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Engineering of Covalently Immobilized Gradients of RGD Peptides on Hydrogel Scaffolds: Effect on Cell Behaviour

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Summary: The aim of this study has been to design a system for the preparation of Polyethylene-glycol (PEG) based hydrogels with a controlled spatial distribution of covalently immobilised RGD adhesion signals in order to control and guide cell response for tissue engineering application. Gradients of immobilised RGD peptides were characterized by confocal microscopy analysis. Moreover, the effect of RGD spatial distribution on cell behaviour was evaluated by using mouse embryo fibroblasts NIH3T3. In particular, we observed cell adhesion and migration of fibroblasts seeded on RGD gradient compared to cells on control hydrogels having an uniform distribution of RGD. Our data suggest that a linear gradient of covalently immobilised adhesion signals affects cell behaviour. In particular, cells feel RGD gradient and oriented themselves and move along gradient direction.

Keywords: gradient; hydrogel; polyethylene-glycol; RGD; scaffold

Introduction

Cell directionality and orientation is critical in many physiological processes, including morphogenesis, the immune response, and wound healing.[1–3] It is well known that, in these processes, cell response can be guided by gradients of various diffusible and non-diffusible chemical signals, such as growth factors, cytokines, chemokines and extracellular matrix (ECM) components.

Also in tissue engineering, it is crucial to control cell behaviour to promote tissue regeneration. Therefore, protein or peptide gradients may also be useful for optimizing engineered tissue formation by enhancing migration and the recruitment of cells into scaffolds.

This study focuses on the realization of polyethylene-glycol diacrylate (PEGDA)-based hydrogels, widely used for tissue-engineering applications, bioactivated with the adhesive peptide, RGD, having a spatially controlled distribution, in order to direct cell behaviour. PEGDA is well known for its anti-adhesive properties.[4] Thus, these scaffolds provide a blank slate to which cell adhesion peptides can be added in a controlled fashion to guide cell interaction.[5,6] RGD is a tripeptide able to promote cell adhesion by interacting with its specific cell membrane receptors, integrins. It has already reported that the presence of covalently immobilized RGD peptides on PEG based scaffold induces cell adhesion and promote cell migration in a dose-dependent manner.[7–9] However, it is still unclear if spatial distribution of these adhesion signals can influence cell behaviour.

The aim of this work has been to realize PEG-based scaffolds with a covalently immobilized RGD gradient and to evaluate

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cell response to this oriented distribution of adhesive signals. To this aim, we set up a novel technique to prepare gradients of RGD PEG-acrylate hydrogel, in order to control scaffold dimension, RGD spatial distribution and gradient steepness. Gradient characterization was performed by confocal microscopy analysis. Cell response to bioactive matrices was evaluated in relation to biological parameters, such as cell adhesion and migration.

Materials and Methods

Reagents

For our experiments, PEGDA (MW = 3400 Da (Shearwater Polymers)) and bioactive conjugated peptide-PEG-acrylate were used. The peptide sequence used was ArgGlyAspLys(Z)β-Ala (here indicated as RGD). The sequence was synthesized by the solid-phase method using standard 9-fluorenylmethoxycarboxyl (Fmoc) chemistry protocol on Rink-amide MBHA resin. Peptide purity and integrity was confirmed by ESI-MS (electrospray ionization mass spectrometry).

Peptide was conjugated to PEG monoacrylate by reacting with acryloyl-PEG-N-hydroxysuccinimide (acryloyl-PEG-NHS, 3400 Da; Nektar Therapeutics, San Carlos, CA) in sodium bicarbonate (pH 8.5) at 1:1 molar ratio over night. The coupled acryloyl-PEG-peptide was dialyzed and lyophilized before use. PEG-peptide was characterized by analytical RP-HPLC chromatography and ^{1}HNMR.

Hydrogel Preparation

Hydrogels were formed by combining 5% (w/w) PEGDA with different concentrations of RGD-PEG-acrylate molecules, ranging from 0.8 to 2.9 μmol/L, in 10 mM HEPES and adding Darocur1173 (Ciba) as photoinitiator (Figure 1). Precursor solutions were irradiated by an UV light at the wavelength of 365 nm for 15 min to allow photopolymerization and hydrogel formation.

RGD Gradient Realization

A continuous linear gradient was formed by using a gradient maker (Figure 2), made in our laboratory, to combine precursor solutions (PEGDA solution + RGD-PEG-acrylate solution) and using photopolymerization to lock the RGD gradient in place. Briefly, two syringes, loaded with precursor solutions at two different concentrations of

