

The influence of the RGD peptide motif and its contextual presentation in PEG gels on human mesenchymal stem cell viability

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Abstract

An investigation was undertaken into the method of delivery of RGD peptide motifs to adult human mesenchymal stem cells (hMSCs) encapsulated in poly(ethylene glycol) (PEG) hydrogel systems. Previous studies have shown that the viability of hMSCs encapsulated in bio-inert hydrogels, such as PEG-based gels, decreases over time. hMSCs are an adhesive-dependent cell type, requiring attachment sites to maintain their survival and function. The incorporation of tethered RGD peptide motifs was found to sustain a high level of hMSC survival in PEG gels. However, previous reports are largely limited to pendently tethered RGD in gels; therefore, further investigation into hMSCs' affinity for and response to the contextual presentation of RGD was studied using varying methods of RGD attachment to the PEG gel, as well as delivery of soluble RGD peptides. Studies with encapsulated hMSCs showed that the constrained RGD peptide, bound via two links to the PEG gel, promoted ~60% cell survival, while tethering the RGD as a pendant group, bound via a single link to the PEG gel, promoted ~80% cell survival. Interestingly, incorporating a glycine spacer arm to the RGD pendant tether further enhanced survival to ~88%. Investigations with solubly delivered peptides resulted in a dramatic decrease in cell viability with time, eventually leading to survival that was similar to that of unmodified PEG gels. Integrin staining for $\alpha v \beta 3$ and $\alpha 4$, as well as focal adhesion staining, correlates to the trends in hMSC survival for covalently-bound RGD motifs and a loss of viability for the solubly delivered peptide systems. Copyright © 2008 John Wiley & Sons, Ltd.

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1. Introduction

In the field of tissue engineering, hydrogels provide a useful platform for the three-dimensional (3D) culture of cells and to study the effects of microenvironmental factors on cell survival, proliferation, migration and extracellular matrix deposition. Often gel environments are composed of synthetic components that lack biological functionalities, such as poly(ethylene glycol) (PEG). While PEG is highly inert, it is useful as a means

to specifically query the effects of epitopes on cell function in 3D culture without confounding effects caused by non-specific interactions. However, when anchorage-dependent cells are immobilized in PEG gels, they are forced into a spherical morphology. In the absence of integrin-binding ligands, the cells undergo an apoptotic progression that results from the lack of matrix interactions (Ruoslahti, 1994; Stupack *et al.*, 2001). Thus, researchers have developed methods to functionalize PEG gels with pendant RGD (arginine–glycine–aspartic acid) groups to improve cell survival (Benoit and Anseth, 2005; Burdick and Anseth, 2002).

To date, numerous studies (Drumheller and Hubbell, 1994; Hern and Hubbell, 1998; Jo, 2000; Shu *et al.*, 2004; VandeVondele *et al.*, 2003) have demonstrated

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the promotion of cell adhesion to two-dimensional (2D) PEG gel surfaces functionalized with RGD, the major binding site in fibronectin. Previous research has shown that cells remain viable, proliferate and migrate on a 2D surface with immobilized RGD binding moieties (Shu *et al.*, 2004; VandeVondele *et al.*, 2003). Further studies suggest that clustering of RGD on the surface enhances cell differentiation and attachment (Maheshwari *et al.*, 2000). 2D cultures are often useful in preliminary experiments, yet 3D culture platforms provide a wealth of knowledge and are critical in tissue-engineering applications. However, the transition from cell survival and attachment on 2D to 3D is not well understood, and investigation into a cell's response to RGD in a 3D culture has not been examined extensively. In a 3D PEG culture system, the cells are forced into a rounded morphology and have limited spreading capabilities, leaving those adhesion-dependent cells to undergo apoptosis (Nuttelman *et al.*, 2005; Ruoslahti and Pierschbacher, 1987). While a rounded morphology is often considered a marker for apoptotic progression in 2D cultures, rounded and immobilized cells in 3D culture may respond to the presence of matrix-bound ligands differently than seen with 2D systems. Recent studies involving the use of adult human mesenchymal stem cells (hMSCs) in PEG hydrogels determined that incorporation of a pendant RGD at 2.8 mM maintained 75% viability after 1 week (Nuttelman *et al.*, 2005). Although hMSCs were shown to remain viable over the short 1 week time course, the presentation and accessibility of this pendant RGD group were not well characterized.

