ADHESION OF MYOBLASTS TO RGD-ALGINATE

by

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STATEMENT BY AUTHOR

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Abstract:

Several types of alginate have been developed, but none of them alone are able to interact with mammalian cells. Alginate does not provide anchorage points that are essential for cell growth and proliferation. As this hydrogel meets many requirements for tissue engineering, modification of alginate was proposed in order to stimulate cell adhesion. After recognition of RGD binding site in ECM proteins, synthetic RGD peptides were coupled with alginate via aqueous carbodiimide chemistry. I applied this conjugation and optimized it in terms of various parameters of the coupling reaction. Mouse skeletal Myoblasts were cultured on the surface of the modified alginate. They were attached, spread and differentiated to form myotubes. This showed that an RGD containing peptide has the ability to mimic ECM molecule binding sites and stimulate adhesion to materials that are otherwise unable to interact with cells. I also demonstrated that RGD density enhanced proliferation and spreading. Increasing crosslinker density made stiffer gels and controlled cell differentiation. Including free Ca^{2+} improved swelling properties of alginate gel, enforced cell attachment and enhanced conversion of myoblasts to myotubes.

Introduction:

The goal of a tissue engineer is the replacement of diseased or damaged tissue by creating living, 3D tissues and organs using cells obtained from patient's own tissue or other available sources. In many cases, the approach is to break the donor material down to the level of individual cells, and then to coax the isolated cells into forming a tissue structure of the appropriate size and/or shape using a physical scaffold to organize cells on a macroscopic scale, and provide molecular cues to stimulate appropriate cell growth, migration, and differentiation.[10]

Tissue engineering presents enormous challenges and opportunities for materials science from the perspective of both materials design and materials processing.[9]

Interest in natural and synthetic hydrogel systems is growing rapidly. Natural polymers such as alginate and agarose are some of the first materials employed in tissue engineering.

Alginate refers to a family of polyanionic copolymers derive from brown sea algae and comprising 1,4-linked β-D-mannuronic (M) and α-L- guluronic acid (G) residues in various proportions. The proportion as well as the distribution of the two monomers determines to a large extent the physiochemical properties of alginate.[24] Alginates are able to form gels by the binding of divalent cations to the G-units.[4] Alginates rich in G form strong and brittle gels, while M-rich alginates form softer & elastic gels.[4]

Alginate undergoes a phase transformation from a polysaccharide solution to a mechanically stable hydrogel under mild, cell friendly conditions. Flexible

mechanical strength, allowing for diffusion of hydrophilic substrates, having up to 40% dry material, biocompatiblity, transparency, abundance in source, low price and easy sterilization with membrane filtration are some other advantages of alginate hydrogels.

Alginate lacks cellular interaction and it has minimal protein adsorption due to the hydrophilic nature of the alginate.

The disadvantage of alginate is its degradation characteristics. Degradation through the loss of divalent cations into the surrounding medium is an uncontrollable and unpredictable process. Crosslinking alginate covalently with various types of molecules and different crosslinking densities precisely control the mechanical, swelling, and degradation properties of alginate.[4]

A common theme in engineering cell and tissue behavior at device surfaces is to modify the material to interact selectively with a specific cell type through biomolecular recognition events. A promising approach is the biomimetic modification of the material in which peptides containing the adhesion domains of ECM proteins are attached to the base material. The central hypothesis of biomimetic surface engineering is that peptides that mimic part of the ECM affect cell attachment to the material, and that surfaces or 3D matrices modified with these active peptides can induce tissue formation conforming to the cell type seeded on the material. In order to stimulate cell adhesion to alginate gels, they can be modified by covalently linking the RGD peptide sequence to the alginate

molecules by utilizing aqueous carbodiimide chemistry. This chemistry is widely used for carboxyl-amine conjugation.[3]

Chemistry

Alginate does not provide anchorage points that are essential for cell growth and cell proliferation. The peptide sequence R-G-D acts as a ligand for cell integrins and can be linked to alginate [Fig. 1], thereby making it suitable for cell attachment.

Aqueous carbodiimide chemistry was applied to covalently conjugate the cell adhesion peptide GRGD to alginate in the bulk. EDC reacts with carboxyl groups to form an intermediate (O-acyl-isourea) that can stabilize with reaction with amine, forming a peptide bond, without space length [Fig.2A]. Unfortunately, this reactive complex is subject to rapid hydrolysis in aqueous solutions, having a rate constant measured in seconds.[8]

If the target amine does not find the active carboxylate before it hydrolyzes, the desired coupling cannot occur. Forming a Sulfo-NHS ester intermediate from the reaction of the hydroxyl group on Sulfo-NHS with the EDC active- ester complex extends the half-life of the activated carboxylate to hours [Fig. 2B]. Since the concentration of added Sulfo-NHS is usually much greater than the concentration of target molecule (alginate), the reaction preferentially proceeds through the longer-lived intermediate. EDC/Sulfo-NHS-coupled reactions are highly efficient and usually increase the yield of conjugation dramatically over that obtainable solely with EDC.[8] A side reaction may take place, notably with carboxyl in

hydrophobic environment, is the internal rearrangement of the O-acyl-urea activated ester of the EDC to N-acyl-urea.[30]

EDC has found much greater favor over the classical N, N'-dicyclohexylcarbodiimide because of its high solubility in aqueous media and the easy removal of the excess reagent and the corresponding urea by washing the conjugated product with water and dilute acid.[8] EDAC has been shown to be impermeable to membranes of live cells.[30]

Note 1: The bulk EDC should be stored desiccated at –20 C. One should warm bottle to room temperature before opening to prevent condensation that will cause decomposition of the reagent over time. The powder should be dissolved rapidly and used immediately to prevent extensive loss of activity.[8]

Note 2: A research in literature revealed that a variety of buffers at different concentrations and at PH 4-5 were employed for carboxyl-amine conjugation. It has been shown that the phosphate buffer commonly used in such conjugation is incompatible with the stability of the acylurea intermediate of EDC. Also buffers such as acetate and citrate cannot be used because these would make mixed anhydrides with the carboxyl reactant and drive the nucleophilic amino reagent into side reactions and by-products.[15] It also has been demonstrated that reaction time are reduced in MES buffer during the EDAC/NHS activation step.[30]

Note 3: For labile biological compounds, especially proteins, it is desirable to perform the conjugation reaction at 4°C to minimize the loss of functional characteristics.[15]

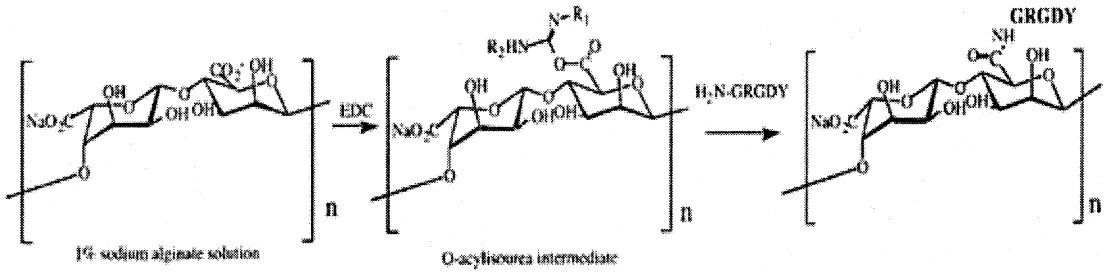


Figure 1. Reaction scheme of peptide coupling to alginate molecules. Amide bond formation is mediated by the carbodiimide through the carboxyl group of the alginate and the N-terminal of the peptide.[2]

