Photocrosslinkable Hydrogel for Myocyte Cell Culture and Injection

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Abstract: Conventional treatment options for myocardial infarction are limited by the inability of mature myocardium to regenerate after injury. Although functional improvements after injection of cells and growth factors have been demonstrated, the clinical utility of this procedure has been hampered by poor cell localization, low survival, and rapid clearance of injected growth factors. The main objective of this study was to evaluate the applicability of a hydrogel, based on photocrosslinkable chitosan and acryloyl-poly(ethylene glycol)-RGDS (Az-chitosan/Acr-PEG-RGD) for myocyte cell culture and myocardial injection. Chitosan was modified with photoreactive azidobenzoic acid and Acr-PEG-RGD was synthesized by reacting YRGDS with an equimolar amount of acryloyl-PEG-*N*-hydroxysuccinimide. For injection and encapsulation each polymer was dissolved in Di-H_2O (pH 6.4), the solutions were mixed and crosslinked by UV application (4 mW/cm^2). C2C12 myoblasts proliferated and differentiated on hydrogels containing 5 m*M* RGD but not on the pure photocrosslinked chitosan. *In vitro*, the crosslinked hydrogels retained 80% of encapsulated VEGF for 24 days. Live/dead staining of neonatal rat cardiomyocytes encapsulated into Az-chitosan/Acr-PEG-RGD hydrogels indicated high cell viability upon UV crosslinking. *Ex vivo*, we localized the hydrogel on the surface and in the ventricle wall of an adult rat heart by brief (2 min) UV light application. © 2006 Wiley Periodicals, Inc. J Biomed Mater Res Part B: Appl Biomater 81B: 312–322, 2007

Keywords: heart; cell injection; cardiomyocyte; myocardial infarction; RGD; photocrosslinkable hydrogel

INTRODUCTION

Nearly eight million people in the United States have suffered from myocardial infarction, with 800,000 new cases occurring each year.[1] Myocardial infarction (MI) results in the death of one billion cardiomyocytes in the infarct zone followed by pathological remodeling of the heart. Upon myocardial infarction, a vigorous inflammatory response is elicited and dead cells are removed by bone marrow derived macrophages. Over the subsequent weeks to months, fibroblasts and endothelial cells proliferate forming granulation tissue and ultimately dense collagenous scar. Formation of the scar tissue severely reduces contractile function of the myocardium and leads to the ventricle wall thinning and dilatation, remodeling, and ultimately heart failure. Conventional therapies are limited by the inability of the myocardium to regenerate after injury and the shortage of organs available for transplantation.[2]

Cell and growth factor based therapies have been considered as novel and potentially curative treatment options.[3] Vascularization and improvement of the left ventricular (LV) function following MI was attempted by sustained release of bFGF[4,5] or injections of VEGF.[5–7] The main limitation in growth factor application was their high diffusibility and short half-life when implanted *in vivo*. Regeneration of infarcted myocardium in animal models has been attempted by transplantation of various cell types including: skeletal myoblasts,[8] fetal and neonatal cardiomyocytes,[9–11] cardiomyocytes derived from embryonic stem (ES) cells,[12] and bone marrow derived mesenchymal stem cells.[13] In all cases, the cells were suspended in saline or culture medium followed by intramyocardial or coronary injection. As a result, significant numbers of cells were washed out from the

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injection site,[14] with as little as 15% of injected cells surviving 12 weeks after transplantation.[9]

In an attempt to improve retention and survival, the cells were injected into the heart using crosslinkable biomaterials. Kofidis and coworkers[15] reported that injection of Matrigel or Matrigel and ES cells into infarcted rat hearts resulted in structural stabilization, prevented wall thinning, and improved fractional shortening of the ventricle. Chirstman et al.[16,17] demonstrated that injection of skeletal myoblasts into myocardial infarcts using fibrin glue increased cell localization within the infarct after five weeks, reduced infarct size and increased vascularization of the scar without causing a significant inflammatory response or foreign body reaction. Similarly, Ryu et al.[18] found that injection of bone marrow mononuclear cells (BMNC) into cryoinjured rat myocardium using fibrin matrix increased the amount of viable tissue, improved microvessel formation and reduced the amount of fibrous tissue in comparison with the injection of BMNC in culture medium or culture medium alone. Recently, it was demonstrated that a synthetic material, self-assembling peptide hydrogel, can also be utilized for cell injection into the myocardium.[19] Upon injection, the peptide formed a nanofibrous structure that promoted recruitment of endogenous cells expressing endothelial markers, and supported the survival of injected cardiomyocytes.

Although the use of hydrogels represents a significant improvement over injections with saline or culture media, limitations in respect to the hydrogel mechanical stability, composition, and the control over crosslinking process still remain. An ideal injectable biomaterial should (i) provide localization of angiogenic factors; (ii) support attachment and differentiation of injected cells; (iii) crosslink fast enough to remain at the desired site upon injection, and (iv) remain mechanically stable enough to provide temporary scaffolding and structural support thereby preventing thinning of the ventricle wall upon MI.

The main objective of this study was to evaluate the applicability of a hydrogel, based on photocrosslinkable chitosan and acryloyl-poly(ethylene glycol)-RGDS (Acr-PEG-RGD) for myocyte cell culture and myocardial injection. Chitosan has been known to be a biocompatible and biodegradable polysaccharide.[20] Photocrosslinkable chitosan has been used to seal experimentally induced lesions in the small intestine, trachea, and thoracic aorta.[21] It accelerated wound closure and healing in a mouse model.[22] Growth factors (FGF-1 FGF-2, VEGF) can be released from the crosslinked chitosan and induce revascularization when implanted subcutaneously in mice.[23] Coating of vascular Dacron grafts with photocrosslinkable chitosan inhibited infection with *E. coli* upon implantation in rabbits. The Acr-PEG-RGD component of this system has been used for both the attachment of cells on the surface and encapsulation of cells within hydrogels.[24] Finally, since the hydrogel forms upon brief (0.5–3 min) exposure to ultraviolet light, its localization can be precisely controlled in a convenient manner during surgery.

In this study, we evaluated the mechanical properties and gelation time of the proposed hydrogel. We determined the ability of the hydrogel to support growth and differentiation of myocyte cells, prevent rapid diffusion of growth factors, and localize at the desired site in the rat heart *ex vivo*.