While these adhesive stem cells respond to the presence of covalently tethered RGD, differences can exist in the extent of cell viability with variations in RGD presentation to the cell. Pendant RGD groups, when presented to an encapsulated hMSC, have been shown to survive; however, natively RGD is presented to the cell via a loop-like extension from the fibronectin molecule (Johansson *et al.*, 1997; Pierschbacher and Ruoslahti, 1984). In this regard, RGD presented to the cell as a loop, via attachments to the PEG backbone on either end of the sequence, may induce higher levels of hMSC survival. Also, creating a distance between the presented RGD and the material backbone can provide better access for the necessary integrin–ligand interactions. Therefore, creating a spacer arm between the RGD motif and the material surface provides mobility and removes steric hindrance for the extension of the adhesive ligand to reach the cell surface integrin (Pierschbacher and Ruoslahti, 1984). Therefore, a number of differences in a cell's affinity toward the RGD motif may exist through variations in presentation of this molecule. It was the goal of this study to investigate the mechanisms involved in hMSC attachment to an RGD moiety, delivered either through covalent attachment to the PEG backbone via a singly attached, pendant tether or via a dually attached, loop-like structure. Here we will determine the effects of the delivery scheme on encapsulated hMSCs and demonstrate how variations in delivery of this molecule

may affect how the cells respond and whether or not they remain viable in these PEG systems.

2. Materials and methods

2.1. Cell harvest and culture

Human mesenchymal stem cells (hMSCs) were obtained from Cambrex Bio Science (Walkersville, MD, USA) and cultured according to Cambrex instructions, in low-glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 1 µg/ml amphotericin B, 50 U/ml penicillin, 50 µg/ml streptomycin and 20 µg/ml gentamicin. All hMSCs used in this study were collected from one donor and encapsulated at passage 3 to reduce the variability from the primary cell isolate.

2.2. Synthesis of poly(ethylene glycol) and peptide motifs

Poly(ethylene glycol) diacrylate (PEGDA) was synthesized by dissolving dried PEG 4600 Da (M_n) in anhydrous methylene chloride and purging the solution for 15 min with argon. Triethylamine (TEA) in a 2:1 molar excess was added to the solution and mixed under argon for 15 min. Acryloyl chloride at a 2.5:1 molar excess was added to anhydrous methylene chloride and added dropwise to the PEG/TEA solution, which was left to react overnight under an argon purge. The reacted PEG was rotovapped and filtered to remove excess salts, precipitated in cold ethyl ether, filtered and dried in a desiccator. Once dried, the PEGDA was redissolved in deionized water (dH₂O) and dialysed (Spectrum, 1000 MWCO) over a 24 h period with two dH₂O exchanges. An average of 88% acrylation was calculated through an ¹H-NMR analysis by comparing acrylate hydrogen to ethylene oxide backbone hydrogen integrations.

The peptide sequences –CRGDSCG (cysteine–arginine–glycine–aspartic acid–serine–glycine, N-terminus to C-terminus), which represents a mono-functionalized and short tethered system; CRGDSCG (cysteine–arginine–glycine–aspartic acid–serine–cysteine–glycine), which represents a di-functionalized and dually attached system; CGRDSG (cysteine–glycine–arginine–aspartic acid–serine–glycine), which represents a scrambled mono-functionalized system); and CGGGGGGGGRGDSCG (cysteine–glycine–glycine–glycine–glycine–glycine–glycine–arginine–glycine–aspartic acid–serine–glycine, N-terminus to C-terminus), which represents a spaced, mono-functionalized RGD system (i.e. RGD system with a spacer arm) – were synthesized using a solid phase peptide synthesizer (Applied Biosystems, Model 433A). The created peptide sequences were cleaved from the resin and deprotected using trifluoroacetic acid (TFA; Sigma), phenol, and triisopropylsilane (TIPS; Sigma).

Sequences were precipitated in diethyl ether and desiccated for 2 days. The dried product was redissolved in dH₂O and lyophilized. HPLC analysis showed that all peptides used in this study were estimated to be ~90% pure. The final product was either delivered in its soluble form or incorporated into the PEG hydrogel network through a photo-initiated thiol–acrylate reaction, as described in detail below. Figure 1 displays the chemical structure of all components used in these studies.

2.3. hMSC encapsulation in peptide-tailored PEG hydrogels

hMSCs were prepared for encapsulation through trypsin removal from tissue culture polystyrene plates used for culture. Cells were encapsulated at a concentration of 2 million cells/ml in all PEG hydrogels systems. Plain PEG hydrogels were used for entrapment of hMSCs and delivery of soluble peptide sequences, while tailored PEG