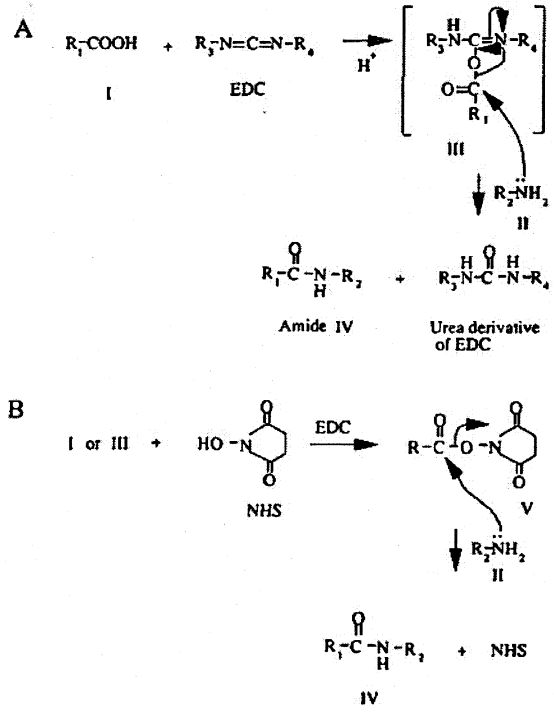


Figure 2. EDC-mediated amidation reaction without (A) and with (B) the assistance of NHS.[15]

RGD & Integrins

The minimal amino acid sequence RGD in fibronectin and other cell adhesion proteins is the most important recognition site for about half of all known integrins. RGD peptides that have not been specifically designed to be selective to bind certain integrins mimic a number of adhesion proteins and bind to more than one receptor. The affinity of these small peptides is relatively low due to high degree of flexibility and loosing synergistic binding sites. [7]

Short peptides containing the RGD sequence can mimic cell adhesion proteins in two ways: When coated onto a surface, they promote cell adhesion, whereas in solution they act as decoys, preventing adhesion.[7]

Integrins are composed of two subunits, α and β, and they hold a cell in place by attaching at one end to proteins of the ECM, like fibronectin, and at the other end to the cytoskeleton, the structural framework of the cell. They connect to the cytoskeleton through a highly organized aggregate of proteins, such as actin filaments, α actin and others [Fig. 3]. Integrins bind to the adhesion domains of the ECM proteins by recognizing specific sites of that domain.[11]

The central hypothesis of biomimetic surface engineering is that short peptides that mimic part of the ECM proteins (like RGD) are recognized by integrins via "specific interactions" and can affect cell attachment to the material.[3] Cells adhere and spread on RGD biomimetic surfaces and surfaces modified with these active peptides can therefore induce tissue formation conforming to the cell type seeded on the material.

Blocking studies demonstrated that myoblasts formed a cytoskeletal attachment to RGD-alginate mediated by integrins ($\alpha5\beta1$, $\alpha6\beta1$, $\alpha7\beta1$, αv have been known).[12] Peptide screening showed that an additional residue included to the linear peptide before the RGD sequence (GRGD) did not change the cell adhesion.[5]

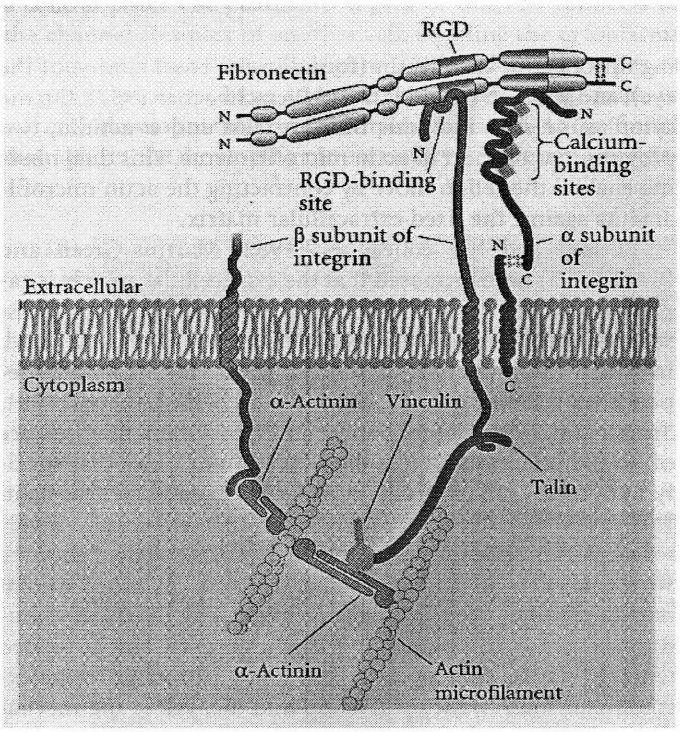


Figure 3. Speculative diagram relating the binding of cytoskeleton to the ECM through the integrin molecule.[28]

Objectives:

The objectives of this study were

1) To modify the alginate by carbodiimide chemistry to interact with myoblasts.
2) To observe the interaction of myoblasts with alginate alone, with fibronectin-coated alginate and with RGD-modified alginates.
3) To determine the effect of peptide density on cellular functions.
4) To determine the effect of cell density on cell spreading.
5) To determine the effect of the crosslinking density on cell attachment.
6) To determine the effect of adding free Ca^{2+} to media.

Materials & Methods

Sodium salt alginic acid prepared from Laminaria Hyperborea was purchased from Sigma (Fluka). According to the manufacturer's data, it has a molecular weight of 100,000 - 200,000 (M = 25-35%, G= 65-70%). $CaCl_2$ was obtained from J. T. Baker. 1-ethyl-3- (3-dimethylaminopropyl) carbodiimide (EDAC) and Glycine-Arginine-Glycine-Aspartic acid tetra peptide abbreviated as GRGD was from Sigma. N-Hydroxysulfosuccinimide (Sulfo-NHS) and dialysis tubing (MWCO: 3.5K) was from Pierce.

1. Chemistry

In order to stimulate cell adhesion to alginate gels, they can be modified by covalently linking the RGD peptide sequence to the alginate molecules by utilizing aqueous carbodiimide chemistry. This modification, developed by Rowley & Mooney at the university of Michigan[2], is widely used for carboxyl-amine conjugation. EDC or EDAC, Water-soluble carbodiimide was used to activate carboxyl groups on alginate. The activated carboxyl groups then react with amino group of H_2N-GRGD to form a stable amide bond. Sulfo-NHS is included to increase the stability of the active intermediate, which ultimately reacts with the attacking amine.

Material processing

Sodium alginate solution 5mM was sterilized through a 0.45-μm filter to get rid of aggregates. GRGD peptide solution was added to the alginate. Coupling reactions

were initiated by adding 5mM NHS and 50mM EDC (Note: this working solution should be used immediately). They reacted over 12 hours by a nutator. Excess reactants were removed by dialysis (3500 MWCO) against MES 2.5 mM after 4 days. Whole reactions took place in 25 mM MES buffer, pH 6.5 at 4°C. This conjugate could be lyophilized until dried to make a desired concentration.

2. Cell Studies

C2C12 mouse skeletal myoblasts were used in this study. C2C12 is a skeletal muscle myoblast cell line of murine origin, which is capable of growing, fusing, and differentiating up to the formation of myotubes in culture. The differentiation of myoblasts into fused myotubes is stimulated by cell contact and by poorly known additional stimuli, such as the lack of some growth factors present in the fetal calf serum. It is possible to promote fusion and differentiation by lowering the fetal calf serum in the medium from 10% to 1% or substitute the calf serum by horse serum.

The growth medium was consisted of low glucose DMEM 400 ml, Fetal Bovine Serum from Hyclone 20%, chick embryo extract 0.5% and penicillin-streptomycin at 100 units per ml. The differentiation medium was DMEM, horse serum 2% and penicillin/streptomycin. The cell preservation medium was DMSO 10% plus FBS in DMEM.

Cells were split after 24-48 h at a dilution 3000 cells/cm^2. Myoblasts differentiate between days 2-3.

Cell attachment assays

Sterile chambered 3-well coverslips with dimension 9.5 mm x 9.5 mm x D 1.0 mm were purchased from sigma to cast the alginate. They were consisted of glass coverslips with gasket-formed wells. I peeled off the gaskets and mounted on microscope glass slides to visualize cells under microscope.