METHODS

Hydrogels

Chitosan, a partially deacetylated chitin (1,4 β-linked *N*-acetyl-D-glucosamine), was modified with photoreactive azidobenzoic acid to make an *in situ* photocrosslinkable hydrogel, based on modification of a previously reported method.[21] Chitosan glutamate (Protasan UP G113; M_w: <200 kDa; degree of deacetylation: 75–90%) was obtained from Novamatrix (Norway). 4-Azidobenzoic acid was obtained from TCI America (Portland, OR, USA). Briefly, 200 mg (1.24 mmol) of chitosan glutamate was dissolved in 15 mL distilled water. *N*,*N*,*N*′,*N*′-Tetramethylethylenediamine (TEMED) 116.2 mg, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) 70 mg (0.451 mmol), and 4-azidobenzoic acid (ABA) 40 mg (0.245 mmol) were added to the chitosan solution. The reaction was conducted at pH 5 overnight, and the modified chitosan was purified by ultrafiltration. The chemical structure of Az-chitosan was elucidated using ^{1}H NMR (Varian Mercury 300 MHz).

To synthesize the acryloyl-poly(ethylene glycol)-RGDS (Acr-PEG-RGD) macromer, Tyr-Arg-Gly-Asp-Ser (YRGDS, 1 mg/mL) was reacted with an equimolar amount of acryloyl-PEG-*N*-hydroxysuccinimide (3400 Da) in 50 m*M* sodium bicarbonate buffer (pH 8.2) for 2 h at room temperature.[24] The macromer was dialyzed against Di-H_2O overnight and lyophilized to obtain a water soluble white powder that was stored frozen prior to use. For gelation, each polymer was dissolved in Di-H_2O. For a mixture of the two polymer solutions, pH of the Acr-PEG-RGD solution was adjusted to 6.4, and then the two polymer solutions were mixed to the desired final concentration (20 mg/mL each). The hydrogels were formed by a free-radical photoinitiated polymerization upon UV illumination (Black-Ray, UVP, radiation range 315–400 nm, peak at 365 nm $\sim$4 mW/cm^2).

Gelation Time

A hundred microliters of Az-chitosan solution (20 mg/mL in Di-H_2O); a mixture of Az-chitosan and Acr-PEG-RGD solution (20 mg/mL in Di-H_2O each) or a mixture of Az-chitosan and PEG (3400 Da, Polysciences) solution (20 mg/mL in Di-H_2O each) was put on a petri-dish (100 $\times$ 20 mm^2, VWR), and a magnetic stirring bar (Teflon fluorocarbon resin, 5 $\times$ 2 mm^2, Fisher Scientific) was placed in the center of the solution droplet. The solution was stirred at 155 rpm using a Corning model PC-320 hot plate/stirrer under UV illumination from a distance of 2 cm. The gelation time was decided when the solution formed a solid globule, which completely separated from the bottom of the dish. The results were reported as averages and standard deviations of five independent measurements per gel.

Rheological Measurement

Cylindrical chitosan gels were prepared by adding gel precursor solutions into a rubber mold sandwiched between two glass slides followed by crosslinking with UV irradiation as described above. The diameter and the thickness of the prepared hydrogel were 8 mm and 2.8 mm, respectively. Gels were then transferred to an AR1000N rheometer (TA Instruments, New Castle, DE). Rheological measurements were conducted using a parallel 8-mm diameter plate at room temperature. Shear modulus, G, was measured by the creep test. The hydrogels were subjected to a constant shear stress (5, 10, 20, or 40 Pa) for 90 s and then allowed to recover for 90 s. After $\sim$60 s in each step, the strain reached a constant value. Shear modulus was determined as a reciprocal of the slope of the strain (read at the end of the recovery step) vs. stress curve. The results were reported as averages and standard deviations of four independent measurements per gel.

Scanning Electron Microscopy

The morphology of crosslinked chitosan gels was examined by scanning electron microscope (JEOL JSM 6060, JEOL USA, Inc., Peabody, MA). The lyophilized gels were fractured after cooling in liquid nitrogen to expose the structures inside the gels. The samples were sputter-coated with palladium and gold (100 Å thick) prior to observation.

Cells

C2C12 cells (ATCC), a cell line derived from murine myoblasts, were subcultured in T-75 flasks (P4–P9) in Dulbecco's modified eagle medium (DMEM) containing 4.5 g/L glucose, supplemented with 10% fetal bovine serum (FBS), 10 m*M* *N*-2-hydroxyethylpiperazine *N*′-2-ethanesulfonic acid (HEPES), 2 m*M* L-glutamine, and 100 units/mL penicillin. For cell attachment studies, C2C12 cells at passage numbers ranging from 4 to 9 were dissociated with trypsin and counted using a hemocytometer.

Cardiomyocytes were obtained from 1 to 2 days old neonatal Sprague Dawley rats, using a protocol approved by the Institute's Committee on Animal Care as previously described.[25] In brief, ventricles were quartered, incubated overnight at 4°C in a 0.06% (w/v) solution of trypsin in Hank's balanced salt solution (HBSS), washed in culture medium, and subjected to a series of digestions (3 min, 37°C, 150 rpm) in 0.1 % (w/v) solution of collagenase type II in HBSS. The first digestate was discarded, and the cell suspensions from the subsequent 4–6 digestions were centrifuged (750 rpm, 4 min), resuspended in HBSS each, pooled, and resuspended in DMEM containing 4.5 g/L glucose supplemented with 10% FBS, 10 m*M* HEPES, 2 m*M* L-glutamine, and 100 units/mL penicillin. Cells were preplated for a 60-min period to enrich for cardiomyocytes (i.e. cells that remained unattached were used for encapsulation).

C2C12 Cell Culture on Crosslinked Hydrogels

The hydrogel ($n = 4$) consisting of 20 mg/mL of Az-chitosan and 5 m*M* (20 mg/mL) Acr-PEG-RGD was placed in 12 well plates (300 μL solution/well, $n = 4$) and crosslinked by UV illumination as described above. The films consisting of Az-chitosan alone (20 mg/mL, $n = 4$) served as controls. Each well was inoculated with C2C12 mouse myoblasts (75,000 or 20,000 cells/cm^2) using 1 mL culture medium/well. Culture medium was replaced by 100% after 24 h and images were taken at 100× and 200× (Nikon Diaphot microscope) to assess cell attachment. From day 0 to day 7, the cells were cultivated in the high glucose DMEM supplemented with 10% FBS, 10 m*M* HEPES, 2 m*M* L-glutamine, and 100 units/mL penicillin. At day 7 (when confluence was reached) the culture medium was switched to 2% FBS to support differentiation into myotubes. Phase contrast images were taken at 100× and 200× every other day and used in conjuction with an image analysis program (Scion Image) to determine the rate of proliferation. To quantify proliferation rate, cell number was determined in each group from images taken at 200× after 3, 4, 5, 7, 9, and 13 days of culture using image analysis. $N = 4$ images per well, with $N = 4$ wells per group were analyzed at each day of culture using ImageJ.