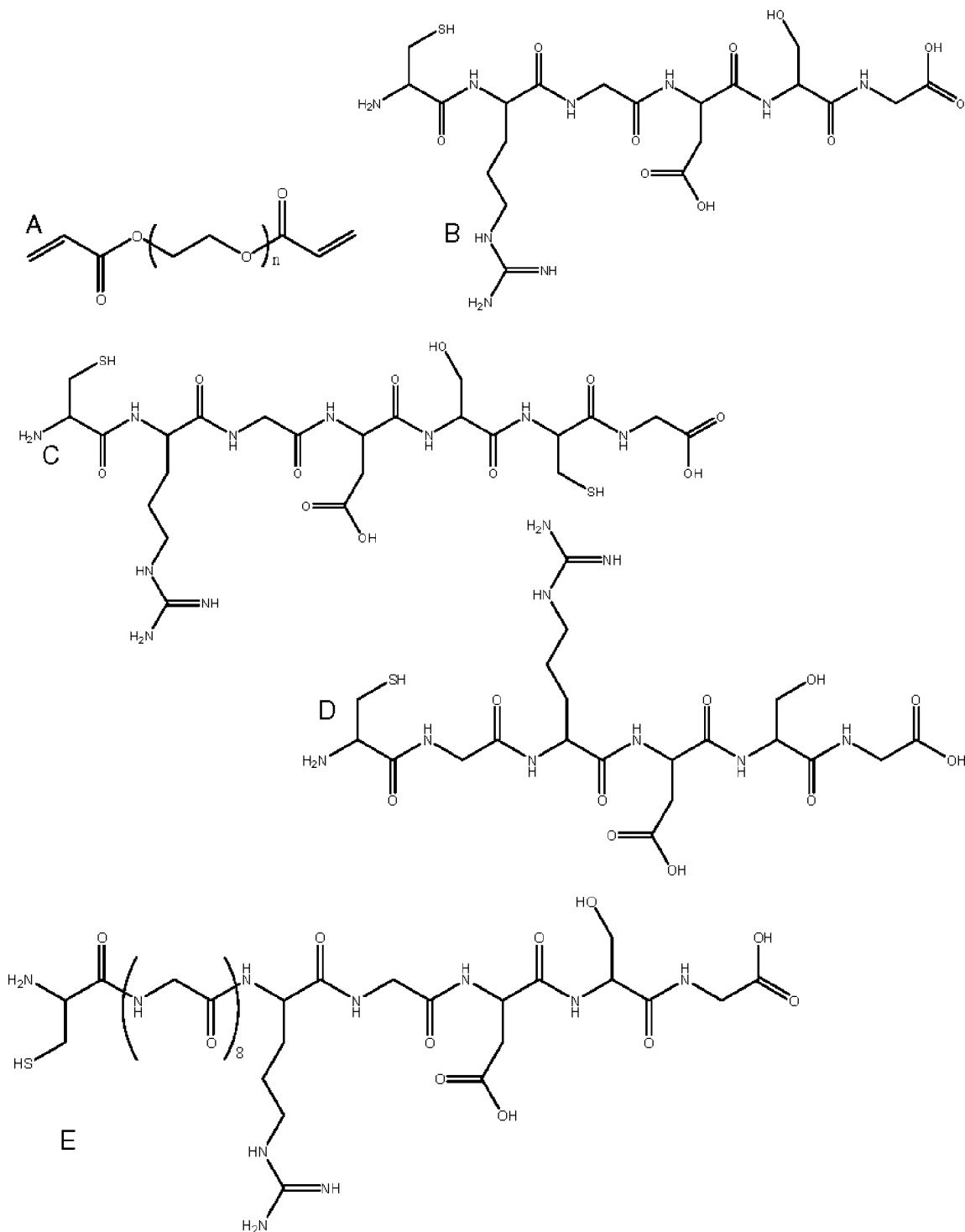


Figure 1. Chemical structures of components used in these studies. (A) PEG4600DA; (B) CRGDSG (mono-thiol); (C) CRGDSG (di-thiol); (D) CRGDSG (mono-thiol scrambled); (E) CGGGGGGGGRGDSG (mono-thiol with a spacer)

gels were reacted with peptide sequences to incorporate the sequences through covalent linkages. hMSCs were encapsulated in a PEG-peptide copolymer system via a photo-initiated thiol-acrylate polymerization. Briefly, the thiol-acrylate reaction proceeds via a photo-initiated polymerization. Thiol groups from the peptide sequence and acrylate groups from the PEGDA are radically activated, leading to a combination of step and chain growth polymerization, resulting in network formation. This technique was used to efficiently and easily tether in the peptide sequence of interest through a robust, cytocompatible polymerization process. In both cases, a solution of 10 wt% PEGDA was dissolved in PBS and the photo-initiator, 4-(2-hydroxyethoxy)phenyl-(2-hydroxy-2-propyl)ketone (I2959), was added at 0.05 wt%. The peptide gels were made at an initial concentration of 1 mM prior to polymerization. The concentrations of covalently attached and solubly delivered peptides were determined based upon amine measurements using the fluoroldehyde assay, which are displayed in Table 1 (Roth, 1971). The macromer solution was filtered and added to an hMSC pellet that had been trypsinized from culture, centrifuged, and counted. The final cell pellet and macromer solution were added in 40 μ l volume aliquots to 1 ml syringes with the tips removed. Each macromer/cell solution was photopolymerized at 365 nm light with an intensity of ~ 5 mW/cm² for 10 min at room temperature. After photopolymerization, the cell-laden gel disks (5 \times 1 mm) were removed and placed in control stem cell media. The constructs were cultured for up to 2 weeks at 37 °C and 5% CO₂ with media changes twice weekly.

2.4. Cell proliferation and matrix production

Cell-gel constructs were collected at 0, 3, 5, 7, 11 and 14 days ($n = 6$). The constructs were digested for 16 h at 60 °C in a papain solution (125 μ g/ml papain, 10 mM cysteine). Double-stranded DNA content was measured via the PicoGreen assay (Pierce) to analyse cell number for comparison between culture platforms.

