Attachment of fibronectin to poly(vinyl alcohol) hydrogels promotes NIH3T3 cell adhesion, proliferation, and migration

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Abstract: Hydrogels have been used in biology and medicine for many years, and they possess many properties that make them advantageous for tissue engineering applications. Their high water content and tissue-like elasticity are similar to the native extracellular matrix of many tissues. In this work, we investigated the potential of a modified poly(vinyl alcohol) (PVA) hydrogel as a biomaterial for tissue engineering applications. First, the ability of NIH3T3 fibroblast cells to attach to PVA hydrogels was evaluated. Because of PVA's extremely hydrophilic nature, important cell adhesion proteins do not adsorb to PVA hydrogels, and consequently, cells are unable to adhere to the hydrogel. By covalently attaching the important cell adhesion protein fibronectin onto the PVA hydrogel surface, the rate of fibroblast attachment and proliferation was dramatically improved, and promoted two-dimensional cell migration. These studies illustrate that a fibronectin-modified PVA hydrogel is a potential biomaterial for tissue engineering applications. © 2001 John Wiley & Sons, Inc. J Biomed Mater Res 57: 217–223, 2001

Key words: tissue engineering; hydrogel; poly(vinyl alcohol); polymer scaffold; fibronectin

INTRODUCTION

Hydrogels are highly hydrated, polymer networks composed of water-soluble macromolecules held together by crosslinks formed via chemical bonds, ionic interactions, hydrogen bonds, hydrophobic interactions, or physical bonds.[1] The molecules of the extracellular matrix, such as proteins and polysaccharides, are generally hydrophilic molecules and, from a material's point of view, are essentially hydrogels.[2] Due to their similarity with the extracellular matrix, synthetic hydrogels have been investigated for uses in biology and medicine since Wichterle and Lim discovered them in the early 1960s.[1,3] Hydrogels possess many characteristics that make them highly attractive for tissue engineering applications. The hydrogel material properties, such as permeability, mechanical strength, and biocompatibility, can easily be engineered for the particular application of choice.[4] Their high water content allows easy exchange of nutrients and wastes with the surrounding environment. In addition, the tissue-like elasticity of hydrogels plays a vital role as these biomaterials are eventually integrated into the human body.

Numerous current tissue engineering efforts are focusing on hydrogel biomaterials as scaffolds for the partial or full regeneration of organs. These materials are based upon natural polymers, including alginates,[5] collagen,[6] gelatin,[7] and agarose,[8] but also synthetic polymers such as poly(hydroxyethyl methacrylate),[9] poly(ethylene oxide),[10] and poly(acrylonitrile-sodium methallyle sulfonate).[11] Our laboratory is investigating the use of poly(vinyl alcohol) in tissue engineering applications.

For most tissue engineering applications, it is a general requirement that cells adhere to the particular support material or scaffold that is being used. Cell attachment has been shown to strongly influence cell proliferation, migration, differentiation, and, of particular importance from a tissue engineering standpoint, extracellular matrix production.[8] Therefore, scaffolds that promote attachment of cells are important; however, cells do not adhere directly to biomaterial surfaces but rather bind through a variety of adhesion proteins, which adsorb onto the biomateri-

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al.[12] Fibronectin, laminin, and vitronectin are important cell adhesion proteins that mediate various aspects of cellular associations with extracellular matrices through a variety of integrin–ligand interactions.[13] Integrins essentially form a "bridge" between adhesion proteins and the cytoskeleton.[14,15] anchoring these cells to the biomaterial or extracellular matrix. This interaction of integrins and surface-adsorbed adhesion proteins is primarily responsible for the ability of cells to adhere to the surface of a biomaterial.