VEGF Encapsulation and Release Profile

Mouse recombinant VEGF165 (170 ng) was encapsulated into 50 μL of Az-chitosan /Acr-PEG-RGD (20 mg/mL each in Di H_2O) hydrogel and cast into 24-well plates ($N = 4$). VEGF stock solution (20 μg/mL) was prepared, and 8.5 μL of the solution was mixed with the rest of gel components to make a final volume of 50 μL. The gel components were thoroughly mixed by pipetting prior to photocrosslinking. To determine release kinetics under nondegrading conditions, the hydrogels were maintained in 1 mL PBS with orbital mixing at 37°C for 24 days (pH 7.4). The PBS was exchanged as indicated and VEGF activity in the supernatant was determined by a sandwiched ELISA kit (R&D systems). The amount remaining in the hydrogel was calculated by subtracting the amount of VEGF in the supernatant (determined by ELISA) from the amount initially encapsulated in the hydrogel (170 ng). Wells with blank hydrogel (no VEGF) served as controls.

Cardiomyocyte Encapsulation and Viability Assay

For encapsulation, 1×10^6 neonatal rat cardiomyocytes were pelleted by centrifugation (1200 rpm, for 5 min), resuspended in 100 μL of photocrosslinkable hydrogel precursor solution (Az-chitosan (20 mg/mL)/Acr-PEG-RGD(20 mg/mL)), and cast into 24 well plates ($N = 4$). The hydrogel was crosslinked by application of ultraviolet light for 1.5 min from a distance of 2 cm.

Immediately following the crosslinking, the viability of encapsulated cells was determined using reduced biohazard cell viability assay (Molecular Probes), according to the manufacturer's instructions. Briefly, the gels were incubated in 1 mL of HBSS containing 2 μL of SYTOX 10 green fluorescent nucleic acid stain and 2 μL of DEAD Red (ethidium homodimer-2) for 15 min at room temperature in the dark. Subsequently, the films were rinsed 3× in HBSS, and the distribution of red (dead) and

live (green) cells was visualized using a fluorescent microscope (Nikon Diaphot) at 20× and a long pass-dual emission filter for simultaneous viewing of both red and green cells.

Feasibility of Hydrogel Injection *Ex Vivo*

Adult female Sprague-Dawley rats were euthanized with pentobarbital injection according to the NIH guidelines. The chest was opened and the hearts were excised and placed into PBS filled petri dishes. To test localization, 100 μL of hydrogel (chitosan (20 mg/mL)/Acr-PEG-RGD (20 mg/mL)) was labeled with blue food coloring and applied to the desired location using gauge 26 needle. The hydrogel was either injected into the ventricle wall or applied to the surface of the heart and crosslinked with the application of UV light for 2 min. Subsequently, the ventricles were rinsed in PBS and photographed (Finepix S1pro, Nikon, Japan). $N = 4$ different locations on the heart surface and within the ventricle were tested in a single adult rat heart.

Statistical Analysis

Significant differences in comparisons between two groups were determined by two-tailed Student's t test, and multiway ANOVA in conjunction with Tukey test for multiple comparisons using SigmaStat 3.1. $p < 0.05$ was considered significant. $N = 4$ unless otherwise indicated. Data are represented as average ± standard deviation.

RESULTS

From ^{1}H NMR spectra, it was estimated that 12% of the amino groups in chitosan were conjugated with azidobenzoic acid: chitosan 6H's (δ 2–4 ppm); aromatic 4H's (δ 7–8 ppm). The gelation time for Az-chitosan/Acr-PEG-RGD (both components 20 mg/mL in Di H_2O) was much shorter than that for solution of Az-chitosan (20 mg/mL in Di H_2O): 25.4 ± 2.2 s ($n = 5$) and 42.9 ± 4.4 s ($n = 13$), respectively ($p < 0.001$, one way ANOVA). The gelation time for Az-chitosan/PEG (41.4 ± 4.2 s, $n = 7$) was comparable to Az-chitosan alone ($p = 0.711$), but was significantly longer than the gelation time for Acr-PEG-RGD ($p < 0.001$).

The average shear modulus of Az-chitosan/Acr-PEG-RGD solution (368.1 ± 86.8 Pa) was slightly but not significantly higher than that of Az-chitosan (337.8 ± 134.8 Pa); p-value: 0.72 on a two-tail t test (Figure 1). Scanning electron microscope observation showed that the crosslinked hydrogels had continuous circular or polygonal pores. The pore sizes in the dehydrated Az-chitosan gel varied significantly depending on the sampled locations: 20–30 μm (upper left panel in Figure 2) or 5–10 μm (lower left panel in Figure 2). The pore sizes in the dehydrated Az-chitosan/Acr-PEG-RGD gel were in the range of 10–20 μm (panel in right, Figure 2).

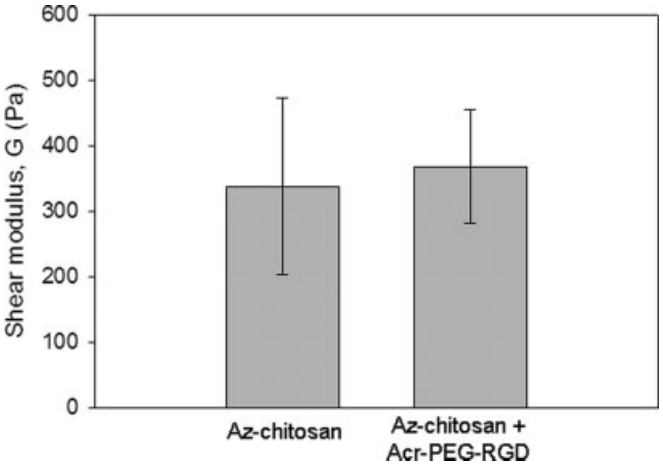


Figure 1. Mechanical properties of the hydrogels Az-chitosan and Az-chitosan/Acr-PEG-RGD hydrogel demonstrated comparable shear moduli. ($N = 4$ per group).

The crosslinked hydrogels were supportive of murine myoblast C2C12 cells attachment, proliferation, and differentiation when RGD was contained in the hydrogel (Figure 3). Crosslinked Az-chitosan hydrogels did not support cell attachment. Three days after inoculating C2C12 skeletal myoblasts into wells containing Az-chitosan hydrogels, only round scattered cells were present on the hydrogel surface. In contrast, the RGD containing hydrogels (5 mM RGD, Az-chitosan/Acr-PEG-RGD) demonstrated high degree of cell attachment and spreading comparable to that observed in the control wells (tissue culture polystyrene, TCP). In high serum culture medium, which encourages proliferation, the Az-chitosan/Acr-PEG-RGD hydrogels supported C2C12 cell proliferation comparable to the control TCP surface, reaching 100% confluence after 7 days of culture. No significant cell proliferation was observed on Az-chitosan hydrogels (Figure 3). Upon switching to the low serum medium (2% FBS), which is a standard protocol to promote differentiation, the cells on Az-chitosan/Acr-PEG-RGD hydrogels and TCP demonstrated formation of elongated differentiated myotubes (Day 13). The first myotubes appeared 24 h after switching to the low serum culture medium. Az-chitosan hydrogels did not exhibit myotube formation.