2.5. Integrin identification and focal adhesion immunostaining

Constructs were collected at the time points mentioned previously and stained either for integrins $\alpha 5\beta 1$, $\alpha v\beta 3$ and $\alpha 4$ or focal adhesion markers. Those cell-gel systems that were stained for focal adhesions also contained a

system with encapsulated full-length fibronectin at a concentration of 20 μ g/ml as a positive control. The cell-gel constructs were fixed in 4% paraformaldehyde overnight and cryosectioned at 40 μ m before staining. All samples were treated with 3% BSA solution for 30 min to avoid non-specific antibody binding. The integrin sections were stained for $\alpha 5\beta 1$, $\alpha v\beta 3$ or $\alpha 4$ using primary mouse-anti $\alpha 5\beta 1$, $\alpha v\beta 3$ or $\alpha 4$ diluted to 1 : 100 for 1 h at 37 °C. All sections were then treated with a secondary goat anti-mouse antibody labelled with Alexa Fluor 488 diluted to 1 : 200 for 1 h at room temperature before being treated with a DAPI counterstain. Focal adhesion staining was achieved similarly through treatment of the sections with TRITC-conjugated Pallodin diluted to 1 : 200 for 1 h at room temperature. The sections were then counterstained with DAPI. All images were visualized on a Nikon Eclipse microscope, using an RT KE Spot camera and related software. Cells were counted via brightfield images and a positive DAPI counterstain. hMSCs staining positive for integrins or focal adhesions were then normalized to the total number of cells. Three images were taken for each culture system (with an average of 40 cells/image).

2.6. Statistics

Data collected throughout this study are presented as a mean \pm standard deviation of three data samples. Student's *t*-test was employed to compare datasets, using $p < 0.05$ to determine significance.

3. Results

3.1. Maintenance of viability of encapsulated hMSCs

Cellular viability within PEG microenvironments of varying composition was determined via the PicoGreen assay, which measures DNA content, over the course of a 2 week study. DNA for each culture platform was normalized to the initial DNA content for that specific construct. These data are presented in Figure 2. The viability of hMSCs cultured in unmodified PEG gels without any added peptide functionality decreased significantly over the course of 14 days, leaving only $7.3 \pm 0.4\%$ viable cells after 14 days. These data coincide with the diminishing survival of hMSCs in PEG gels as compared to previous reports (Nuttelman *et al.*, 2005; Salinas *et al.*, 2007). However, when RGD is covalently attached to the network, maintenance of at least 70% viable cells is achieved after 14 days of culture. In both cases, the scrambled RGD sequence follows the same trend in the time course of cell survival as that of PEG hydrogels. The RGD with a spacer arm sequence, when covalently tethered to the PEG network as a pendant functionality, provided the highest survival rate of hMSCs, reaching $\sim 84\%$ after 2 weeks in culture, which was significantly higher than the survival seen with dually attached RGD.

Table 1. Determination of final peptide concentration in hydrogels

Peptide sequence	Final concentration (mM)
CRGD SG	0.95 ± 0.02
CRGD SCG	0.97 ± 0.01
CGRD SG	0.93 ± 0.03
C(G) ₈ RGD SG	0.94 ± 0.02

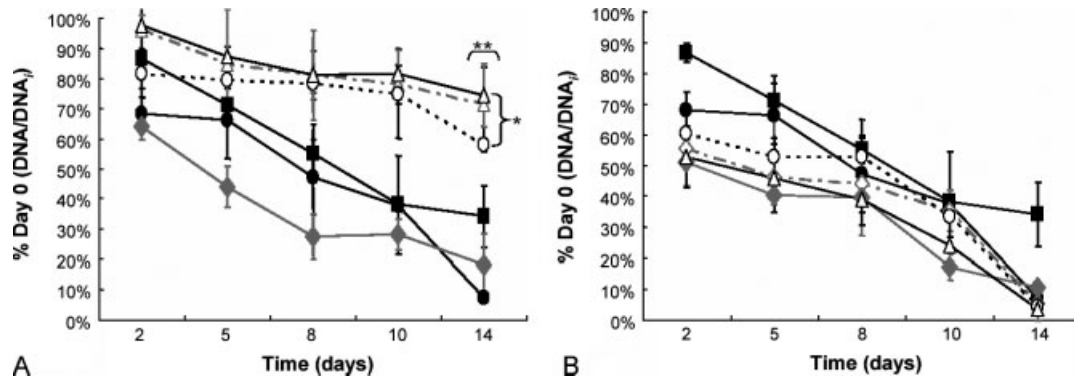


Figure 2. DNA content as a measure of cell viability using the PicoGreen assay over the course of 2 weeks in culture. Percentage viability was determined as the DNA content at each time point was normalized to that of day 0. The graphs depict viability for cells entrapped in PEG gels with exposure to no peptide (●), 20 $\mu\text{g}/\text{ml}$ fibronectin (■), short tethered RGD (◇), dually attached RGD (○), a scrambled RGD sequence (◆) and a tethered RGD with a spacer arm sequence (Δ). (A) Sequences that were covalently attached to the network; (B) peptide sequences that were delivered solubly. * $p > 0.05$; ** $p > 0.01$

A mono-functionalized, short tethered RGD sequence provided $\sim 79\%$ survival, while the di-functionalized, dually attached RGD supported $\sim 61\%$ after 14 days. The solubly delivered peptides showed no significant variation in cell survival, regardless of the thiol functionality, and those systems resulted in $\sim 6\%$ viable cells after 14 days, similar to that of pure PEG gel systems.

