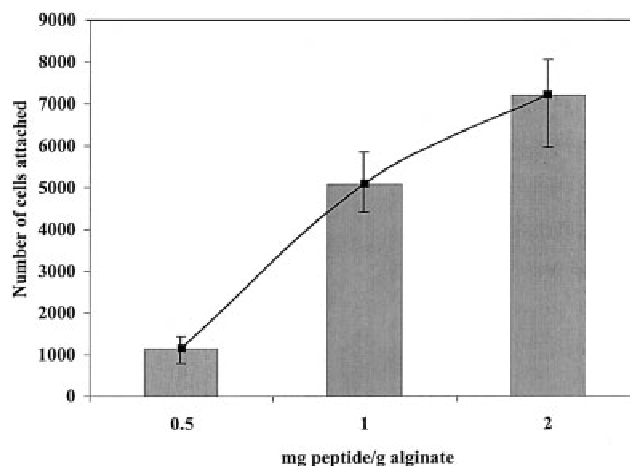
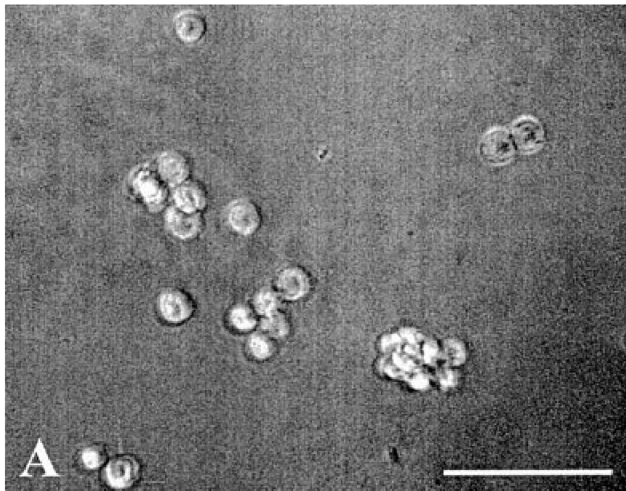


Figure 2. Adhesion of NB2a cells on YIGSR–alginate surfaces with different peptide densities (alternate cell counting method). These experiments were conducted on small disks of gels of modified alginate. At the end of the culture phase, the disks were dissolved by treatment with EDTA and the cells were counted using a Coulter Multisizer. The trends observed in this case are very similar to those observed with the original method, as shown in Figure 1. The attachment of NB2a cells increases dramatically between peptide–alginate ratios of 0.5 mg/g and 1 mg/g, but not as much at a peptide–alginate ratio of 2 mg/g. Each data point represents the average of three different samples, with each sample being counted thrice.



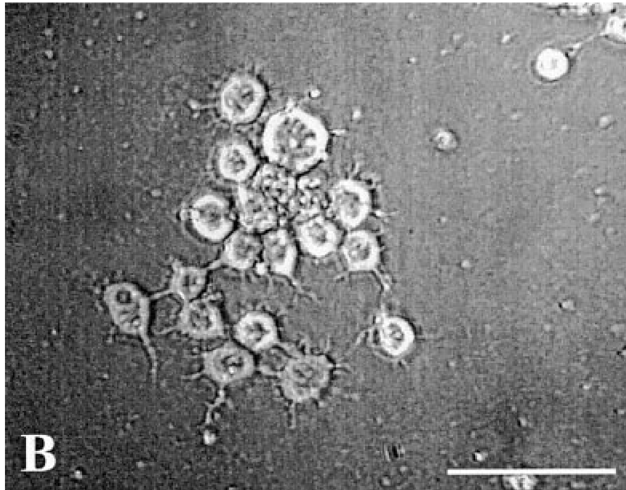
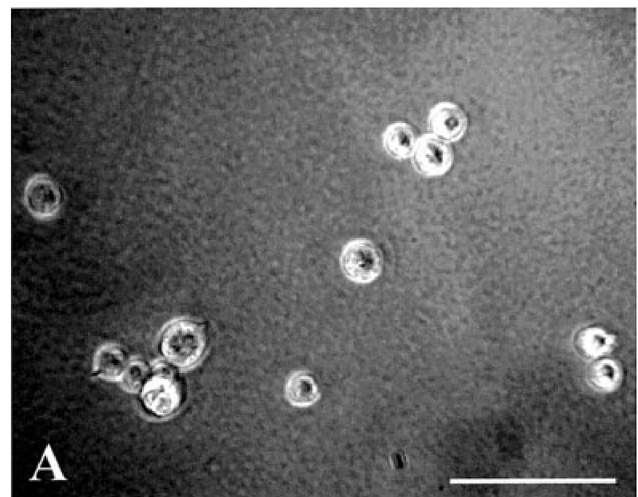