The cell density data obtained via image analysis confirmed the observed trend of cell proliferation followed by differentiation on the Az-chitosan/Acr-PEG-RGD hydrogels and control TCP (Figure 4). Az-chitosan/Acr-PEG-RGD hydrogels showed lower cell density than the TCP control during the first 5 days in culture. However, cell proliferation rate on Az-chitosan/Acr-PEG-RGD increased significantly between day 5 and 7 resulting in high cell density at day 7, which was comparable to that of TCP control. As a result, there was no statistically significant difference in cell density between TCP and Az-chitosan/Acr-PEG-RGD hydrogel from day 7 to day 13 in culture. In contrast, cell density was approximately 10 times lower on the Az-chitosan surfaces than on TCP or Az-chitosan/Acr-PEG-RGD indicating poor

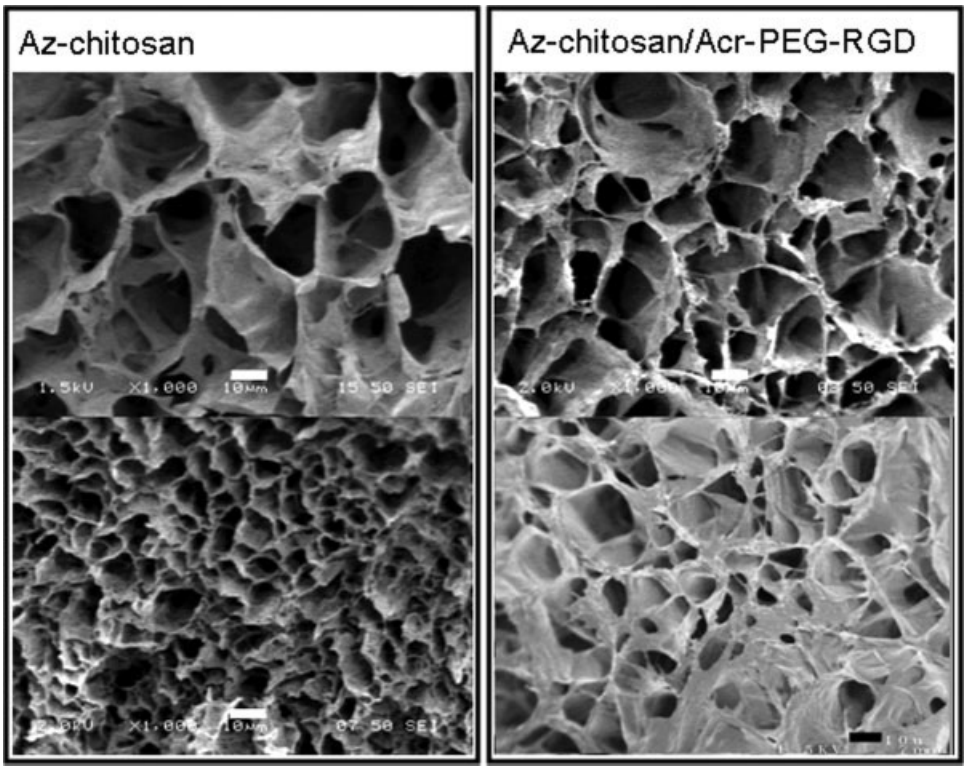


Figure 2. Scanning electron micrographs of lyophilized photocrosslinked gels. Two representative images were shown for each sample. Bars indicate 10 μm.

cell coverage and no significant proliferation. After day 7 in culture, we observed a slight decrease in cell density on the TCP and Az-chitosan/Acr-PEG-RGD surface. This is consistent with cell fusion, a part of differentiation process, where two of more individual myocyte cells fuse to form multinucleated myotubes. Mulitway ANOVA indicated that culture time ($p < 0.001$) and type of surface (i.e. presence of RGD, $p < 0.001$) affected cell density, as well as that there was an interaction between culture time and type of surface ($p < 0.001$). The interactive effect is reflected by the faster proliferation rate on RGD containing hydrogels.

Cardiomyocytes were resuspended in the Az-chitosan/Acr-PEG-RGD hydrogel, cast into well plates and crosslinked by application of UV light for 1.5 min. The hydrogel crosslinking process via UV illumination was cytocompatible as indicated by high viability of encapsulated cardiomyocytes immediately after encapsulation in a live/dead assay (Figure 5, green cells).

To test the ability of Az-chitosan/Acr-PEG-RGD to maintain high local concentration of growth factors, we encapsulated 170 ng of VEGF into 50 μL of hydrogel cast into 24-well plates. The gels were maintained under nondegrading conditions (i.e., with no enzymes to digest chitosan gels such as lysozyme) in 1 mL of PBS at 37°C with orbital mixing. The PBS was exchanged at time points indicated, and the VEGF concentration was determined in the supernatant by ELISA. The amount remaining in the gel was determined by subtraction. The results [Figure 6(A)], indicated a peak of VEGF release after 24 h followed by a low release rate over the subsequent 24 days. Approximately, 80% of the encapsulated VEGF was maintained in the hydrogel under nondegrading conditions, indicating that the hydrogel was capable of localizing the growth factor and preventing rapid clearance by diffusion normally observed in aqueous solutions and upon injection [Figure 6(B)].

In a feasibility study, we tested the ability of the Az-chitosan/Acr-PEG-RGD hydrogel to remain localized following application on or within the adult rat heart *ex vivo*. Two specific locations were tested: surface of the heart (epicardium) and within the ventricle wall, both of which are relevant for treatment of myocardial infarction. One hundred microliters of hydrogel containing a water-soluble dye was applied to the heart and crosslinked via 2 min application of the UV light. The hydrogel demonstrated excellent localization on both the ventricle surface and within the wall as evident from the dye remaining in the locales even after extensive washing in the PBS (Figure 7).

DISCUSSION

Injection of cells and growth factors has been studied previously as a treatment option for infarcted myocardium. Although significant progress has been made in testing the effect of various cell types,[26] major challenges remain with respect to the localization and the survival of the injected cells. Similarly, application of growth factors was hampered by their fast diffusion out of the injection site.