3.2. Determination of cellular response to delivery of RGD motifs

Immunostaining techniques were employed to investigate the cell's response to the presence of RGD in their

environment, especially as a function of the RGD attachment, functionality and spacer length. Cell-gel constructs were analysed at 2, 5, 7, 9, 11 and 14 day time points and stained for the cell surface integrin $\alpha\text{v}\beta 3$, specific to binding the RGD peptide motif. In Figure 3A, only an early time point, day 2 (black bars), and a later time point, day 11 (grey bars), are shown to illustrate the quantitative changes in integrin expression of the hMSCs over the course of the study and as a function of the gel chemistry. Three images were taken for each culture system (with an average of 40 cells/image), and the cells were counted based upon DAPI staining (indicative of cell nuclei). Positive cells, staining for $\alpha\text{v}\beta 3$, were normalized to the total cell number, and

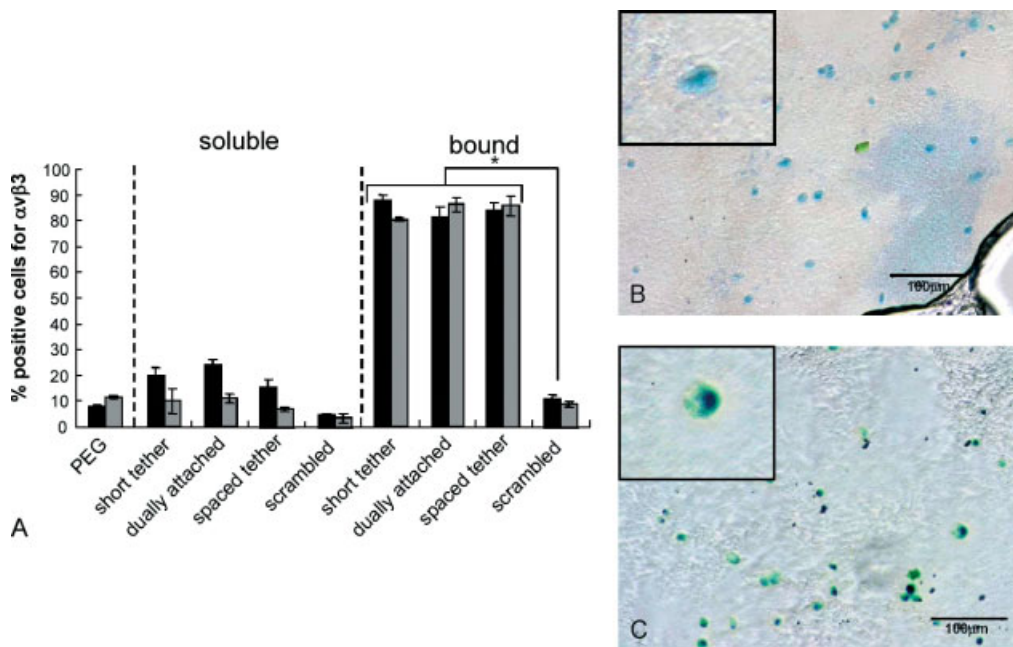


Figure 3. hMSCs were stained for $\alpha\text{v}\beta 3$ cell surface integrin, resulting in a fluorescent green staining, and counterstained with DAPI (blue cell nuclei). The positive $\alpha\text{v}\beta 3$ cells were counted and normalized to the total cell number for each system and presented in graphical form (A) for days 2 and 11. Acronyms for the peptide sequences are as follows: CRGDSG, short tether; CRGDSC, dually attached; C(G)GRDGS, spaced tether; CGRDSG, scrambled. (B, C) Micrographs of cells staining for DAPI and $\alpha\text{v}\beta 3$ on day 11; (B) is that of a pure PEG system and (C) is that of a di-functionalized attached RGD system. * $p > 0.01$