0.1 ml of modified alginate was pipetted into individual wells, followed by a layering of 46 mM $CaCl_2$. The alginate set for 24 h at 4°C. Alginate was gelled to form a continuous sheet. Extra $CaCl_2$ was aspirated and rinsed with MES the next day. Myoblasts were pipetted into individual wells on the surface of modified alginate (1) with varying RGD density (2) with varying crosslinker density. I also added few mM $CaCl_2$ in media to characterize its effect on myoblasts. I applied different cell density. Cell plus media was 0.1 ml in each experiment. Plates were incubated at 37°C in a humidified atmosphere containing 5% CO_2. Cells were visualized with a Nikon microscope, and Images were obtained on a Leitz DM RXE microscope equipped with a Leica DC500 camera and IM50 imaging software. All images are at 10x magnification.

Results

1. Chemistry

Several different parameters and conditions were examined to obtain the maximum yield for the coupling of alginate to GRGD peptides (all are based on molar ratio)

Alginate Conc	RGD: alginate	EDC: alginate	EDC: NHS
3.75 mM	1:1000	1:10	1:1
5 mM	1:100	10:1	2:1
20 mM	1:10	20:1	10:1
	1:2		15:1
	1:1		
	5:1		

The following shows the sufficient amounts for this conjugation.

$M_{alginate}$	RGD: alginate	EDC: alginate	EDC: NHS
1 mg / ml ~ 5 mM	1:10	10:1	10:1

2. Cell studies

Myoblasts attached and began to spread on glass wells and glass wells which were covered with fibronectin (1μg/cm^2), while no myoblasts adhesion was observed on the alginate surface alone or alginate with a cover of 2μg/cm^2 fibronectin [Fig. 4].

In general myoblasts proliferated between day 1-2 at 3000 cell/cm^2, and cell number decreases after 3 days as a result of the fusion of myoblasts into multinucleated myofibrils, which shows skeletal muscle differentiation [Fig. 5].

Step 1. Effect of RGD density on cell attachment

Varying molar ratios (0.001, 0.01, 0.1, 0.5, 1, 5) of RGD/alginate were used for RGD coupling and the rest of parameters were according to the below table.

Alg	EDC: Alg	EDC: NHS	$CaCl_2$	Cell #
3.75 mM	20:1	15:1	46.2 mM	10,000 & 20,000

My results showed that increasing peptide density strongly increases cell attachment and in higher concentrations cells look bigger. I found the ratio of 0.1 and 20,000 cell/cm^2 was sufficient to promote cell attachment and spreading [Fig. 6,7].

Step 2. Effect of different concentration of $CaCl_2$ as a crosslinker

0.1 ml of varying concentration of $CaCl_2$ (11.57, 23.15, 46.2, 69.4, 92.6, 115.7 mM) which are in equal molar ratio to 0.5, 1, 2, 3, 4, 5 (%w/v) of alginate solution respectively were crosslinked with RGD-alginate surfaces that were made according to the below table.

Alg	RGD: Alg	EDC: Alg	EDC: NHS	Cell #
1 mg / ml ~ 5mM	1:5	10:1	15:1	20,000

Crosslinker density significantly altered myoblast interaction with modified alginate between 11.57 – 46 mM $CaCl_2$, but over that cell attachment started to decrease. At 46 mM $CaCl_2$, cells were large, elongated with stress fibers just after 24 h [Fig 8].

Step 3. Effect of different concentration of $CaCl_2$ in substrate

Ionically crosslinked alginate gels lose mechanical properties over time in vitro, due to out flux of crosslinking ions into the surrounding medium. Including few mM free Ca^{2+} in the substrate media to keep Na:Ca $\leq$25:1 for high G alginates help to prevent gel dissociation.[16] 4, 8, 12, 16, 20, 24, 28 mM of $CaCl_2$ were added to the media to determine its effect on cell attachment and spreading. The RGD-Alginate surfaces were according to the below table.

Alg	RGD: Alg	EDC: Alg	EDC: NHS	$CaCl_2$
3.75 mM	1:2	20:1	15:1	46.2 mM

The result showed that including 4-28 mM Ca^{2+} increased cell spreading [Fig. 9].

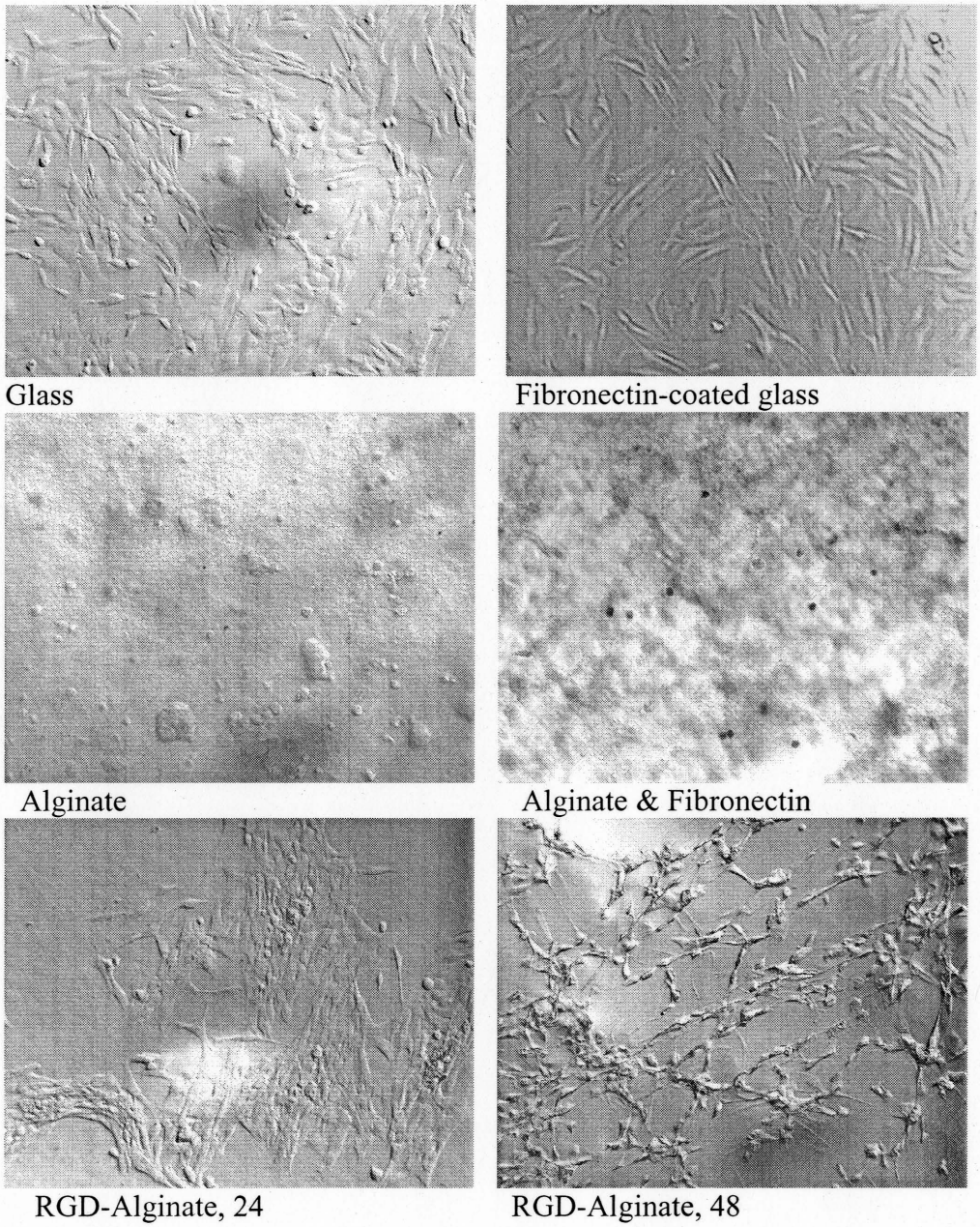


Fig.4: The control experiments show cell attachment to a glass slide surface, Fibronectin-coated glass, Alginate alone, Alginate-coated fibronectin, RGD coupled alginate after 24, and 48 h for 20,000 cells.