Figure 3. Differentiation of NB2a on the Ca–alginate surface coated with laminin. NB2a cells attached on laminin-coated Ca–alginate surfaces (A) and showed very little neurite outgrowth after differentiation for 7 days with 10 μM db cAMP (B). The loss of neurite outgrowth-promoting activity could be a result of binding of excess Ca^{2+} from the alginate gel. (Bar = 100 μm.)

seen that no or very minimal neurite outgrowth is observed in the attached cells. With 1 mg peptide/g alginate, the NB2a cells attached on YIGSR–alginate [Fig. 5(a)] and had a slightly larger fraction (<5%) of the cells showing neurite outgrowth and some of the cells even had significant neurite outgrowth [Fig. 5(b)]. Figure 6 shows differentiation of NB2a cells on the highest peptide density studied. The cells attached on the modified alginate surface [Fig. 6(a)] extend neurites that are considerably longer [Fig. 6(b)] compared to the alginate gels with lower peptide densities. Moreover, a much larger fraction (~10–15%) of the cells showed neurite extension compared to the modified alginate gels with lower peptide densities. Also, there was evidence of some branching of the neurites produced in this case [arrow, Fig. 6(b)]. In contrast, cells attached to the unmodified alginate gels showed no neurite extension (data not shown). In fact, the number of cells attached to the unmodified alginate surface was observed to decrease with time, and could be a result of the removal of loosely attached cells during the washes applied between changes of medium.

These results indicate that the YIGSR–alginate conjugate can provide a surface that is permissive to neurite outgrowth in neuroblastoma cells. Also, the observation of a pronounced amount of neurite outgrowth on YIGSR–alginate with the highest peptide density and little growth at low density suggests that peptide density plays an important role in inducing differentiation of the neuroblastoma cells. There also



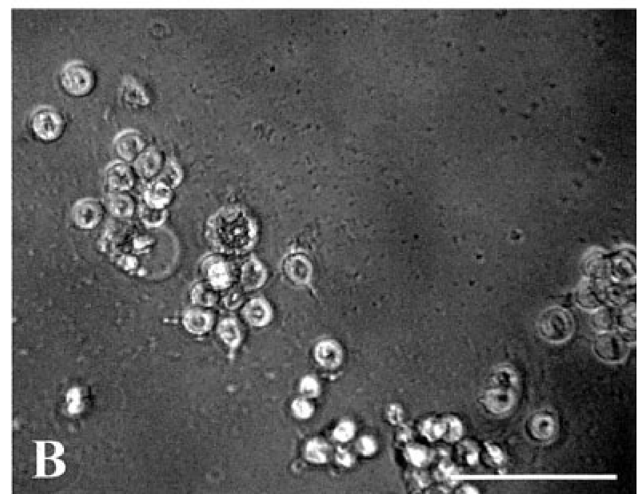
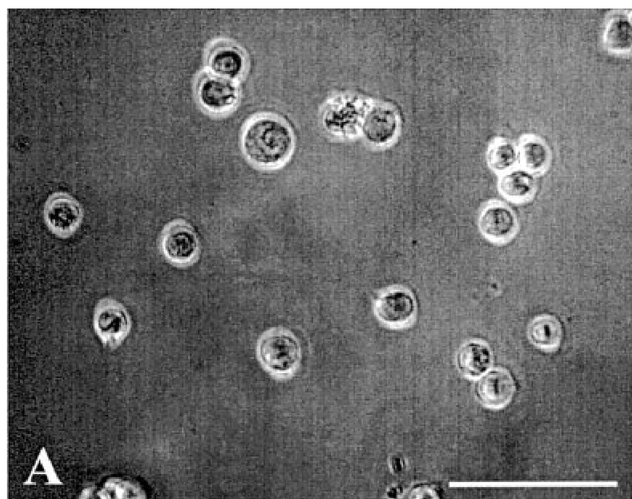


Figure 4. Differentiation of NB2a on the YIGSR–alginate surface with a low peptide density. NB2a cells attach on the modified alginate surface with a peptide–alginate ratio of 0.5 mg/g (A) but a very small amount of neurite outgrowth is observed after differentiation for 7 days (B). (Bar = 100 μm.)