The attachment of cells to various polymeric substrates is often dictated and controlled by the ability of adhesion proteins to adsorb onto the polymer surface; cells cannot adhere to a surface unless cell adhesion proteins are present. In general, the driving force for protein adsorption to hydrophilic surfaces is enthalpic, whereas the driving force for adsorption to hydrophobic surfaces is entropic.[16] Water molecules near a hydrophobic surface are in a highly ordered structure, and protein adsorption to hydrophobic surfaces disrupts this order, causing an increase in entropy and a thermodynamically favorable driving force. As a result, protein adsorption to hydrophobic surfaces is usually irreversible, as opposed to the reversible adsorption of proteins to hydrophilic surfaces. Researchers[17–19] have shown that maximal cell adhesion to polymeric surfaces occurs on surfaces of moderate water wettability. Surfaces of high or low water wettabilities discouraged cell adhesion. For example, the highly hydrophobic polymer poly(tetrafluoroethylene) promoted less cell adhesion than the moderately hydrophobic polystyrene.[17,18] In contrast, poly(ethylene glycol) does not support cell attachment due to its highly hydrophilic nature. Although cell adhesion proteins bind stronger to surfaces with greater hydrophobicity,[19] there is an associated loss of activity. For example, on a highly hydrophobic surface, fibronectin may unwind into an inactive filamentous structure, and this denatured form of fibronectin promotes less cell adhesion.[20,21] Recently, fibronectin was covalently linked to the end groups of the triblock copolymer Pluronic F-108, and used as a means to immobilize fibronectin onto the surface of a PEO-containing surface.[22] Surface-bound fibronectin promoted sensory neurite outgrowth, which was not observed in the absence of bound fibronectin.

Our laboratory is investigating the use of hydrogels based on poly(vinyl alcohol) (PVA) as scaffold materials for a variety of tissue engineering applications, including bone, cartilage, and the aortic heart valve. Because it is well known that hydrogels discourage protein adsorption, we wanted to first determine the extent to which proteins adsorb onto PVA hydrogels. We then conducted experiments to see if we could promote attachment of a cell line, NIH3T3 fibroblasts, onto PVA hydrogels by attaching the important cell adhesion protein fibronectin. Finally, we measured the proliferation rate and migration behavior of fibroblasts on a fibronectin-modified PVA hydrogel.

MATERIALS AND METHODS

Hydrogel synthesis

A 20% (w/w) solution of poly(vinyl alcohol) (6,000 MW, Polysciences) in dH_2O was prepared at 80°C and cooled to room temperature. Glutaraldehyde (25 wt % solution in dH_2O, Sigma) was added to a final concentration of 1 wt %. Three milliliters of the polymer/glutaraldehyde solution was placed in a scintillation vial (Fisher) and 10 drops of 2 *M* HCl was added. Upon addition of the HCl, gelation occurs quickly (within several minutes), so the polymer solution was immediately stirred, placed between two glass slides, and left for 10 min. Disks of diameter 14 mm were cut from this hydrogel sheet (~1 mm thick) and used in subsequent studies.

Protein adsorption studies

To evaluate the adsorption of serum proteins to PVA hydrogels, a method developed by Faucheaux et al.[23] was used. The glutaraldehyde-crosslinked PVA hydrogels were equilibrium swollen in phosphate buffered solution (PBS, Gibco) by rinsing several times over 2 h. The disks were then incubated in fetal bovine serum (Gibco) (2 mL, 50% solution in PBS) at 37°C for 45 min in a 12-well plate (Falcon). After the prescribed incubation time, the disks were rinsed with three consecutive washes of 1 mL of dH_2O for 20 min each at room temperature; all washes were removed and placed in 1 mL eppendorf tubes. Subsequently, each disk was incubated with graded isopropanol solutions (10, 30, 50, and 70% in dH_2O, 1 mL per rinse) at room temperature for 20 min. The washes were decanted and transferred to 1 mL eppendorf tubes. All eppendorf tubes were left open and placed in a vacuum oven overnight to remove isopropanol. The samples were then frozen and lyophilized for analysis.

SDS-PAGE analysis

The products of the water rinses and graded isopropanol washes were evaluated for protein content by performing sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE). By running a protein gel, the adsorption strength of serum proteins to PVA can be determined. A Gibco Mini-V 8/10 Vertical Gel Electrophoresis System and power supply were used to produce the protein gel. The separating gel buffer consisted of 500 mL dH_2O, 90.8 g Tris-Base (Fisher), pH 8.8 with HCl, and 25 mL 10% sodium dodecyl sulfate (SDS, Gibco). The stacking gel buffer was made of 250 mL dH_2O, 15.1 g Tris-Base, pH 6.8 with HCl, and 12.5 mL 10% SDS. Running buffer consisted of 600 mL

dH_2O, 2.35 g Tris-HCl (Fisher), 11.25 g glycine (Fisher), and pH 8.3 with NaOH. The acrylamide solution was made of 29% (w/v) acrylamide (Gibco) and 1% (w/v) *N,N′*-methylenebisacrylamide (Gibco) in dH_2O.