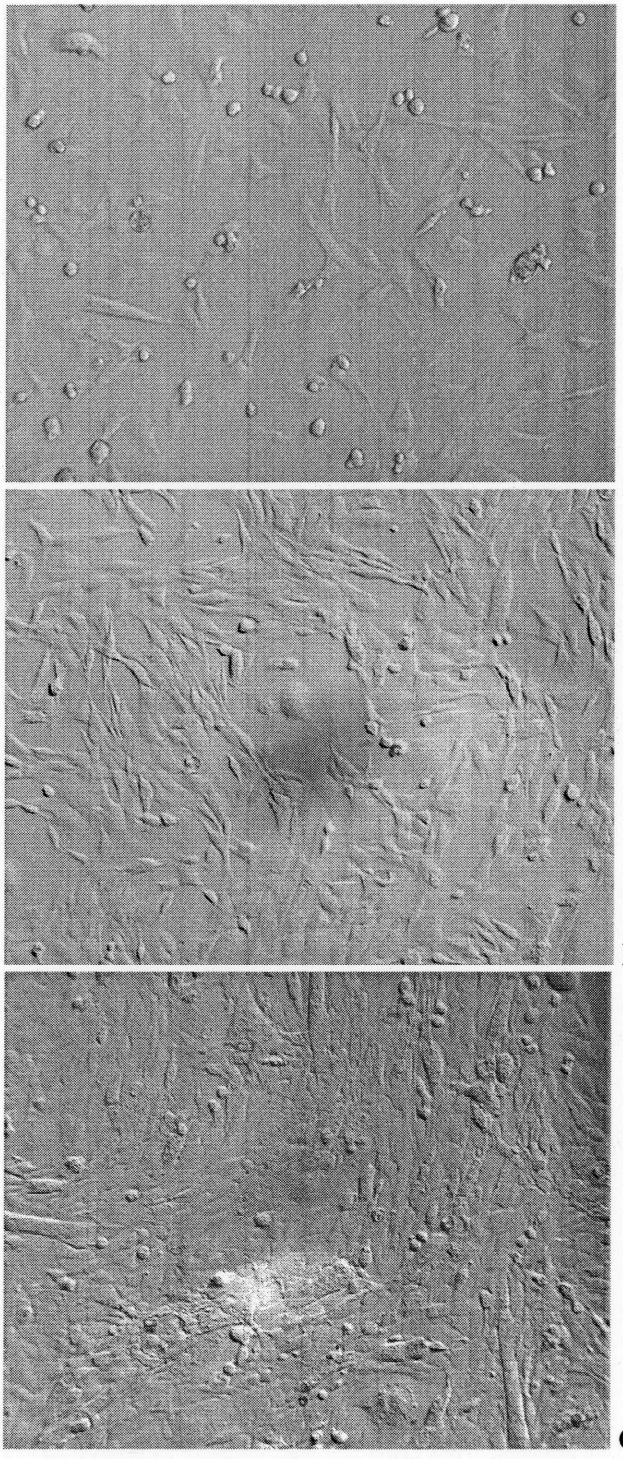
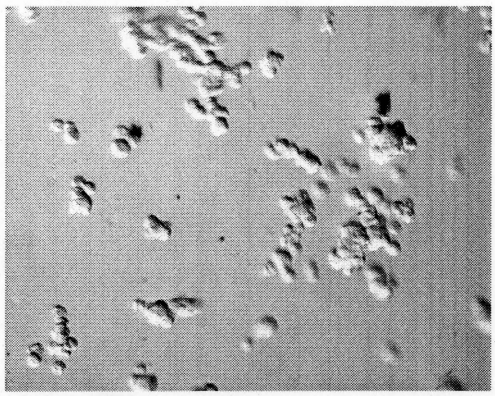
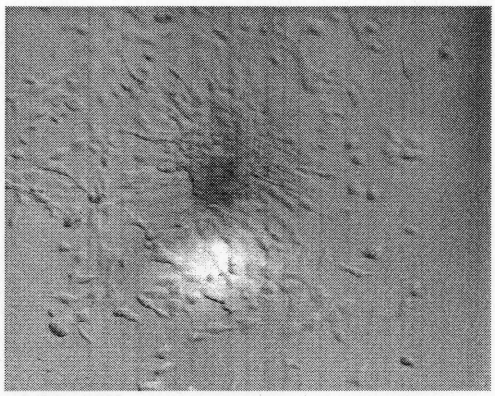


Figure 5. Cell proliferation day 1 (A), day 2 (B) and differentiation on day 3 (C) on glass wells.

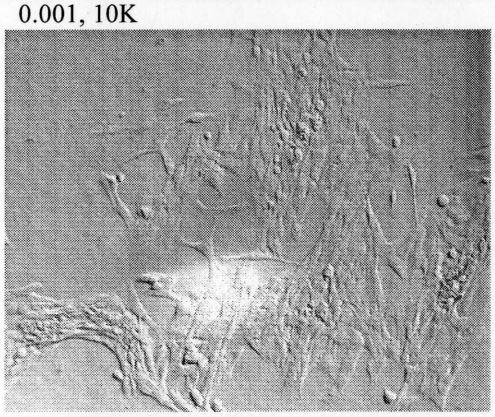
A)



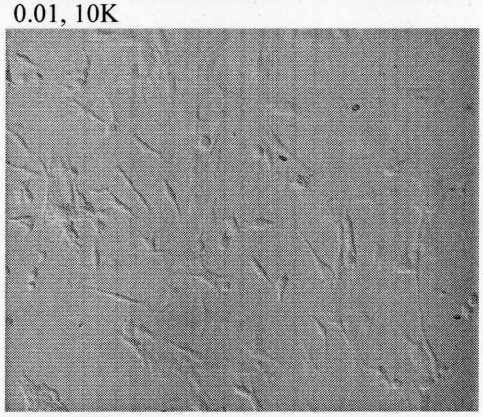
0.001, 10K



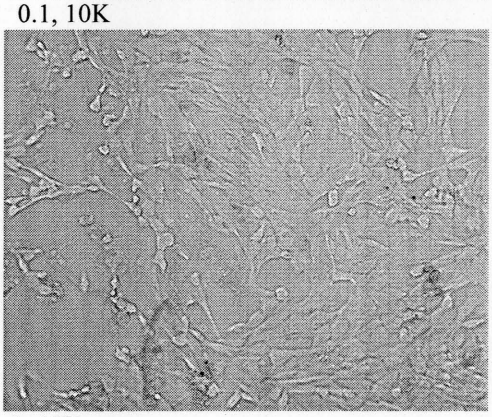
0.01, 10K



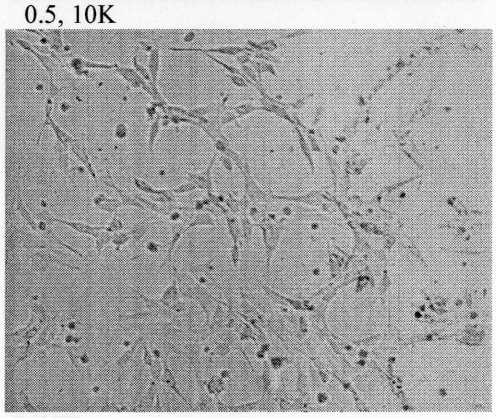
0.1, 10K



0.5, 10K



1, 10K



5, 10K

B)