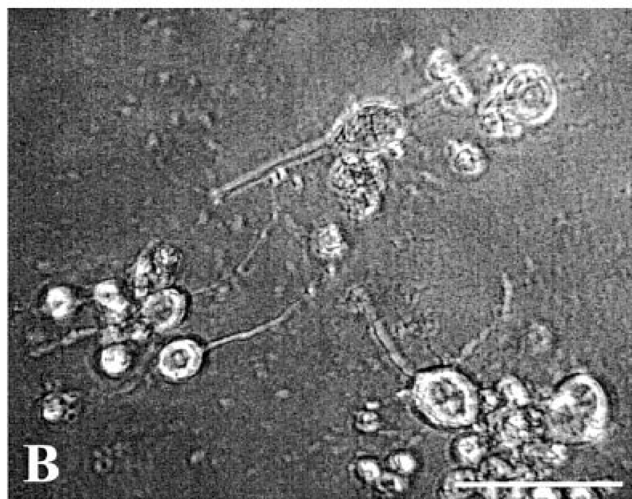


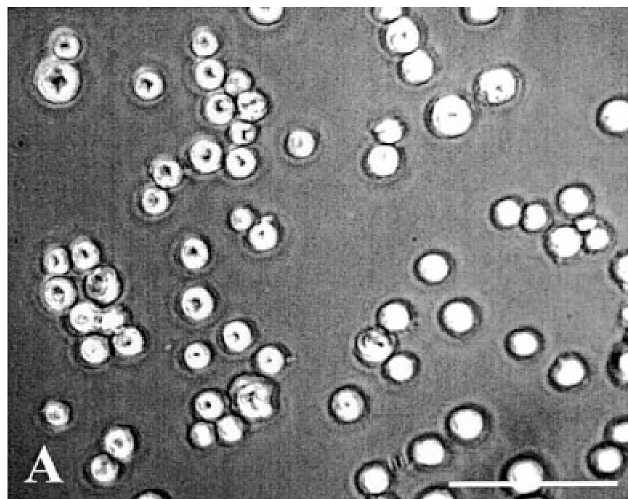
Figure 5. Differentiation of NB2a on the YIGSR–alginate surface with a medium peptide density. NB2a cells attached on the modified alginate surface with a peptide–alginate ratio of 1 mg/g (A) and show marked neurite outgrowth after differentiation for 7 days (B). Moreover, a larger fraction of the attached cells developed neurites. (Bar = 100 μm.)

appears to be an optimal peptide density, because we saw a dramatic increase in cell attachment between the conjugates prepared with 0.5 mg peptide/g alginate and 1 mg peptide/g alginate (Figs. 1 and 2), and a less than proportionate increase with the conjugate prepared with 2 mg peptide/g alginate.

The total length of neurite outgrowth was measured for all visible cells within different, randomly selected fields of view under the microscope and the resulting average neurite outgrowth induced by the modified alginate gels was calculated and plotted in Figure 7. The fields were selected at random due to the difficulty of visualizing large numbers of cells on the culture plate within a single field of view under higher magnification without compromising the ability to measure accurately, which would have resulted from use of a larger filed of view at lower magnification. At 1 mg peptide/g alginate, the average neurite outgrowth per cell was about 145 μm, whereas the average neurite outgrowth per cell increased to approximately 340 μm at a peptide density of 2 mg peptide/g alginate. These data clearly indicate a dependence of neurite outgrowth on peptide density.