Protein gel samples were prepared by reconstituting the lyophilized samples with 25 μL dH_2O and combining with 25 μL 8 *M* urea (Gibco), 10 μL 0.1 *M* dithiothreitol (Gibco), and 6 μL bromophenol blue (Sigma) solution (2.5 mg bromophenol blue, 2.5 mL glycerol, 5 mL stacking gel buffer). These samples were boiled for 5 min prior to loading in the gel. Samples were run on an 8% polyacrylamide denaturing gel (separating gel: 2.46 ml acrylamide solution, 2.31 mL separating gel buffer, 4.69 mL dH_2O, 50 μL 10% ammonium persulfate [APS, Polysciences], and 7.5 μL TEMED [Gibco]; stacking gel: 0.25 mL acrylamide solution, 0.625 mL stacking gel buffer, 1.62 mL dH_2O, 7.5 μL 10% APS, and 5.0 μL TEMED). Five microliters of markers (Benchmark standards, Gibco) and 10 μL of each sample were run on the protein gel. The first water rinse was diluted 20 times to decrease the intensity, but all others were run undiluted. The gel was run at constant voltage (80 V) for several hours until the dye front had reached the bottom of the gel. Coomassie blue [Gibco, 0.25% (w/v) in 50% acetic acid] was used to stain the gel for 10 min at 4°C, and then the gel was destained over the course of several days using a destaining solution (10% acetic acid, 10% methanol in dH_2O).

Fibronectin-modified PVA hydrogel surfaces

PVA hydrogel disks were modified with fibronectin to promote cell attachment. First, a long alkyl chain containing an acid group was linked to the hydroxyl groups of PVA. These acid groups were then activated towards nucleophilic attack by amine groups found on fibronectin. Four hydrogel disks were added to 10 mL of 3.0 *M* NaOH in a 20 mL scintillation vial. 11-Bromoundecanoic acid (11-BUDA, Aldrich) was added (100 mg), and the vial was stirred at 37°C for 2 h. The disks were rinsed several times with water to remove unreacted 11-BUDA and reaction byproducts. The disks were then placed in a vacuum oven overnight to dry completely. Acetone (10 mL) and carbonyl diimidazole (CDI, Aldrich) were added to four disks in a scintillation vial, and the CDI was allowed to react with the pendant acid groups for 2 h at room temperature. Disks were then rinsed several times with acetone, allowed to dry, and sterilized under ultraviolet light in a sterile hood for several hours. A sterile fibronectin solution (50 μg/mL in 0.1 *M* sodium carbonate buffer, pH 9–10) was prepared, and this solution was added to disks in a 10 mL conical tube. This coupling reaction of the fibronectin to the PVA hydrogel occurred at room temperature for 2 h. The disks were then rinsed several times with PBS to remove any unreacted fibronectin. A control was performed in which every step was followed identically to the above, except CDI was left out of the CDI activation step.

Verification of fibronectin covalent attachment

To verify covalent linking of the fibronectin to the PVA surface, immunostaining was utilized. The fibronectin-modified PVA hydrogels were blocked with block solution [3% bovine serum albumin (BSA, Sigma), 2% normal goat serum (NGS, Sigma)] for 15 min at room temperature. A primary antibody directed against fibronectin (host animal: mouse, Sigma) was diluted in block solution (1:100) and incubated with the PVA hydrogel in a humidity chamber for 1 h at room temperature. The disk was then rinsed with PBS three times for 5 min each, and subsequently incubated with a secondary antibody (Rhodamine RedX-conjugated goat anti-mouse, Jackson ImmunoResearch) for 1 h at room temperature. Again, the disk was rinsed three times with PBS for 5 min each. Finally, the disk was visualized using fluorescence microscopy. Prior to antibody staining, the disk was washed with 70% isopropanol to desorb any adsorbed proteins. Thus, any immunofluorescence is due to covalent attachment of fibronectin to the surface region of the PVA hydrogel. Fibronectin's rate of diffusion through the gel is small compared to its rate of reaction at the surface, so it is thought that most of the fibronectin is found either on or within a small distance of the gel surface. Control hydrogels (CDI activation step omitted) were also stained in a similar manner.