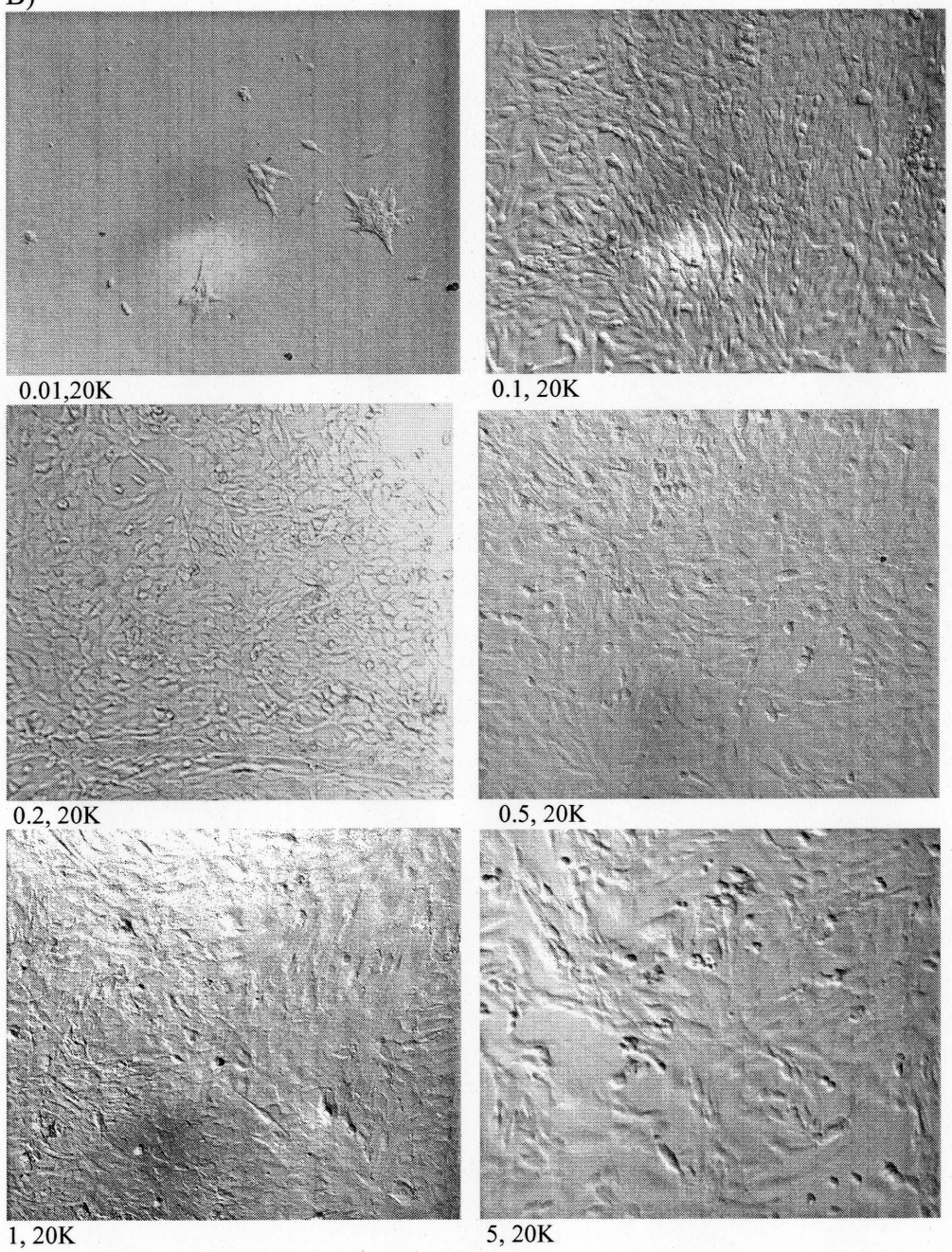


Figure 6. The effect of RGD density on cell attachment and spreading in 10K (A) and 20K (B) cells after 24 h. First number indicates the ratio of RGD/ Alg. The concentration of crosslinker ($CaCl_2$) was 46mM.

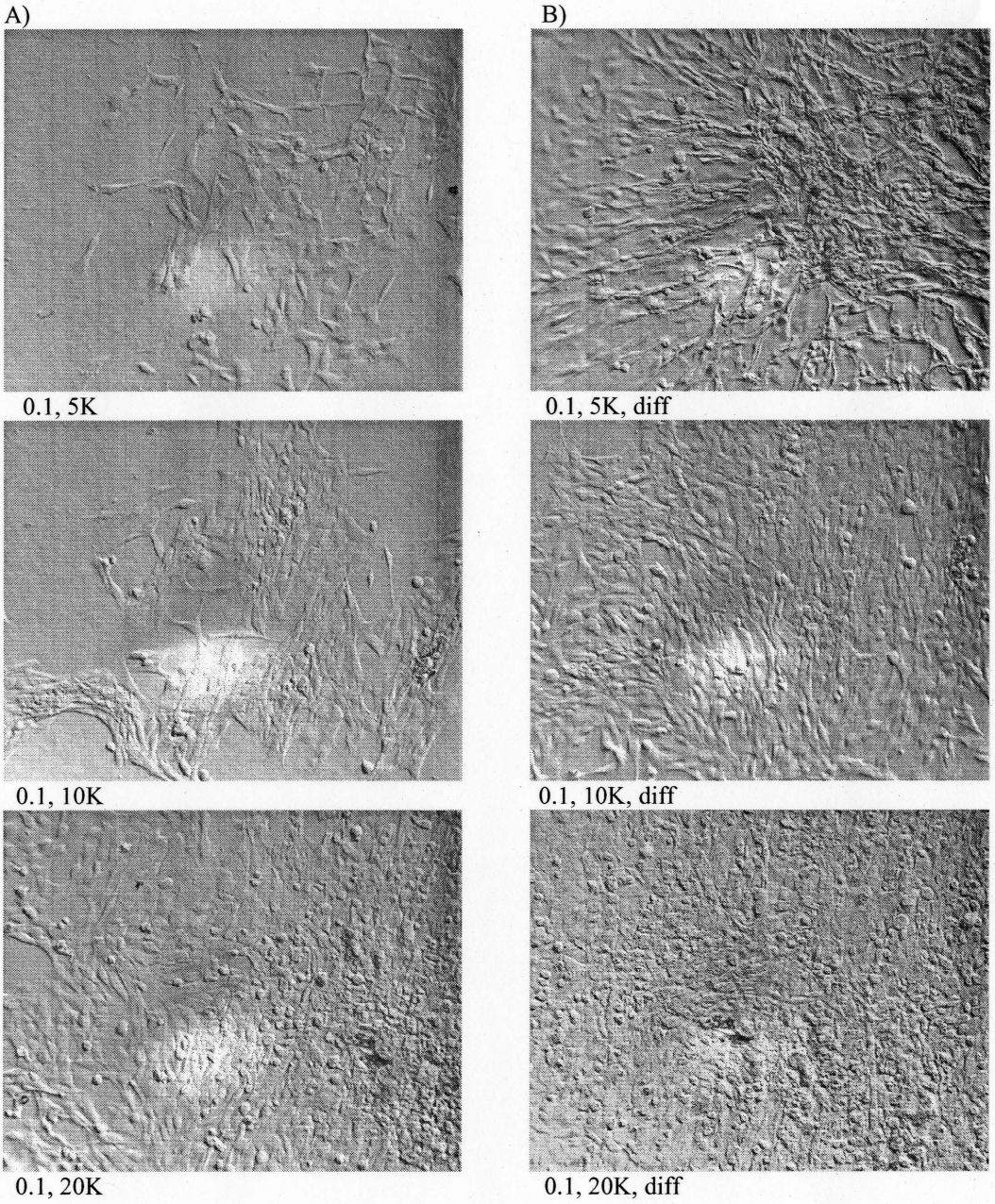


Figure 7. The effect of cell number on the morphology of differentiated myoblasts on RGD-alginate after 24 (A) and 48 h (B). First number indicates the ratio of RGD/Alg; the concentration of crosslinker ($CaCl_2$) was 46mM.