these data were then averaged over all images for a given gel system. The data are shown as percentages of positive cells staining for $\alpha v\beta 3$. Unmodified PEG gels show $\sim 10\%$ of the hMSCs staining positive for the $\alpha v\beta 3$ integrin over the entire course of the study, while all gels with the attached RGD motifs, with the exception of the scrambled sequence, maintain a much higher percentage of $\alpha v\beta 3$ -positive cells at $\sim 86\%$ over days 2 and 11. The delivered RGD sequences, again with the exception of the scrambled sequence, stain for a higher percentage of positive cells on day 2, at about 25% positive, yet this percentage of positive cells decreases by day 11 to $\sim 12\%$ for the peptides delivered in solution. In both cases, whether covalently attached or solubly delivered, the scrambled sequence provided approximately 9% positive cells for both days 2 and 11. Figure 3B depicts little to no staining as seen with a typical PEG gel at day 11, whereas Figure 3C shows heavy green staining for the dually attached system on day 11, indicating that there is a high percentage of cells positive for $\alpha v\beta 3$ cell surface integrin. Similar results were seen for cells staining positively for $\alpha 5\beta 1$ (data not shown). Interestingly, the percentage of cells that stained positive for $\alpha 5\beta 1$ represented a slightly smaller population. This finding can be due to the fact that $\alpha 5\beta 1$ binds to RGD and its synergistic site PHSRN with a much higher affinity than to RGD alone. Therefore, the $\alpha v\beta 3$ staining was depicted, as it better represents the cell's interaction with RGD specifically.

Furthermore, a second cell surface integrin, $\alpha 4$, which is known to bind fibronectin at a site other than the RGD motif, was also investigated in these systems.

Figure 4A depicts the data for $\alpha 4$ -positive cells. These data were collected and processed similarly to the procedure previously described, with analysis of three images (with an average of 40 cells/image) taken for each system. Interestingly, PEG gels show a high percentage of positive cells on day 2 at $\sim 46\%$, which then decreases to $\sim 20\%$ by day 11. This same trend is seen with all of the attached RGD motifs, with the exception of the tethered RGD with a spacer arm sequence, where day 2 shows positive $\alpha 4$ cells at a $\sim 40\%$ level reducing to $\sim 10\%$ by day 11. The attached RGD with a spacer arm sequence led to relatively equivalent and low levels of positive cells, $\sim 12\%$, for both day 2 and day 11. Furthermore, the solubly delivered peptides correlated to high levels of positive cells on day 2, similar to that of the attached RGD systems, but expression of $\alpha 4$ either remained constant or slightly increased by day 11. The short tether RGD system showed $\sim 45\%$ positive cells on day 2 and $\sim 49\%$ by day 11. The RGD with a spacer arm system led to lower levels, but was fairly constant at $\sim 32\%$ over days 2 and 11. The scrambled system was relatively constant, with $\sim 13\%$ positive cells for both days shown. Finally, the dually attached system had a positive cell level of $\sim 45\%$ on day 2, which then increased to $\sim 60\%$ by day 11. Figure 4B is representative of the limited staining observed for cells in PEG gels at day 11, whereas Figure 4C depicts the staining typically seen for cells with the mono-functional short tethered RGD system at day 2. These images qualitatively demonstrate the differences observed in the cells positive for the $\alpha 4$ cell surface integrin on surfaces with high and low levels of expression.