Cell seeding

The attachment and proliferation of NIH3T3 fibroblasts (American Type Tissue Collection) on fibronectin-modified PVA hydrogels were evaluated. Cells were seeded onto the hydrogel surfaces at 25,000 cells/cm^2 in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (Gibco), gentamicin (Gibco) and Pen-Strep (Gibco). At various time points, disks were removed from culture conditions, rinsed briefly in PBS, and fixed in a 10% formalin solution. Photomicrographs of the cell attachment and spreading were taken, and the number of cells attached was averaged over several views of the same polymer surface to provide cell attachment as a function of time. Cell counting at each time point was performed in triplicate.

Cell migration

The ability of cells to migrate two-dimensionally on the surface of these fibronectin-modified hydrogels from a highly confluent area to a less populated area was analyzed. A glass slide was clamped onto a disk so that half of the gel surface was blocked whereas half was not. NIH3T3 fibroblasts were seeded onto the unblocked area at 25,000 cells/cm^2, and were allowed to become a confluent cell layer. Subsequently, the glass slide was removed and the ability of cells to migrate was evaluated. Photomicrographs of the migration behavior of cells from confluent areas to unpopulated areas of the hydrogel surface were obtained.

RESULTS AND DISCUSSION

Gel synthesis

PVA hydrogels were made by crosslinking aqueous solutions of poly(vinyl alcohol) with glutaraldehyde.

The aldehyde groups present on each molecule of glutaraldehyde react rapidly under acidic conditions with the abundant hydroxyl groups found on PVA to form stable acetal linkages.

Protein adsorption

Protein adsorption to PVA hydrogels was evaluated using SDS-PAGE. Glutaraldehyde-crosslinked PVA hydrogels were incubated with serum, and nonadsorbed serum proteins were removed by rinsing three times with water. Essentially, any proteins removed by the water rinses were considered unadsorbed to the polymer. To desorb any adsorbed proteins, varying concentrations of isopropanol were used. Isopropanol desorbs any adsorbed proteins; a high concentration of isopropanol is required to remove strongly adsorbed proteins. Figure 1 shows the SDS-PAGE results to examine the adsorption of proteins from fetal bovine serum onto a glutaraldehyde-crosslinked PVA hydrogel, and it is representative of several repeats of the same experiment. Protein molecular weight standards are in lane 1. Lanes 2–4 are proteins removed from the hydrogel using water rinses at room temperature. Lanes 5–8 show proteins removed by the graded isopropanol rinses (10, 30, 50, and 70%). As can be seen in Figure 1, the first two water washes contain proteins, but there are no proteins in the third water wash or any of the graded isopropanol rinses. In light of these results, it can be concluded that serum proteins do not adsorb strongly onto PVA hydrogels. It is likely that the important cell adhesion proteins, such as fibronectin and vitronectin, are not adsorbing onto these hydrogels; thus, cells are unable to adhere to the PVA hydrogel.

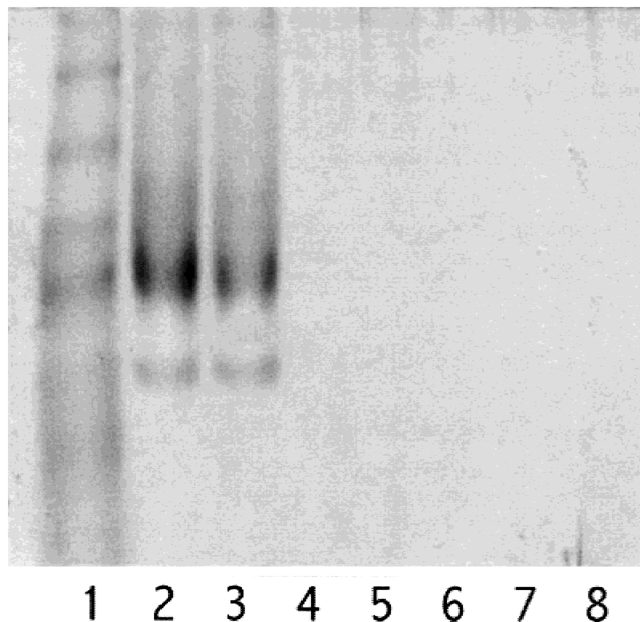


Figure 1. Serum protein adsorption experiment. Serum proteins were incubated with PVA-GA disks and rinsed three times with dH_2O (lanes 2–4) followed by graded isopropanol rinses (10, 30, 50, and 70%; lanes 5–8). Standards are in lane 1.