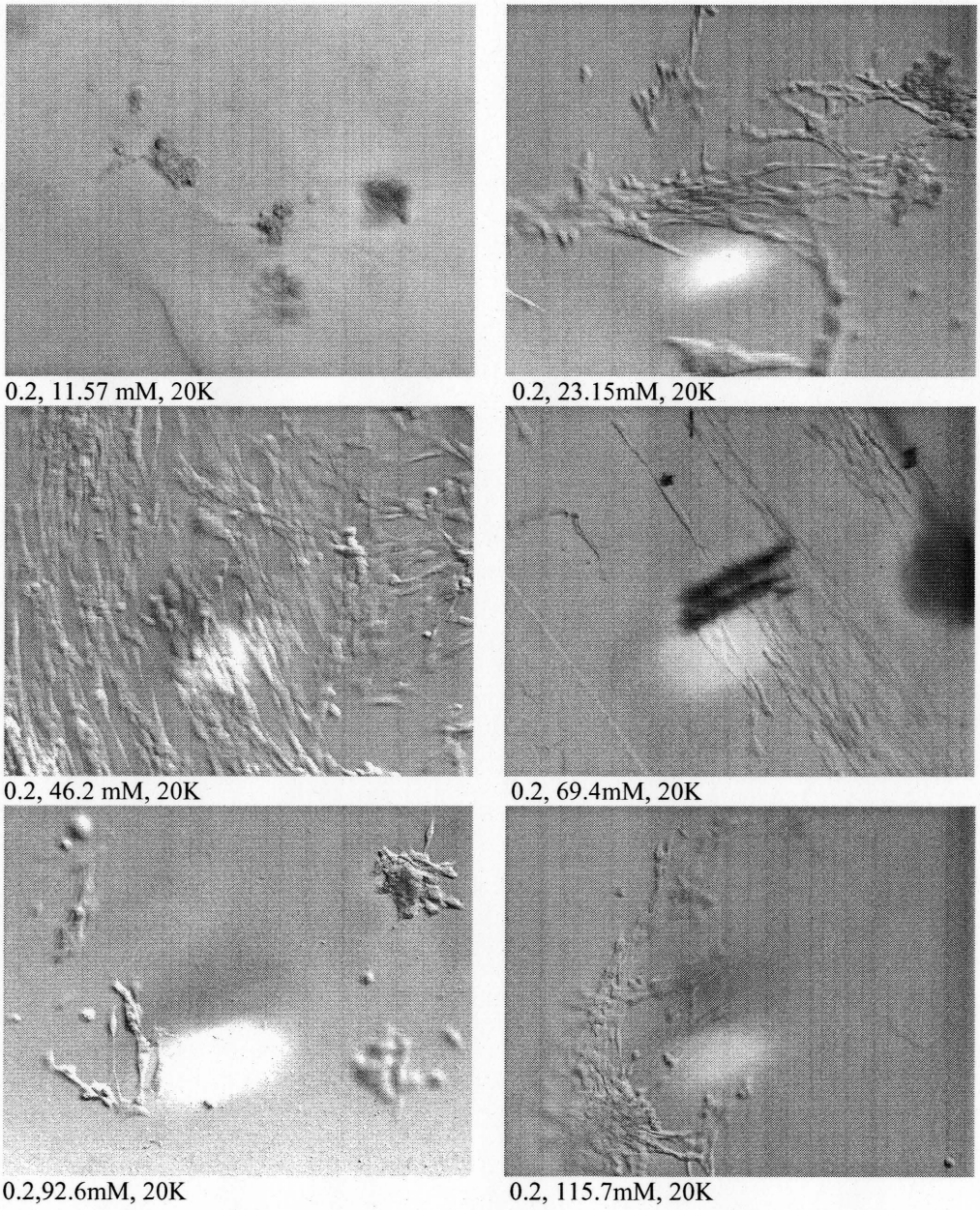
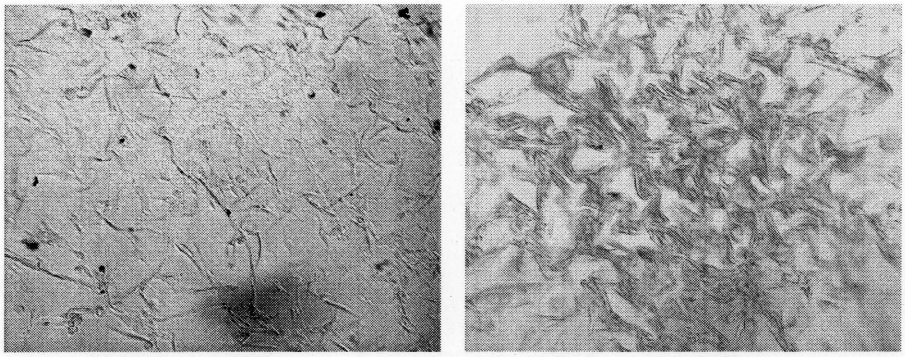
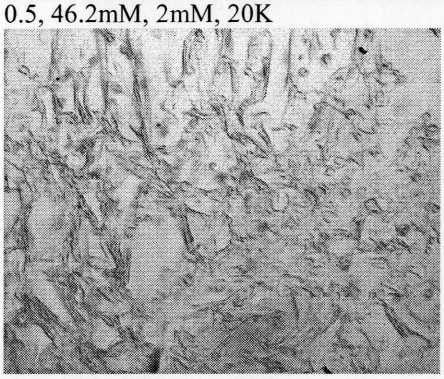


Figure 8. The effect of different conc. of $CaCl_2$ as a crosslinker after 24 h. First numbers are the ratio of RGD/Alg and the second numbers are the concentration of $CaCl_2$.

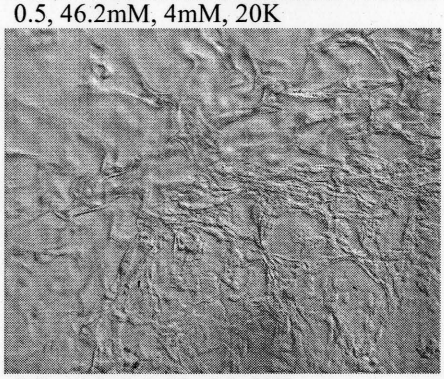


0.5, 46.2mM, 2mM, 20K

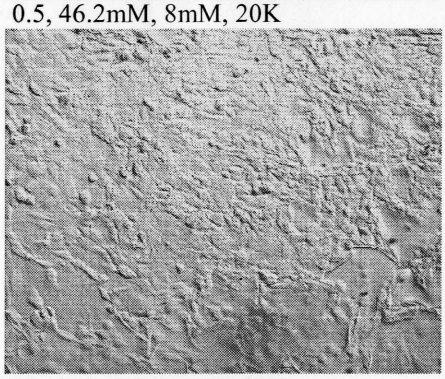
0.5, 46.2mM, 4mM, 20K



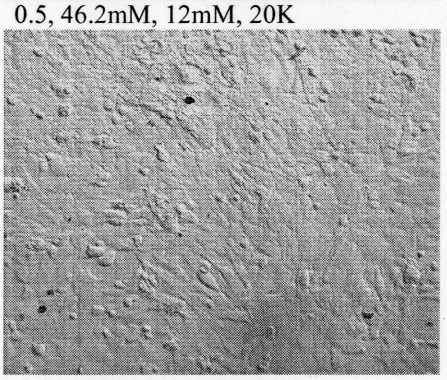
0.5, 46.2mM, 8mM, 20K



0.5, 46.2mM, 12mM, 20K

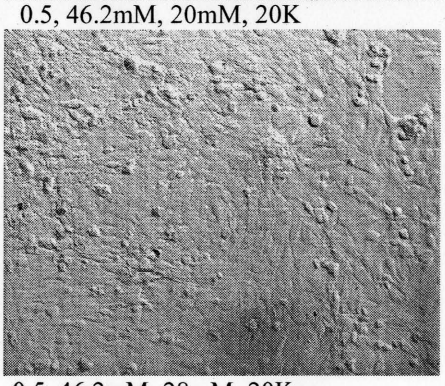


0.5, 46.2mM, 16mM, 20K



0.5, 46.2mM, 20mM, 20K

0.5, 46.2mM, 24mM, 20K



0.5, 46.2mM, 28mM, 20K

Figure 9. The effect of different concentration of $CaCl_2$ in media after 24 h.