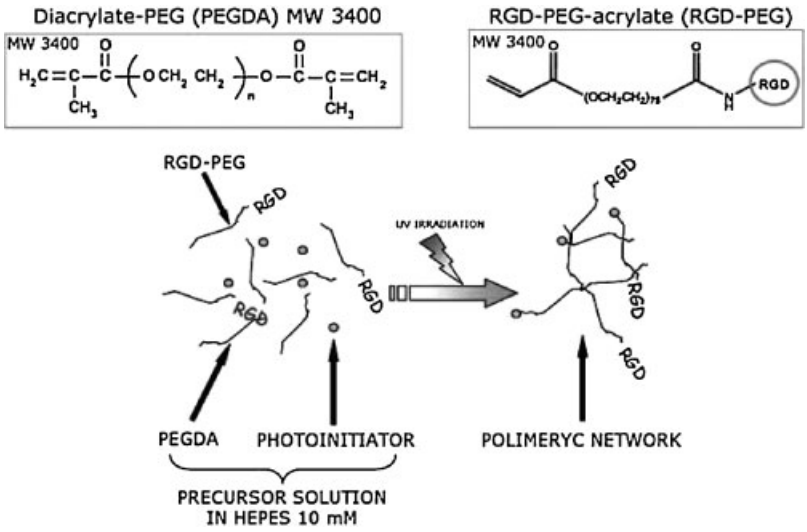

Figure 1.
Scheme of photopolymerization reaction.

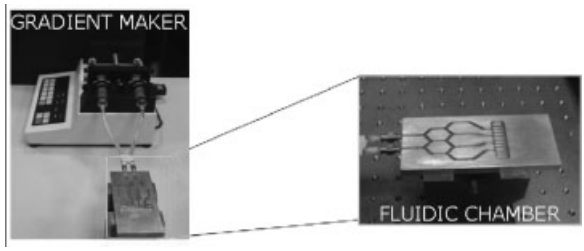

Figure 2.
Gradient maker.

RGD-PEG-acrylate, were positioned on a syringe pump. The outgoing flow was pumped into a fluidic chamber where precursor solutions were mixed. The resulting solution was collected in a mold and photopolymerized by UV irradiation. A RGD gradient with a slope of 1 mM/cm was obtained. Moreover, PEGDA hydrogels with uniform distribution of RGD [2.9 mM] were prepared as control.

RGD Gradient Characterization

In order to characterize RGD spatial distribution, gradient scaffolds were prepared using fluorescent RGD-PEG-acrylate molecules. RGD concentration along the gradient was evaluated as a function of variation of fluorescence intensity along the sample measured by confocal microscopy.

Cell Culture

Mouse embryo fibroblasts NIH3T3 were maintained at 37 °C and 5% CO_2 on Dulbecco's modified Eagle medium (DMEM) supplemented with 10% fetal bovine serum (FBS, BioWhittaker, Walkersville, MD), 2 mM l-glutamine (Sigma, St. Louis, MO), 1000 U/l penicillin (Sigma, St. Louis, MO) and 100 mg/l streptomycin (Sigma, St. Louis, MO).

Cell Adhesion Experiments

For cell adhesion experiments, $1^{*}10^{5}$ NIH3T3 cells were seeded on RGD-PEGDA gradients and on control hydrogels, pre-incubated in serum free medium for 6 h, to avoid unspecific cell adhesion depending on serum protein adsorption, and then incubated in DMEM-10% FBS for the analyses. Cell morphology was evaluated by optical microscope observations.

Cell alignment along the gradient was analysed by measuring cell angle relative to RGD gradient direction through an image analysis software (GIMP2.2). Cell angles were determined for at least 100 cells on each hydrogel surface, and then these angles were categorized into five different groups based on their relative angle to the RGD gradient. These groups included cells positioned relative to the RGD gradient within 0° to 20°, 20°to 40°, 40° to 60°, 60° to 80°, or 80° to 90°.

Cell Migration Experiments

The measurement of cell migration parameters (cell speed "S" and persistence time "P") was carried out by using a time-lapse videomicroscopy for 18 h. The images were collected every 15 minutes. The coordinates of cell displacement were calculated in order to evaluate the parameters of cell migration following the Stokes-Lauffenburger theoretical model [Stokes and Lauffenburger, 1991], described by this equation:

$$\langle D^2 \rangle = n \cdot S^2 \cdot \left[P \cdot t - P^2 \cdot \left(1 - \exp\left(-\frac{t}{P} \right) \right) \right]$$

where the mean square cell displacement $\langle D^2 \rangle$ is expressed as function of number of dimensions, n, in which cells migrate during the time, t, cell speed, S, and persistence time, P.

The centroid position of a single cell gives rise to two vectors X and Y that

represent the input of Matlab software, that provides a good fit of the experimental data. The resulting plot shows the cell displacement, D, in function of time. The parameters Speed (S) and Persistence length (P) were then calculated as outputs of the fitting using an Image Analysis Software (Metamorph, USA).

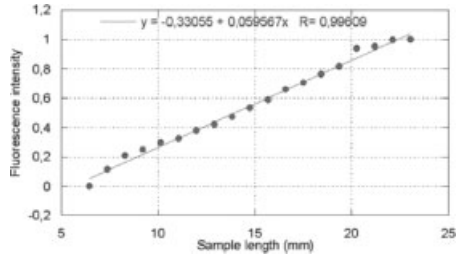

Figure 3.
Variation of fluorescence intensity of fluorescinated RGD peptides along PEG hydrogel length.