Covalent attachment of fibronectin

Cell attachment to the polymer scaffold is important for tissue engineering applications. Because cell adhesion proteins do not adsorb to PVA hydrogels, it is necessary to modify the polymer scaffold to promote cell adhesion. Using several steps, fibronectin was covalently linked to the surface of glutaraldehyde-crosslinked PVA hydrogels. Figure 2 shows the chemistry of this modification. The first step involves linkage of an 11-carbon spacer to the PVA backbone. Under basic conditions, the alkyl halide 11-bromoundecanoic acid reacts with the hydroxyl groups of PVA to form a stable ether linkage. The terminal acid group is then activated with carbonyl diimidazole (CDI). The activated acid group, a highly reactive *N*-acylimidazole, is much more susceptible to nucleophilic attack than the original acid. Subsequent reaction with fibronectin occurs most efficiently in buffered solution (pH 9–10) primarily through the nucleophilic primary amine groups found at the *N*-terminus and on the lysine residues of fibronectin. Figure 3 shows indirect immunofluorescence of the fibronectin-modified PVA hydrogel. Also shown is a control experiment in which the steps were exactly identical except that the CDI activation step was left out. To alleviate concerns with potential protein adsorption rather than covalent attachment of the fibronectin, the gel was treated with 70% isopropanol to desorb any adsorbed proteins prior to performing the immunostaining. However, fibronectin adsorption to the gel was not expected, based on the protein adsorption studies presented above. Results show no fibronectin attachment in the negative control, and widespread attachment of fibronectin to the activated PVA surface with regions of varying attachment density.

NIH3T3 fibroblast attachment

Fibroblast cells were seeded on unmodified and fibronectin-modified PVA hydrogels, and their attachment behavior is shown as a function of time in the photomicrographs presented in Figure 4. After 1 h, cells have begun to attach to the FN-modified gel, and the cells show excellent spreading after 8 h. At 16 h, the cells have proliferated and are almost confluent. Compared to the control gels at all time points, which show little attachment, there is a dramatic improve-

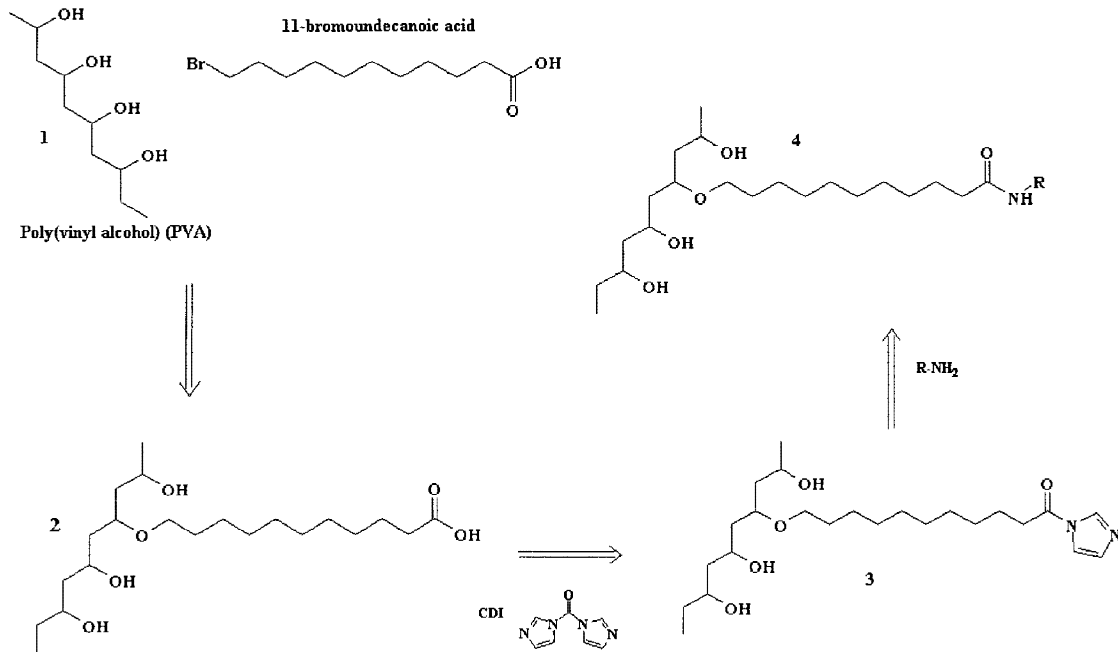


Figure 2. Chemistry of attachment of fibronectin to poly(vinyl alcohol) hydrogels. The hydroxyl group is first converted to an acid group using 11-bromoundecanoic acid, which is then converted to a highly reactive *N*-acylimidazole using carbonyl diimidazole (CDI). The amine groups found on fibronectin rapidly react with the activated acid group, providing a stable link between fibronectin and the polymer chain.

ment of cell adhesion and spreading on the fibronectin-modified gels. Moreover, cells that do attach to the control surfaces are unable to spread out on the surface; instead, they "clump" together and they appear to adhere to each other rather than the hydrophilic PVA surface. From these results, the covalently bound fibronectin is likely presented to the cells in an active form and is promoting cell adhesion.