Discussion

Cellular response to RGD-alginate surfaces:

Interaction of myoblasts with the alginate gel undergoes several important parameters.

- The amount of EDC present in the reaction had a strong effect on peptide incorporation. Two separate reactions need to be done on the carboxyl groups of the alginate: some are coupled to the EDC and some are crosslinked with $CaCl_2$. The order of these two reactions and the number of the G residue in the alginate determines the correct amount of EDC in the reaction. I checked with three different molar ratios of EDC : alginate subunits 1:10, 10:1 & 20:1. In the ratio of 20:1, there is excess EDC available to activate carboxyl groups, and it allowed good cell attachment and spreading. The ratio was reduced to 10:1 as the result of limitation in ionic gelling capacity of the alginate, rearrangement of the O-acyl urea to stable N-acyl urea in the alginate backbone, and difficulty of dialyzing the large amount of isourea by-product. The result showed that cells were elongated and organized because of making stiffer alginate gels [Fig. 10]. The ratio of 1:10, I did this experiment in my early work with the 0.02 RGD/alginate ratios, so no notable cell attachment was observed. It was probably due to the shortage of RGD peptide. I would think in case of consuming higher RGD ratio (≥ 0.1) it would make a considerable cell attachment. Some protocols reported 0.05-0.4 mg EDC/mg carboxyl-group compound for protein-peptide conjugation[30], and some mentioned at least 10 molar excess to the amount of carboxyl-group[8].

▪ The density of the adhesion ligand is another parameter that supports different cellular responses. The morphology of attached cells is dependent on the density of immobilized RGD. By varying the peptide density from 0.001-5 (the ratio of GRGD/Alginate), myoblasts spread to an increasing extent, and cell attachment area increased with increasing RGD density [Fig. 6] and cells looks bigger [Fig. 11 & 6]. At ratios of ≥ 5, peptides started to precipitate and at ratios ≤ 0.01, only a few cell attachment spots were observed. I concluded that a minimum ratio of 0.1, corresponding to a surface density of 50 $\mu M/cm^2$ was sufficient to promote cell spreading.

▪ Another parameter is cell density. Myoblast differentiation (conversion to myotubes) is stimulated by cell contact. Much evidence supports the idea that integrin adhesion receptors require aggregation for proper signal generation. Varying number (5,000, 10,000, 20,000 cell/cm^2) of cells seeded on the surface of GRGD-coupled alginate (in equal RGD density like RGD: Alginate 1:10) to compare cell morphology.

At 20,000, cells fused and aligned together and covered whole the surface like a tissue form while at 10,000, they are still like individual cells that were just align together. At 5,000 cell/cm^2 cells were almost confluent and started contracting in the spots where they found enough cells for interaction [Fig. 7].

▪ The rate and amount of attached myoblasts will be regulated by the mechanical properties of the alginate. Increasing the crosslinker density may have several effects on the alginate gel including: decreasing molecular mobility; changing

charge density; and increasing stiffness. Reports indicated high extracellular calcium content influences integrin affinity and binding.[20]

Cell differentiation on stiffer gels is very evident. Cells on stiffer gels show more organization than cells on either rigid glass or soft gel [Fig. 12].

Crosslinker density alters myoblast interaction with modified alginate surfaces. 1mg/ml of alginate gelled with 46 mM $CaCl_2$ made sufficient stability for cell attachment. Stiffer gels up to 46 mM $CaCl_2$ increased the rate, magnitude, and degree of spreading of myoblast adhesion to modified alginate [Fig. 8].

Mauck showed that different crosslinker species didn't change cell adhesion, and it appeared that alginate mechanics was the main factor to control cell attachment.[29] Mann demonstrated that the strong adhesion of cells to modified alginate had a negative effect on the production of ECM proteins as well as proliferation and migration of smooth muscle cells.[28]

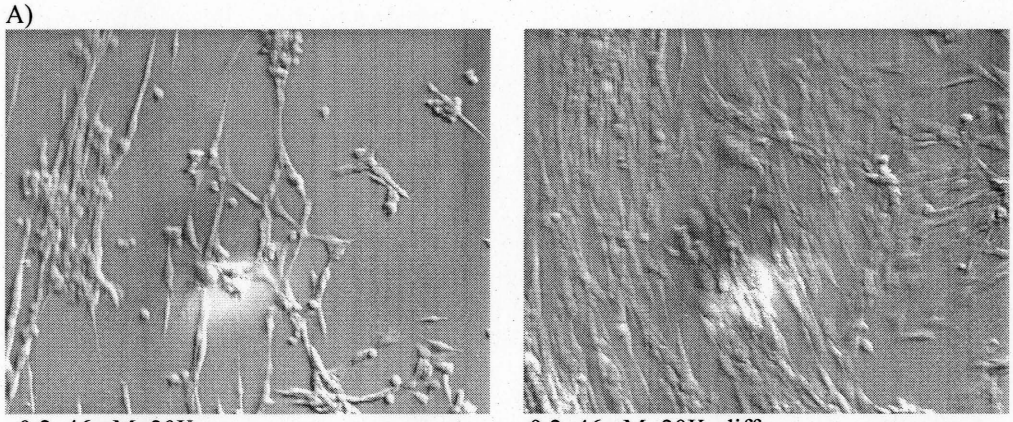
- The last parameter was to determine the effect of free Ca^{2+} ions on myoblasts. As the alginate gel is sensitive to chelating compounds such as phosphate, lactate, citrate, and presence of anti-gelling cations such as Na^{+} or Mg^{2+}, including free Ca^{2+} in media helps to prevent gel dissociation. The DMEM had1.8 mM $CaCl_2$ and 0.15 M NaCl.

Shoichet monitored the strength of cell containing cross linked gels for a 90 days period. Gel strength decreased 40% within 9 days compare to 30% control and decrease 70% within 30 days compare to 57% control and an equilibrium gel strength was achieved after 30 days.[21] This is due to the loss of divalent ions from

the hydrogels by ion exchange with monovalent ions (Na^+) in the surrounding fluid.

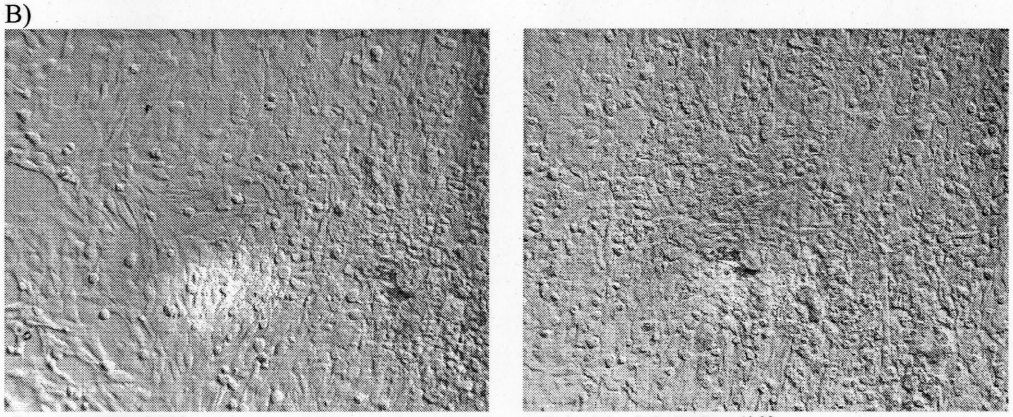
Various ways to overcome this limitation have been suggested. The simplest is to keep the gels in a medium containing a few milimolar free calcium ions [Fig. 9]. In terms of molecular biology, adding Ca^{2+}in media improved cell attachment because a) The integrin α subunit binds divalent cations (e.g. Ca^{2+}, Mg^{2+}), which are essential for integrin ligand binding function [Fig. 1].[11] b) In conversion of myoblasts into muscles, cells align together and fuse to form the multinucleated myotubes. As in most membrane fusions, calcium ions are critical, and fusion can be activated by calcium ionophores that carry ions across cell membranes.[26]