DISCUSSION

The use of alginate has been well established over the years in the encapsulation of various drugs, pro-



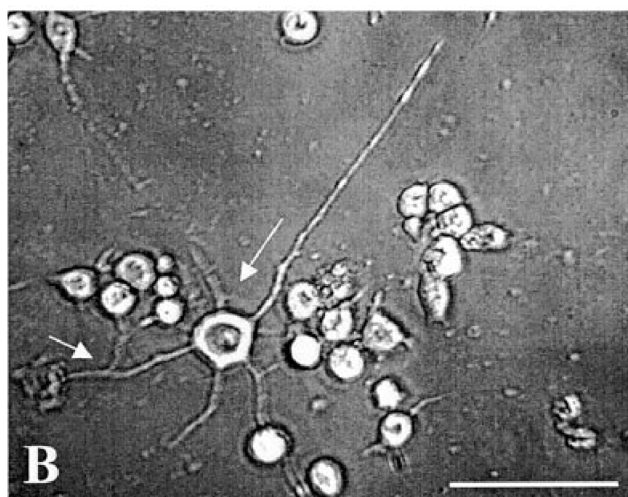


Figure 6. Differentiation of NB2a on the YIGSR–alginate surface with a high peptide density. NB2a cells attached on modified alginate surface with a peptide–alginate ratio of 2 mg/g (A) and showed extensive neurite outgrowth (B) after differentiation for 7 days compared to that observed on surfaces with low and medium peptide–alginate ratios. A large number of the cells developed neurites, and the number of neurites developed per cell was also higher. Arrows indicate branching. (Bar = 100 μm.)

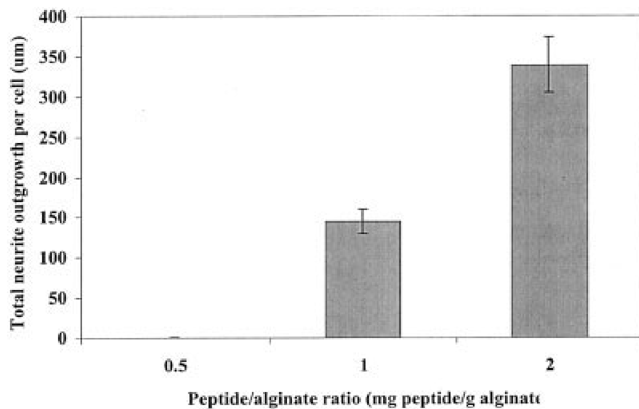


Figure 7. Average length of neurite outgrowth per cell induced by YIGSR–alginate gels for the different peptide-to-alginate ratios studied. Negligible neurite outgrowth was seen in cells seeded on gels with the lowest peptide-to-alginate ratio. However, higher peptide-to-alginate ratios resulted in neurite outgrowth, and the total average neutrite outgrowth increased significantly ($p < 0.001$) when the peptide-to-alginate ratio was doubled from 1 mg peptide/g alginate to 2 mg peptide/g alginate. (Error bars = ±standard error.)

teins, and cells. In recent, years there has been increased attention towards the use of alginate in tissue engineering applications such as tissue scaffolds,[7] wound healing,[8,9] bridges for peripheral nerves and the spinal cord (unpublished results),[10–13] and tissue bulking agents.[16] Alginate in its native form does not permit cell adhesion but the conjugation of a biologically active peptide can change that. Rowley et al.[14] showed that covalent attachment of GRGDY peptide to alginate hydrogels allowed attachment and proliferation of C2C12 skeletal myoblasts. Also, an alginate hydrogel linked with a synthetic peptide derived from bone morphogenetic protein (BMP) has been shown to induce ectopic bone formation *in vivo*.[15]

We have previously shown that alginate-encapsulated fibroblasts that are genetically engineered to produce BDNF survive inside the spinal cord, continue to secrete biologically active BDNF and induce sprouting of neurons at the site of the injury (unpublished results).[5] However, the sprouting of neurons was observed only along the periphery of the capsules and not inside the capsules. Previous reports have shown axons growing into grafts of freeze dried alginate gel in peripheral nerves of cats,[10] peripheral nerves of rats,[11] and transected rat spinal cord.[12,13] The difference, we believe, is a result of the poly-L-ornithine (PLO) coating applied to the alginate microcapsules used in our study, which prevented the axons from penetrating into the alginate matrix. The PLO coating was applied as a means to protect the encapsulated cells from the host immune system while allowing secretion of the BDNF and eliminating the need for immune suppression. Our objective here was to provide a surface that promoted neurite outgrowth from the sprouting neurons in an effort to enhance the effectiveness of the therapeutic strategy.