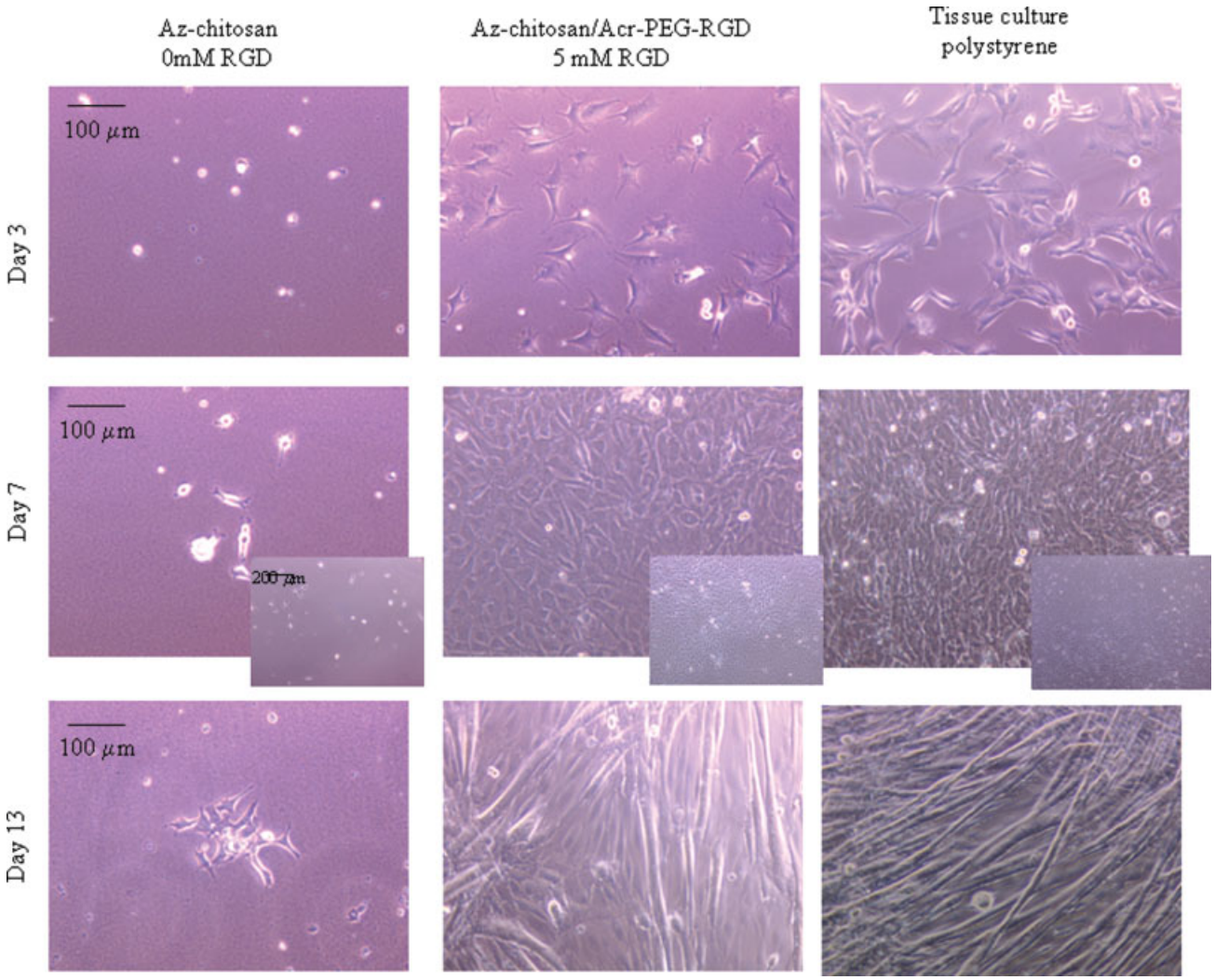


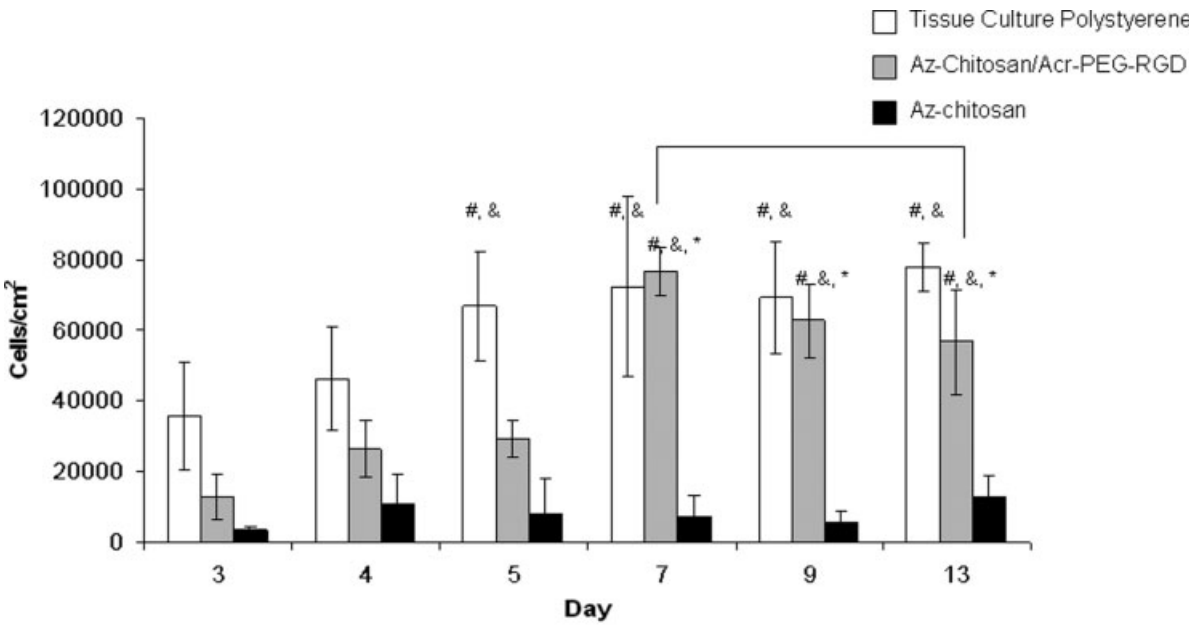
Figure 3. Attachment, proliferation, and differentiation of C2C12 cells is RGD dependent. Phase contrast micrographs during culture. Az-chitosan hydrogels contained only scattered and mostly round cells throughout the cultivation with no signs of differentiation into myotubes. In contrast, Az-chitosan/Acr-PEG-RGD hydrogels supported cell attachment, proliferation, and differentiation similarly to the tissue culture polystyrene. ($N = 4$ per group). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

This study was aimed at developing a hydrogel, for delivery of cells and growth factors, that will promote localization upon injection. For that purpose, we chose to focus on photocrosslinkable hydrogels, since the crosslinking process is rapid and the hydrogel localization can be precisely controlled by the application of UV light. For eventual *in vivo* application minimally invasive endoscopic light sources can be utilized.[27]

Upon myocardial infarction, a vigorous inflammatory response is elicited and dead cells are removed by marrow derived macrophages. Over the subsequent weeks to months, fibroblasts and endothelial cells proliferate forming granulation tissue and ultimately dense collagenous scar. Formation of scar tissue severely reduces contractile function of the myocardium and leads to ventricle wall thinning and dilatation, remodeling and ultimately heart failure. The best regeneration strategy thus depends on the time post-infarction.