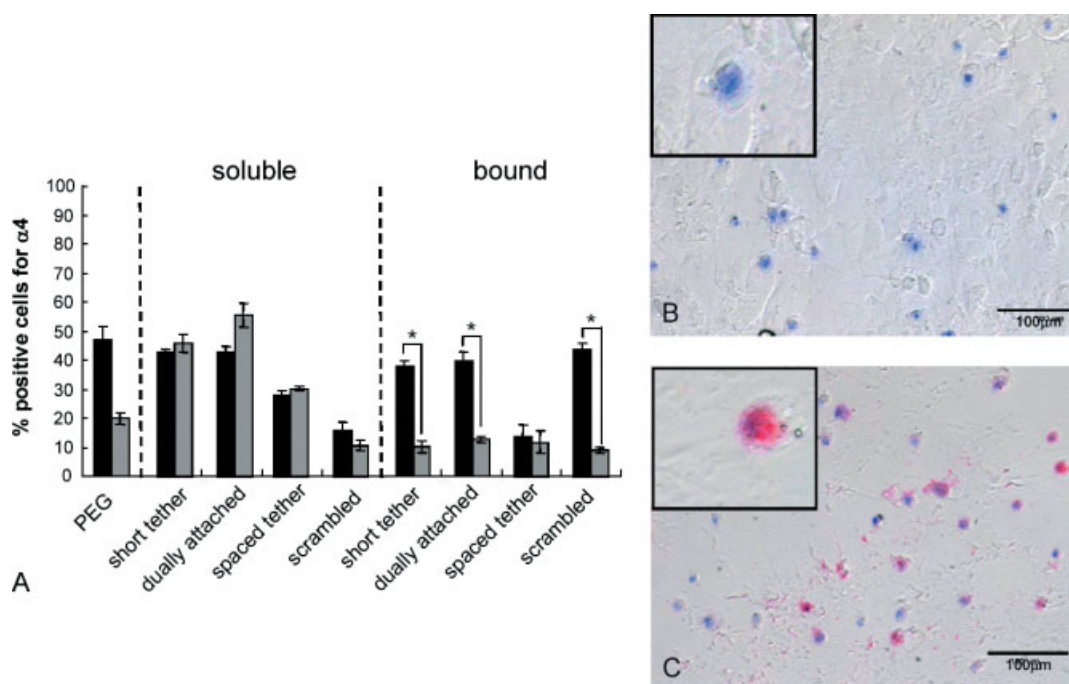


Figure 4. hMSCs were stained for $\alpha 4$ cell surface integrin, resulting in a fluorescent red staining, and counterstained with DAPI (blue cell nuclei). The positive $\alpha 4$ cells were counted and normalized to the total cell number for each system and presented in graphical form (A) for days 2 and 11. (B, C) Micrographs of cells staining for DAPI and $\alpha 4$; (B) is that of a plain PEG system on day 11 and (C) is that of a mono-functionalized attached RGD system on day 2. * $p > 0.05$

3.3. Investigation of focal adhesion indicating hMSC adhesion to RGD motifs

Focal adhesions were also examined to determine if the encapsulated hMSCs were interacting in an adhesive fashion with the various modes of RGD presentation in the gel systems. In this study, full-size fibronectin proteins were also entrapped in a PEG gel, as a positive control for focal adhesion staining. Figure 5A depicts the number of cells staining positively for focal adhesions on day 14 of culture. Here, the unmodified PEG gels showed only ~8% of the cell population staining positive for focal adhesions. A similar response was observed for the solubly delivered peptide systems, where ~9% of the cells exposed to short tethered RGD, dually attached RGD, and RGD with a spacer arm sequences in solution stain positive for focal adhesions. The scrambled sequence stained for ~5% positive cells, which was less, but not significantly less, than the other solubly delivered RGD motifs. In contrast, cells cultured in the PEG gels with the attached RGD motifs stained positive for focal adhesions at a much higher percentage by day 14. Specifically, cells in the fibronectin-containing gels were ~75% positive; while cells in the dually bound RGD system were ~68% positive. The tethered short RGD and RGD with a spacer arm sequences led to $85 \pm 3\%$ and $82 \pm 2\%$, respectively, of positive cells. In both cases, the scrambled peptide sequence served as a negative control and only ~8% of the total population was positive for focal adhesions. Figure 5B indicates the low staining observed for cells in a PEG system on day 14, whereas Figure 5C shows the

typical positive staining of cells and their focal adhesions on a tethered and RGD with a spacer arm system by day 14 of culture.

4. Discussion

hMSC survival in PEG gels decreases with culture time, due to a lack of matrix interactions, leading to ~45% survival after only 2 weeks in culture (Nuttelman *et al.*, 2005). hMSCs are an adhesion-dependent cell type and require cues from adhesion proteins to promote their survival in a 3D environment (DeLise, 2000; Hersel *et al.*, 2003). Previous reports have demonstrated the effectiveness of promoting a higher degree of hMSC survival in PEG gels by incorporating an adhesive peptide sequence, specifically RGD (Nuttelman *et al.*, 2005; Salinas *et al.*, 2007). While RGD provides cues and interactions for hMSC survival in PEG gels, the mechanism for this cellular response is less understood. Therefore, this study aimed to examine differences in the presentation of the RGD functionality, as well as different modes of attachment on hMSC integrin interactions and survival when encapsulated in PEG gels.

The hMSC viability for the unmodified PEG gel decreased dramatically after 2 weeks of culture, agreeing with previously published results for hMSC survival in PEG gels over an extended culture period (Nuttelman *et al.*, 2005). Further, the scrambled GRD sequence led to no enhancement in hMSC survival or integrin upregulation, illustrating the specificity of the integrin interactions to