Cell proliferation

Fibroblast proliferation was measured as a function of time on tissue culture polystyrene (as a control), unmodified PVA hydrogels, and fibronectin-modified gels. Figure 5 shows the proliferation of cells with time on these substrates. As can be seen, fibroblasts proliferate and divide more rapid on the fibronectin-modified gels than on tissue culture polystyrene. Cells seeded on the unmodified PVA gels have extremely low proliferation rates.

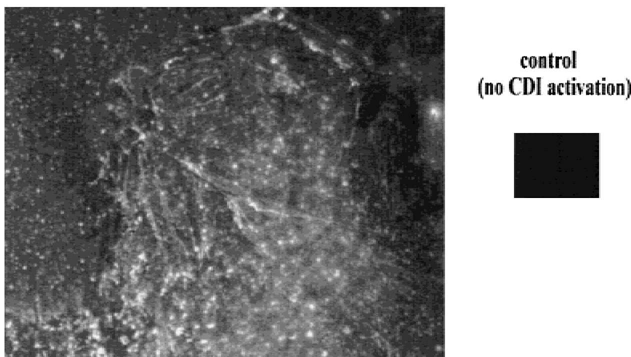


Figure 3. Indirect immunofluorescence to verify covalent attachment of fibronectin to the hydrogel surface. The surface was treated with isopropanol prior to staining to desorb any adsorbed proteins.

Fibroblast migration

Figure 6 shows a schematic of the fibroblast migration experiment, and a picture obtained during the experiment. A glass slide was used to mask half of the gel, and cells were seeded onto the unmasked side. After confluency, the glass slide was removed and the cells were able to migrate two-dimensionally to the unpopulated area. Figure 6 shows migration 3 h after removal of the glass slide. The initially unpopulated area eventually becomes confluent with cells. These experiments show that fibroblasts are able to migrate two-dimensionally on fibronectin-modified PVA hydrogels from an area or high cell density to one of low cell density.

CONCLUSIONS

PVA hydrogels have many advantages that make it an ideal scaffolding material for tissue engineering

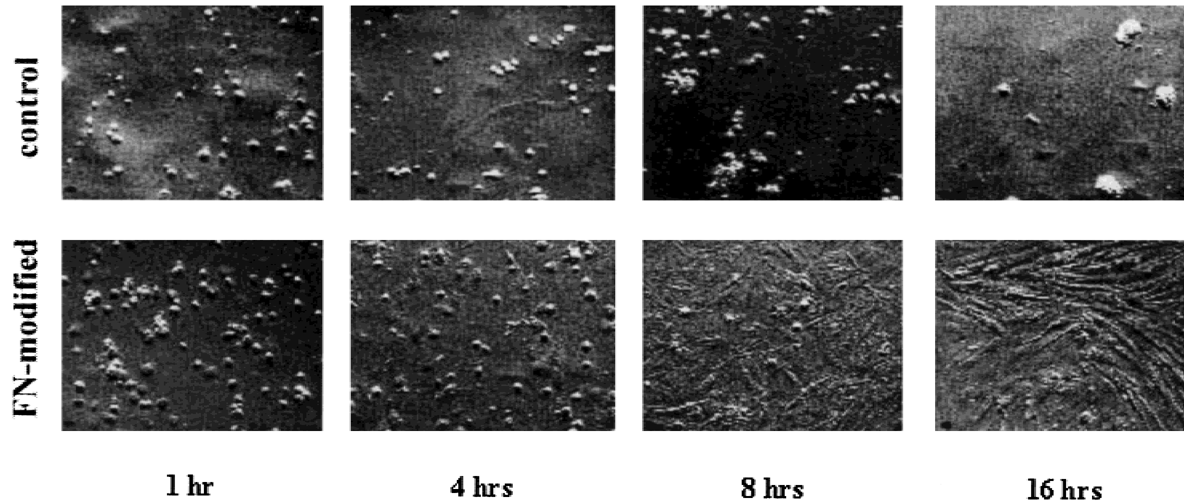


Figure 4. NIH3T3 skin fibroblasts plated at a density of 25,000 cells/cm^2 on a control substrate of glutaraldehyde-crosslinked PVA hydrogel compared to a fibronectin-modified PVA hydrogel. All micrographs are shown at 100× original magnification, except the 1-h experiment, which is 200×.