Cell/growth factor injection strategies with hydrogels as delivery vehicle will work best if applied shortly after MI. Application of cells and growth factors within hours and days after MI using a hydrogel has a potential of directing the wound repair process so that the minimum amount of scar tissue is formed, the contractile function is maintained in the border zone, and pathological remodeling is attenuated. Tissue engineering strategies may be necessary after scar has formed to replace the scarred tissue, and this is potentially where a scaffold based approach may be most useful.

The design criteria for the envisioned hydrogel formulation are (i) to crosslink upon brief application of UV light forming a mechanically stable hydrogel, (ii) to support attachment, proliferation, differentiation, and viability of myocyte cells, (iii) to prevent diffusion of angiogenic factors, and (iv) when injected to remain localized at the desired site within the heart.



Day	TCP vs. Az-chitosan	TCP vs. Az-chitosan/Acr-PEG-RGD	Az-chitosan vs. Az-chitosan/Acr-PEG-RGD
3	P<0.001	P<0.001	NSD
4	P<0.001	P=0.003	P=0.023
5	P<0.001	P<0.001	P=0.002
7	P<0.001	NSD	P<0.001
9	P<0.001	NSD	P<0.001
13	P<0.001	NSD	P<0.001

Figure 4. Change in density of C2C12 cells on crosslinked Az-chitosan, Az-chitosan/Acr-PEG-RGD, and tissue culture polystyrene (TCP) during culture. Cell density was determined by image analysis with $N = 4$ per surface, 4 images from each well were analyzed per time point. Individual and interactive effects of surface type (crosslinked Az-chitosan, crosslinked Az-chitosan/Acr-PEG-RGD, and TCP) determined by multiway ANOVA. Significant differences in pairwise comparisons were determined by Tukey test ($p < 0.05$). Significant differences between cell density on each surface at each day in culture are indicated in the table. Significant differences for each surface with respect to time are indicated in the graph. #, Significantly different than the cell density on a given surface at day 3; &, Significantly different than the cell density on a given surface at day 4; *, Significantly different than the cell density on a given surface at day 5. Line indicates significant difference between the two groups (day 7 and day 13 samples for Az-chitosan/Acr-PEG-RGD).

The hydrogel of choice was a photocrosslinkable chitosan, because of its known biocompatibility, mechanical strength, and use in controlled release. Az-chitosan solutions were polymerized to form hydrogels by UV irradiation. Azide groups are known to be converted to highly reactive nitrene groups releasing N_2 upon UV irradiation. The short-lived nitrenes react extremely rapidly with the surrounding chemical environment, especially with amines of the other Az-chitosan molecules resulting in polymerization. We used the low-intensity long-wave UV as a light source, which was shown to be benign to cell viability.[28]

We used murine myoblast cell line C2C12 to test ability of the hydrogels to support cell attachment, proliferation, and differentiation. We demonstrated previously that the attachment properties of C2C12 cells are predictive of the attachment of cardiomyocytes.[29] In addition, skeletal myoblasts are currently in clinical trials for restoration of contractile function upon myocardial infarction.[26] The crosslinked Az-chitosan hydrogels did not support cell attachment, proliferation, and differentiation (Figure 3). Since the hydrogels are composed of mostly water (>95%), they are hydrophilic and do not support adsorption of the proteins necessary for cell adhesion. In addition, the number of primary amines, which might attract cells through electrostatic interaction, was reduced by crosslinking.

We thus chose to functionalize the hydrogel by addition of RGD groups known to promote cell attachment and proliferation, introduced as Acr-PEG-RGD. The conjugation reaction of YRGDS to Acr-PEG-NHS has been thoroughly characterized previously by Hern and Hubbell,[30] who showed that the reaction occurred on the N-terminal tyrosine (Y) residue of YRGDS and not the arginine. They also demonstrated that 85% of the peptide YRGDS reacted with the Acr-PEG-NHS to form Acr-PEG-RGD. In addition, since the amino group in tyrosine is consumed to form amide bond with Acr-PEG-NHS during

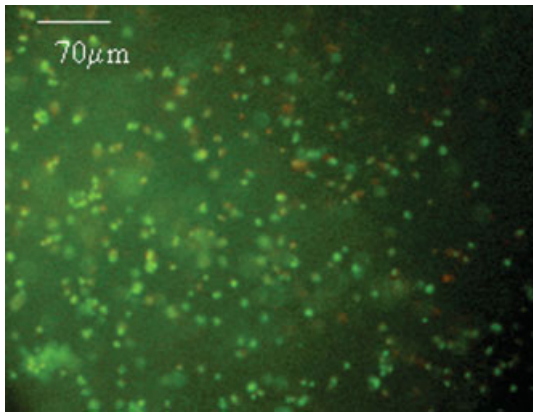


Figure 5. Viability of neonatal rat cardiomyocytes encapsulated into Az-chitosan (20 mg/mL)/Acr-PEG-RGD(20 mg/mL) hydrogel *in vitro*. For encapsulation, 10^6 neonatal rat cardiomyocytes were pelleted by centrifugation (1200 rpm, for 5 min) resuspended in 100 μL of photocrosslinakble hydrogel, and cast into 24 well plates. Viability was determined using Reduced Biohazard Cell Viability Kit (Molecular Probes) ($N = 4$). [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

synthesis of Acr-PEG-RGD, it is unlikely that Acr-PEG-RGD would crosslink with each other through tyrosine–tyrosine crosslink. Also, we have not observed noticeable color change after UV treatment in this study.

Acr-PEG-RGD and Az-chitosan were homogeneously mixed in solution prior to gelation. We have not observed apparent phase separation after gelation at least on an optical microscopic scale (~ 10 μm). Although we cannot exclude the possibility that Acr-PEG-RGD can be removed from the hydrogel surface, we note the significant effect on cell attachment and proliferation when mixed with Az-chitosan (Figures 3 and 4). The Az-chitosan/Acr-PEG-RGD (at 20 mg/mL each) supported proliferation and differentiation of C2C12 myoblasts as comparable to the control TCP (Figures 3 and 4). The concentration of each component (20 mg/mL each) was chosen to result in 5 m*M* RGD, which was found optimal for attachment of osteoblasts in previous studies.[24] It was also demonstrated that the scrambled peptide in Acr-PEG-RDG had no effect on osteoblast attachment.[24]

The gelation time was significantly shorter in Az-chitosan/Acr-PEG-RGD group (20 mg/mL each) when compared with the Az-chitosan only (20 mg/mL). The accelerated crosslinking in Az-chitosan/Acr-PEG-RGD could come from (a) higher total concentration in Az-chitosan/Acr-PEG-RGD (40 mg/mL) compared with the Az-chitosan alone (20 mg/mL) or (b) higher concentration of reactive species. Specifically, Acr-PEG-RGD introduces the unsaturated double bond of the acrylate group and the amine on the arginine group of the RGD sequence that may act as insertion sites for the nitrene. To investigate this further, we measured the gelation time of Az-chitosan/PEG (20 mg/mL each). The PEG used as a control for gelation is of M_W 3400. It is identical to the PEG used for synthesis of Acr-PEG-RGD except that no functional groups (acrylate or RGD) were present. There were no significant differences in the gelation time between Az-chitosan (42.9 $\pm$ 4.4 s) and Az-chitosan/PEG (41.4 $\pm$ 4.2 s, $p = 0.711$) while the gelation time for Az-chitosan/Acr-PEG-RGD (20 mg/mL each) was significantly lower (25.4 $\pm$ 2.2 s, $p < 0.001$). The results indicate that the acceleration is consistent with the higher concentration of reactive species.