Results and Discussion

RGD Gradient Characterization

In order to verify the realization of an uniform linear gradient of covalently immobilised fluorescinated-RGD peptides on PEGDA hydrogels, the variation of fluorescence intensity along the sample was measured. Confocal microscopy analysis showed that intensity of fluorescence of fluorescinated-RGD molecules linearly increased along the hydrogel length indicating the formation of a linear and continuous gradient (Figure 3).

Cell Adhesion and Morphology on RGD Gradient

Cell behaviour was influenced by spatial distribution of RGD peptides. In particular, after 5 h from seeding, mouse embryo fibroblasts responded to covalently immobilized RGD gradients by changing their morphology. Indeed, NIH3T3 cells, adhered on RGD gradient, were fusiform and aligned along direction of the gradient (Figure 4A). Conversely, on control matrices cells were well-spreaded and randomly distributed (Figure 4B).

These observations were further confirmed by the analysis of cell alignment, performed as previously described in Materials and Methods. Results showed that the percentage of cells aligned along the gradient was higher than control surfaces (Figure 5).

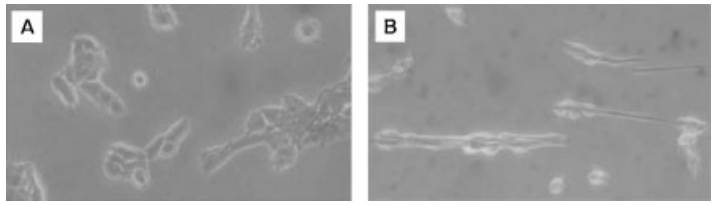

Figure 4.
Cell morphology on PEG hydrogel with uniform distribution of RGD (A) and on RGD gradient (B). Magnification 10×.

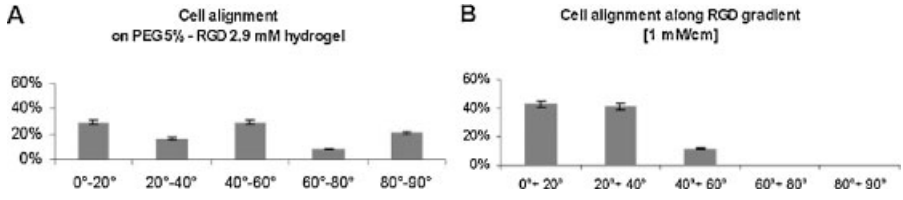

Figure 5.
Analysis of percentage of cells aligned along RGD gradient (B) and random distribution of cells seeded on control surface (A).

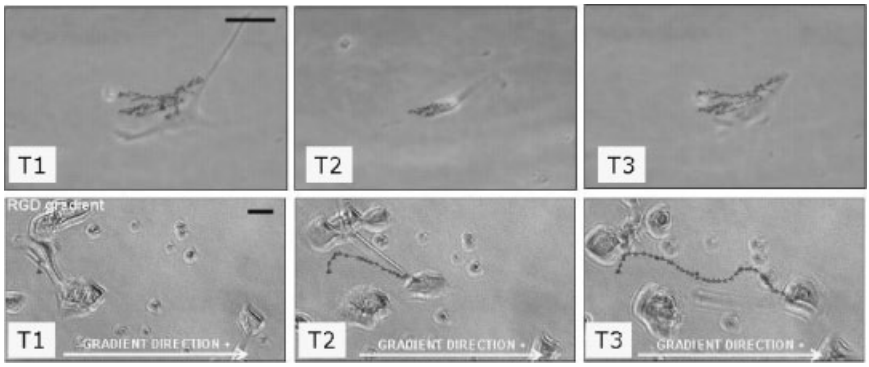

Figure 6.
Movie frames indicating trajectory (black line) of cells migrating on PEG hydrogel with uniform distribution of RGD (top panel) and on RGD gradient (bottom panel). Bar 10 μm.

Table 1.
S and P values of cells migrating on RGD gradient and on control surface.

	S [μm/min]	P [min]
RGD uniformly distributed hydrogel	0,6	68,5
RGD gradient	2,1	85,0

Effect of RGD Gradient on Cell Migration

Cell movement was also affected by covalently immobilized RGD gradients. As observed in Figure 6, cells migrating on RGD gradient move along gradient direction. Conversely, on PEGDA hydrogels with uniform RGD distribution, cell movement was random.

Moreover, cell migration parameters, S and P, were higher for cells migrating on RGD gradient, as shown by Table 1.

Conclusions

Our results show that cell behaviour can be influenced by the spatial distribution of adhesive signals. In particular, cells were aligned along the covalently immobilized RGD gradients. This study provides an example that well-controlled adhesiveness substratum can yield into basic cell biological principles essential for deciphering the cell-ECM signalling complexity for tissue-engineering biomaterial design. Even though many other aspects need to be considered in controlling cell behaviour, this hydrogel scaffold may be useful to study protein gradient effects on cell behaviour and for directing cell migration to improve the formation of engineered tissues.

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