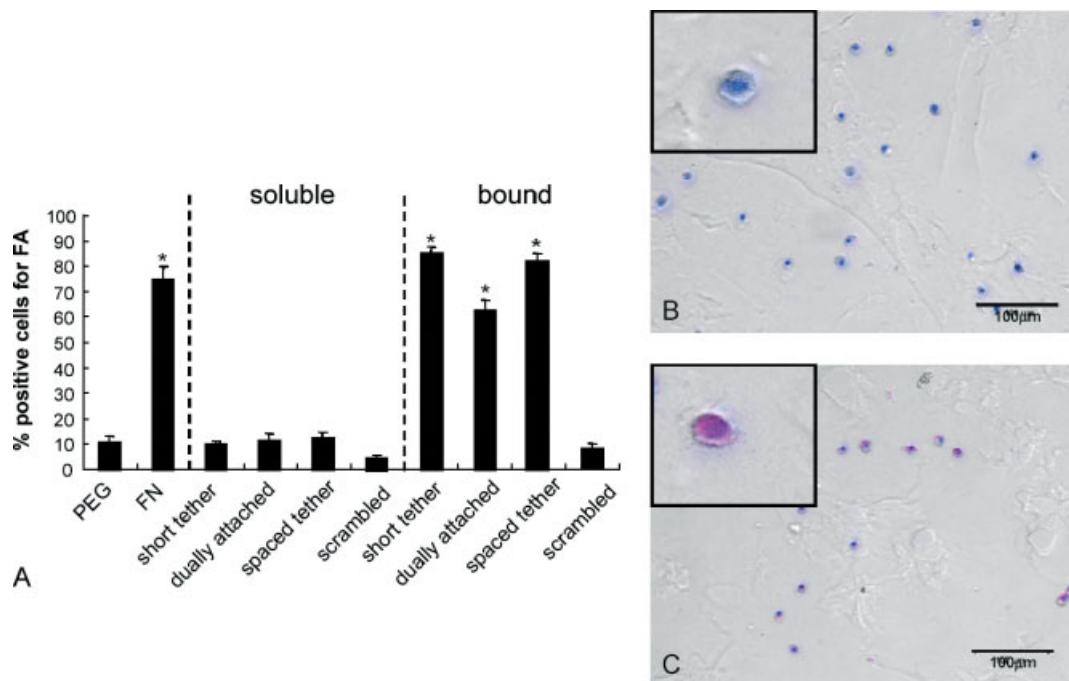


Figure 5. hMSCs were stained for focal adhesions (FA) on the various gel microenvironments. Actin filaments were stained with a fluorescent purple marker (colour modifications were made for image analysis purposes) and cell nuclei were stained blue with DAPI. The cells that were positive for focal adhesions were counted and normalized to the total cell number for each system and presented in graphical form (A) for day 14. (B, C) Typical micrographs of cells staining for DAPI and focal adhesions; (B) is that of an unmodified PEG system on day 14 and (C) is that of a tethered and spaced RGD system on day 14. * $p > 0.05$

the RGD peptide motif. The solubly delivered peptide sequences, despite their ability to bind with the cell's surface integrins, led to a decrease in cell survival over the course of the study and lack of integrin production for attachment purposes. As seen in Figure 2B, the fibronectin entrapped in the hydrogel provided some stimulation for cellular survival at the beginning of the study, but that survival decreased with time. This decrease in survival is related to the large size of the fibronectin and the limited ability for a great population of cells to sense its adhesion properties. Previous studies regarding cellular attachment on 2D PEG surfaces indicated that when RGD is delivered solubly, it has been seen to interact with the cell surface integrins, such as $\alpha v\beta 3$ or $\alpha 5\beta 1$, and block the cell's binding to the coated surface (Hersel *et al.*, 2003). A similar response was seen with cells in 3D culture, whereby in blocking these receptor sites with solubly delivered RGD, the hMSCs are left unadhered to their surroundings. Even if the encapsulated hMSCs were producing fibronectin on their own, the overwhelming exposure to soluble RGD will continuously block the adhesive integrins causing the hMSCs to apoptose. This is further supported by the results for the bound RGD sequences, where a much higher degree of survival was observed compared to the solubly delivered RGD.

The attached RGD motifs, on the other hand, provide a slight mechanical stimulus, in as much as the PEG network provides some structural support. Here, the hMSCs can interact with these peptide sequences via their cell surface integrins and expend their energy on other cellular activities aside from generating their own fibronectin. The dually attached RGD system provided the lowest level of viable cells and attachment integrins, such as $\alpha v\beta 3$, among the attached RGD peptides (excluding the scrambled sequence). While this peptide is thought to be able to attach at both ends, creating a loop-like structure, mimicking the native presentation of RGD, steric hindrance of the dually attached RGD system makes it harder for a cell to extend its integrin to bind to the RGD motif. The short RGD tether system resulted in slightly higher levels of cell survival and integrin upregulation, while the RGD with a spacer arm sequence reached the highest level of cell viability and $\alpha v\beta 3$ production, in these systems, although not significantly different from the short tethered RGD sequence. Hypothetically, the cells are more likely to interact with the RGD with a spacer arm sequence as it increases the accessibility of the RGD through a small extension of the RGD away from the PEG backbone. However, our results indicate that there are very slight differences between the spaced sequence and the short tether in terms of cellular attachment through the RGD. This is most likely due to the limitations in cell mobility and the tightly crosslinked network, which forces cells to interact with those peptides in their surrounding environment, whether they be close to or spaced away from the PEG backbone.

These data generate answers to some of the questions about the proper way to deliver the adhesive RGD motif to encapsulated cells in order to induce the best cellular

response. As seen here, the bound RGD provides not only an adhesive site for binding cells, but also gives some mechanical support to the cell through its covalently attachment to the PEG network. Studies conducted by Beer, investigating the addition of glycine spacers on cell attachment on a 2D PEG gel, concluded that adhesion integrins can bind to an RGD moiety extending out from a material surface by 11–32 Å (Beer *et al.*, 1992). The results obtained from this study verify this conclusion in a 3D environment, whereby cells respond to a higher degree to RGD that is spaced away from the PEG backbone. Decreasing the steric hindrance effects by extending a spacer arm can increase the likelihood of integrin–ligand interaction. Further investigations using a longer spacer arm will determine whether a higher degree of cell survival can be achieved through limiting steric hindrance effects, for both singly and dually attached RGD.

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