The largest factor in the modulus of the hydrogels is related to the overall crosslinking of the gels. Essentially, our results (Figure 1) indicate that the modulus and ultimately the crosslinking density is similar between the two gels. The results indicate that although Acr-PEG-RGD may contribute to the network formation, it mostly acts as a tether to the network formed of Az-chitosan molecules.

SEM micrographs indicated the presence of the continuous porous structure within the hydrogels. The polygonal structures in SEM micrographs (Figure 2) indicate that the hydrogels were crosslinked,[31] as such structures were not observed previously in the uncrosslinked Az-chitosan processed in the same way[32] (Figure 2).

Given the improved attachment and proliferation of C2C12 cells upon addition of RGD, our studies aimed at evaluating retention of growth factors, viability of the encapsulated cardiomyocytes, and localization at the desired site in the heart were performed with the Az-chitosan/Acr-PEG-RGD hydrogel.

Upon exposure to light azide groups from Az-chitosan convert to highly reactive nitrene groups that may react nonspecifically with surrounding environment including cells, which may compromise their viability, as well as ECM components, which may affect hydrogel localization. Viability assay was performed immediately after photoencapuslation, to determine whether the photoencapsulation process and the exposure to UV induced cardiomyocyte damage and death (Figure 5). Under the conditions used in the *in vitro* studies, no decrease in viability of encapsulated cardiomyocytes was observed (Figure 5). These results are consistent with the previously reported cytocompatibility at similar crosslinking conditions *in vivo*[33] and for osteoblast cell encapsulation.[24] Further *in vivo* studies are required to assess the extent of nonspecific binding to the ECM components.

We chose to use VEGF as a model growth factor, because of its effect on the proliferation of endothelial cells, capillary sprouting, and extensive use in studies aimed at angiogenesis. Data in Figure 6 show that Az-chitosan/Acr-PEG RDG hydrogel (20 mg/mL each) was able to efficiently maintain 81% of the encapsulated VEGF for 24 days under nondegrading conditions. Within the first 24 h, 18.5% of the encapsulated VEGF was released from the hydrogel (81.5% remaining in the hydrogel), followed by a slow release of additional 0.2% from day 1 to day 24. This data is consistent with the ability of Az-chitosan/Acr-PEG-RGD to retain VEGF under nondegrading conditions, and thus prevent rapid diffusion that would otherwise be observed in an aqueous solution. This property would have a beneficial effect on a potential *in vivo* application as the rapid diffusion of growth factors out of the injection site was identified as one of the major obstacles to growth factor delivery and motivated the development of continuous infusion approaches.[34]