A)



0.2, 46mM, 20K 0.2, 46mM, 20K, diff

B)



0.1, 46mM, 20K 0.1, 46mM, 20K, diff

Figure 10. The effect of EDC : alginate ratio in cell attachment A) 10:1, B) 20:1

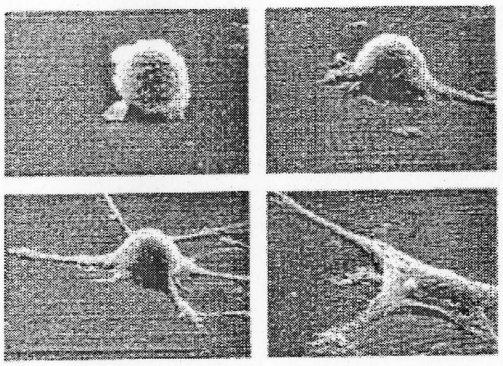
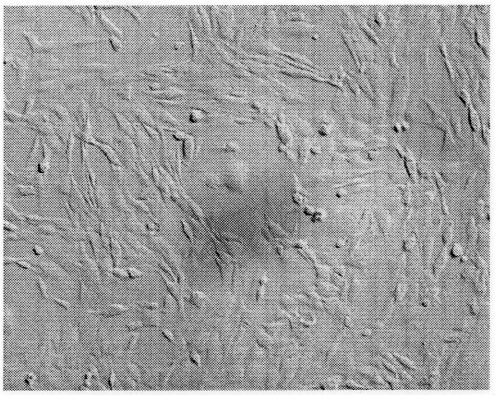
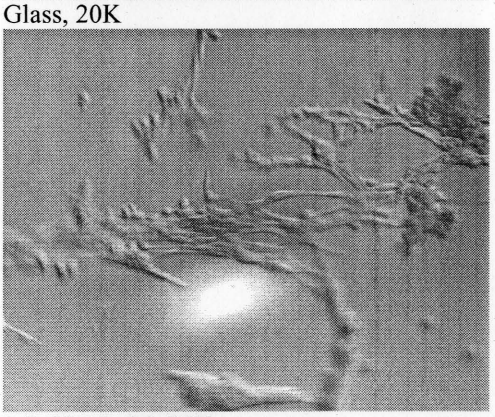


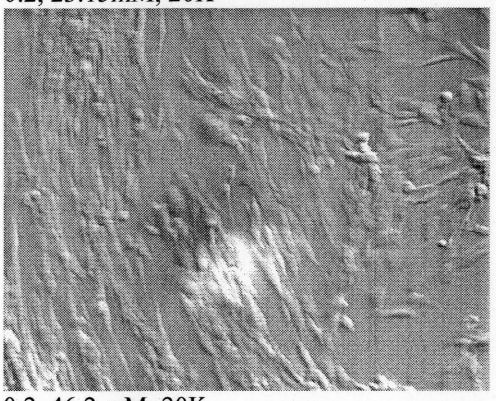
Figure 11. Cells adherent on substrates with increasing concentration of attached RGD.[29]



Glass, 20K



0.2, 23.15mM, 20K



0.2, 46.2 mM, 20K

Figure 12. Comparison of myoblast attachment in glass and in two different gel stiffness 23.15 & 46.2 mM $CaCl_2$.

Appendix A

Wild type C2 cell survival manual:

- The growth medium consisted of low glucose DMEM (Gibco Cat No. 11885) which was supplemented with glutamine and pyruvate 400 ml, Fetal Bovine Serum from Hyclone 20%, chick embryo extract 0.5% and penicillin-streptomycin at 100 units per ml.
- The differentiation medium was DMEM, horse serum 2% and penicillin/streptomycin.
- The cell preservation medium was DMSO 10% plus FBS in DMEM.

Thawing cells:

a) Thaw cells quickly by holding vial in 37° C water bath.

b) Wipe vial with 70% Ethanol.

c) Transfer cells to a 15 ml conical tube containing 10 ml growth medium.

d) Centrifuge for 3 min at 600 rpm and remove medium with Pasteur pipette connected to a vacuum line.

e) Add 10 ml growth medium and plate into a 100 ml dish.

f) Feed the next day with fresh growth medium.

Splitting cells:

To remain cells healthy over several consecutive passages, it is important not to let them grow too dense and to feed them at the same time. They do not like to be passed often at low density. Best is to keep them below 60% confluent and split no more than 1:40.

Split the cells using 0.05% trypsin-0.02% EDTA in saline (STV).

a) Aspirate off the growth medium.

b) Wash the cells with phosphate buffer serum (PBS 1X).

c) Add STV and wait 30 sec, aspirate the STV.

d) Put in incubator for 1 min, let the cells round up and detach.

e) Add growth medium to inactive the trypsin

f) Pipette the cells up and down to break up any clusters of cells.

g) Replace in a new plate at a dilution of 3000 /cm^2.

h) Feed cells daily or every other day.

Differentiating the wild C2:

a) Plate about 3000-cells/ cm^2 on day 1.

b) Feed with growth medium the next day or add extra medium and feed on the second day.

c) On day 3, remove the growth medium and replace by differentiation medium

In general, fusion really becomes apparent 24 hr after switching to differentiation medium (day 4), with good myotubes on the next two days. If the tubes start contracting, they are healthy. Unfortunately, they also have a tendency to come off the dish. C2C12 cells differentiate after 48 h in growth medium as well.

Freezing the wild C2 for posterity:

a) Label cryovials with cell line, passage number, date, and initials.

b) Trypsinize and count the cells.

c) Spin down; remove as much medium as possible.

d) Tap to disperse cells, resuspend the cells with cell preservation medium (CPM) (0.2 ml/200,000 cells).

e) Wrap in a blue diaper inside of a Styrofoam box and leave to slowly freeze in the –80°C overnight.

f) Transfer to liquid N_2 for long-term storage.

Appendix B

Previous works

- The generalized protocol for this water-soluble carbodiimide is:

Dissolve carboxyl-group compound in MES buffer at the concentration of 1-10 mg/ml; add amine-group peptide at least a 10 molar excess to the amount of carboxyl-group for small molecules; add EDC to obtain at least 10 molar excess of carboxyl-group (0.05-0.1M); add NHS to make final concentration to 5mM. The parameters of this generalized protocol may have to be modified to retain solubility or activity of the resulting conjugate.[8]

- Sehgal achieved high efficient water–soluble carbodiimide method with optimizing various parameters of the coupling reaction. The optimum condition for the conjugation of carboxyl group of butyric acid to the amino function of Affi-Gel 102 at ratio 1:10 (linear increase up to 1:5 & slightly increase up to 1:12) appeared to be pH 6.5, 25mM MES buffer and an NHS/EDC ratio of 1:15 for 12 h or longer at 4°C giving a maximum yield of up to 90%. These parameters showed the same result on N-carboxypentyl-1-deoxynojirimycin & Affi-Gel.[15]

- Rowley modified preformed-alginates (3M $CaSO_4$ was added to 0.1M Na-alginate) using carbodiimide chemistry. All reactions were performed in 0.1 M MES buffer containing 0.3 M NaCl at pH 6.5 for a peptide density of 1 mg GRGDY per gram alginate, alginate/EDC ratio of 20:1, NHS/EDC molar ratio of 1:2 to react for 20 h at room temperature with a reaction efficiency of 78%. They reported that penetration of reactants was 50 nm and RGD surface density was

1nmol/cm^2. Heavy-chain myosin marker was stained positively for this marker, confirming the differentiated gene expression of the Myoblasts on the surface-modified alginate gels. They also demonstrated that the soluble RGD blocked cell adhesion in a dose-dependent manner.[2]

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