Laminin-coated alginate gels did not induce significant neurite outgrowth in the NB2a cells despite the known properties of laminin. We believe that the loss of neurite outgrowth promoting activity of laminin after coating on alginate gels could be a result either of steric hindrance of the active site, or of interaction with the Ca^{2+} present in the gels. It has previously been observed that Ca^{2+} interacts with laminin, enhancing the formation of large polymers of laminin.[35] Also, we have observed that alginate gels crosslinked with Ca^{2+} (in the absence of the traditional PLO coating) dissolved upon coating with laminin while gels crosslinked with Ba^{2+} or Al^{3+} did not (unpublished results), which demonstrated that laminin possesses a great affinity for Ca^{2+}. Another reason for the anomalous behavior of laminin upon application to alginate gels could be that while the laminin coating on alginate allowed adhesion of cells, the regions that promoted neurite outgrowth were obscured and not available for interaction with the cells. This is conceivable that the propensity to promote attachment is less affected by alginate than the propensity to promote neurite extension because free laminin is known to have several cell adhesion sites.[42,43]

Peptides such as the YIGSR peptide of laminin have been shown to promote neurite activity, are commercially available, and can be covalently linked to alginate via aqueous carbodiimide chemistry. We hypothesize that this process could be used to increase the growth permissive nature of alginate microcapsules. EDC, a water-soluble, zero-length crosslinker was used to form amide linkages between the amine terminus of the peptide and the carboxyl groups on the alginate. The reaction was carried out under slightly acidic conditions (pH 6.5) because EDC is more reactive under those conditions. Sulfo-NHS was added to stabilize the highly reactive intermediate formed between the EDC and the carboxyl groups of alginate. Addition of sulfo-NHS greatly reduces hydrolysis of the intermediate and increases the efficiency of the conjugation.[38] EDC equivalent to 5% uronic acid activation was used to minimize side reactions associated with increasing amounts of EDC, which could affect the gelation properties of the alginate and could have undesirable effects *in vitro* as well as *in vivo*.

Our experiments have shown that conjugation of the YIGSR peptide to the alginate allows attachment of cells on these alginate gels and that cell adhesion increases with increasing peptide density. Also, we saw that the gels made from YIGSR–alginate conjugate promoted neurite outgrowth from the attached NB2a cells and neurite outgrowth increased with increasing peptide density on the alginate. Although we saw a significant increase in cell attachment between

the conjugates prepared with 0.5 mg peptide/g alginate and 1 mg peptide/g alginate (Figs. 1 and 2), these peptide densities did not promote extensive neurite outgrowth that was observed only in the case of the conjugate prepared with 2 mg peptide/g alginate. This suggests that although a lower peptide density might be appropriate for cell adhesion, a higher peptide density is required for the attached cells to differentiate. It has been shown that an RGD ligand spacing of 440 nm is sufficient for cell attachment but a ligand spacing of 140 nm is required for other functions such as motility and stress fiber formation.[44] It is conceivable that a similar requirement leads to the observation of increased neurite outgrowth at a higher peptide density. It must be kept in mind, however, that increasing peptide density beyond a certain point might actually result in an inhibition of neurite outgrowth.

The development of neurite outgrowth-promoting alginate–peptide conjugate is a promising avenue in the study of peripheral nerve and spinal cord repair. This YIGSR–alginate conjugate can be used as a growth permissive surface in combination with an alginate based strategy to deliver neurotrophic factors to the spinal cord (unpublished results)[5] or as a growth-permissive bridge for resected nerves. Future work should involve studying and comparing the effectiveness of alginate–peptide conjugates using different peptides, either alone or in combination, with regard to their ability to promote neurite outgrowth *in vitro* as well as *in vivo*. Also, geometries such as tubes and bundles, with gradients of neurite outgrowth promoting entities are being investigated in an effort to achieve directional neurite outgrowth that could play an important role in spinal cord repair.

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