purposes, including its tissue-like elasticity, high water content, and ease of fabrication and sterilization. In addition, the abundant hydroxyl groups found along the PVA backbone can be easily modified to incorporate a wide range of molecules throughout the scaffold. However, due to the inability of adhesion proteins such as fibronectin to adsorb onto PVA, it is essential to modify the PVA hydrogel such that it promotes cell adhesion.

In this work, we first showed that proteins do not adsorb to glutaraldehyde-crosslinked PVA hydrogels. We then described a new method for attaching proteins to the surface of these hydrogels, and further showed that covalent attachment of the cell adhesion protein fibronectin dramatically increases the attachment of NIH3T3 fibroblast cells. Furthermore, we showed that covalent attachment of fibronectin to the hydrogel surface significantly improves cell proliferation and encourages cellular migration on fibronectin-modified PVA hydrogel surfaces. These studies show that the covalent linkage of the important cell adhesion protein fibronectin can facilitate the attachment of cells to PVA hydrogels, and this modification will play a vital role as we use this material for tissue engineering applications.

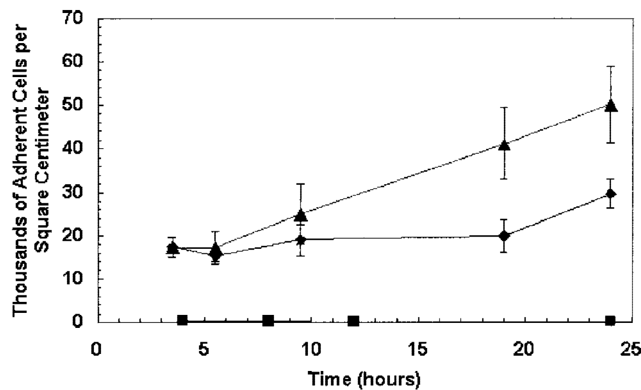
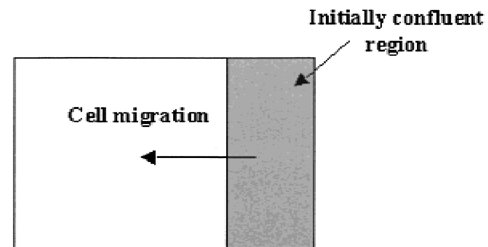


Figure 5. Proliferation of NIH3T3 fibroblasts on tissue culture polystyrene (♦), unmodified PVA hydrogels (■), and FN-modified PVA hydrogels (▲). Cells were initially seeded at ~25,000 cells/cm^2.



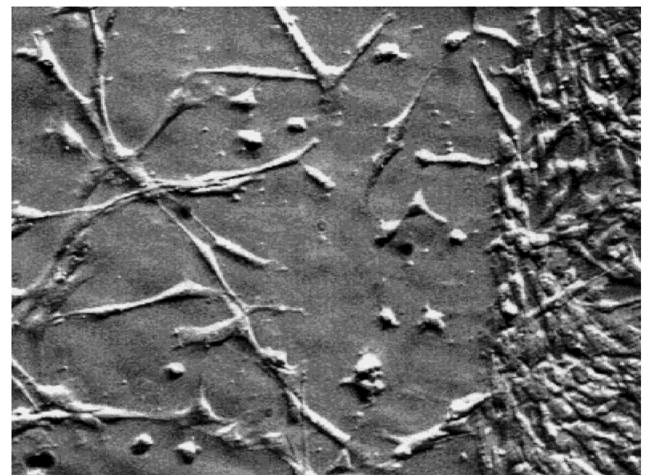


Figure 6. NIH3T3 fibroblasts initially seeded onto a semi-masked FN-modified PVA hydrogel (right side of micrograph) and grown to confluence. The mask was removed and cell migration was monitored as a function of time (3-h time point shown here) (original magnification 50×).

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