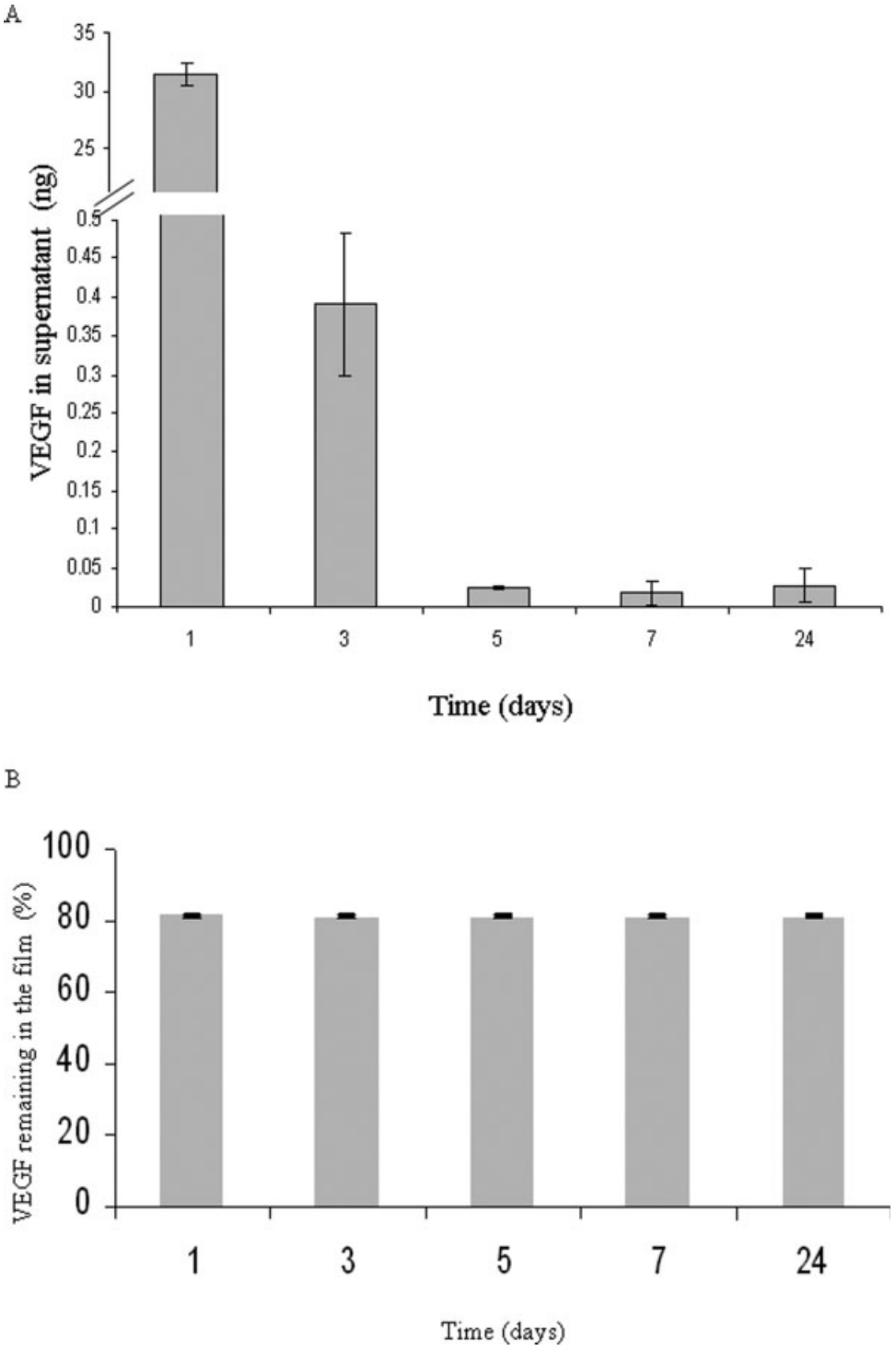


Figure 6. VEGF release profile. VEGF (170 ng) was incorporated into Az-chitosan (20 mg/mL)/Acr-PEG-RGD (20 mg/mL) hydrogel and maintained under nondegrading conditions in PBS for 24 days with orbital mixing (50 rpm) at 37°C. PBS was exchanged by 100% after 1,3, 5, 7, and 24 days, and the amount of VEGF was determined by ELISA. (A) The amount of VEGF in the supernatant (ng). Under nondegrading conditions 18.5% of incorporated VEGF was released within the first 24 h, followed by release of only 0.2% from day 1 to day 24. (B) The percent of VEGF remaining in the film. Under nondegrading conditions, Az-chitosan/Acr-PEG-RGD films maintain over 80% of incorporated VEGF for as long as 24 days. ($N = 4$).

Ishihara et al. reported similar findings for the encapsulation of VEGF in the photocrosslinked chitosan hydrogel, where over 75% of encapsulated VEGF was retained in the hydrogel under nondegrading conditions.[22] The same group demonstrated that the encapsulated VEGF could be released under degrading conditions (with chitinase and chitosanase added to

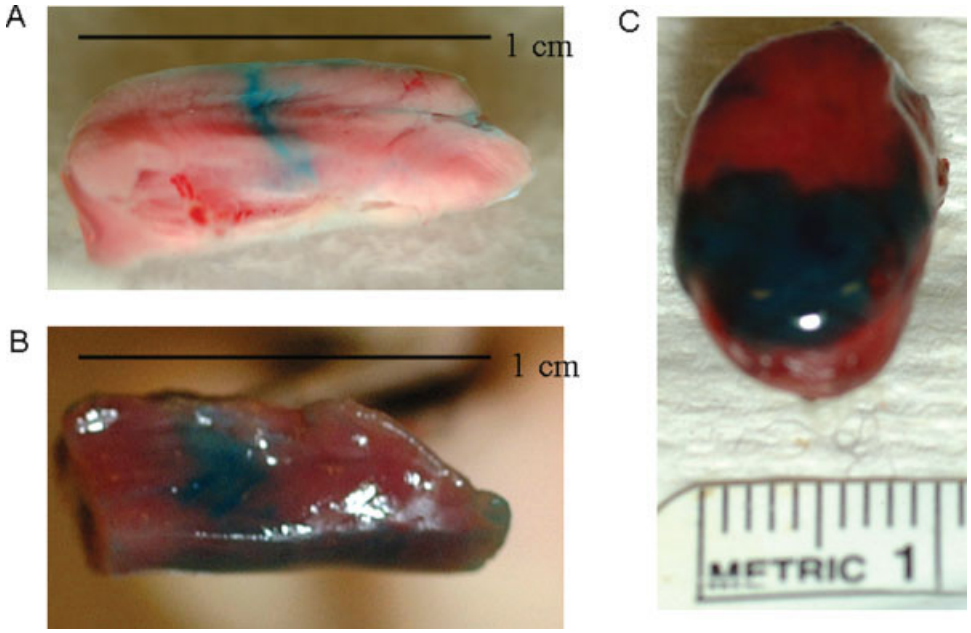


Figure 7. Hydrogel localization *ex vivo*. The hydrogel was labeled with blue food coloring, and 100 μL was injected using a G26 needle into the ventricular wall (A,B) or on the surface of a rat heart (C) *ex vivo*. The hydrogel was crosslinked with the 2 min application of UV light, the ventricles were rinsed in PBS, cross-sectioned with a razor blade and photographed. $N = 4$ different surface and intraventricular locations tested in a single rat heart. [Color figure can be viewed in the online issue, which is available at www.interscience.wiley.com.]

the culture medium) and that released growth factor maintained the ability to enhance HUVEC proliferation. Given the opposite charges of VEGF and chitosan gel, electrostatic interaction was postulated as a mechanism of the retention.[22] However, we cannot exclude potential crosslinking between VEGF and Az-chitosan and/or Acr-PEG-RGD during the UV illumination. *In vivo*, as previously reported,[23] chitosan would be degraded by the endogenous lysozyme; thus, the growth factor load is expected to be fully released over this time period.

The fact that the growth factor release and cell attachment are governed by two independent components of the hydrogel is an important advantage over the current systems. In the systems previously used for intramyocardial cell injection (Matrigel or Fibrin), cell attachment sites were lost as the hydrogel degraded. In the proposed hydrogel, growth factors (VEGF) can be released by chitosan degradation without the loss of attachment sites for endothelial cells and injected cardiomyocytes provided by the Acr-PEG-RGD. Since lysozyme is present in the serum (9–17 μg/mL), the growth factor release would occur faster under the degrading *in vivo* conditions compared with the *in vitro* release kinetics.[23] In addition to enzyme concentration, the rate of degradation will be the function of hydrogel concentration. Ishihara et al.[23] found that close to 85% of photocrosslinkable chitosan (15 mg/mL) implanted subcutaneously in mice degraded over the period of 14 days.

Finally, in a feasibility study, we demonstrated that Az-chitosan/Acr-PEG-RGD can be placed and stay at the heart surface and within the ventricle wall (Figure 7). Upon *in vivo* injection, this property will ensure that the hydrogel delivered material remains at the site of application, whereas material delivered by saline or culture media usually leaks out of the injection site. We have observed that UV could penetrate the tissue to some degree and crosslink the hydrogel, thus leading to the localization of the hydrogel on the heart surface as well as within the ventricle wall (Figure 7). Elisseeff et al.[35] used UV light for transdermal photopolymerization of injectable poly(ethylene oxide) dimethacrylates. They demonstrated that although UV light intensity decreased significantly within the first 2 mm of pig or human skin, photopolymerization was still possible.

In summary, we demonstrated that photocrosslinkable Az-chitosan/Acr-PEG-RGD hydrogel supports attachment, proliferation, and differentiation of C2C12 myocytes and retains VEGF over the period of 24 days under nondegrading conditions. Upon photoencapsulation, the cardiomyocytes remained viable in the hydrogel. In addition, we successfully localized the hydrogel at the surface of the adult rat heart or in the ventricle wall *ex vivo*. Therefore, the hydrogel may be useful for delivering cells and growth factors to the injured myocardium. The regenerative effect of this delivery strategy is a topic of our future studies.

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