

# Bioactive Hydrogel Substrates: Probing Leukocyte Receptor–Ligand Interactions in Parallel Plate Flow Chamber Studies

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**Abstract**—The binding of activated integrins on the surface of leukocytes facilitates the adhesion of leukocytes to vascular endothelium during inflammation. Interactions between selectins and their ligands mediate rolling, and are believed to play an important role in leukocyte adhesion, though the minimal recognition motif required for physiologic interactions is not known. We have developed a novel system using poly(ethylene glycol) (PEG) hydrogels modified with either integrin-binding peptide sequences or the selectin ligand sialyl Lewis X (SLe<sup>X</sup>) within a parallel plate flow chamber to examine the dynamics of leukocyte adhesion to specific ligands. The adhesive peptide sequences arginine–glycine–aspartic acid–serine (RGDS) and leucine–aspartic acid–valine (LDV) as well as sialyl Lewis X were bound to the surface of photopolymerized PEG diacrylate hydrogels. Leukocytes perfused over these gels in a parallel plate flow chamber at physiological shear rates demonstrate both rolling and firm adhesion, depending on the identity and concentration of ligand bound to the hydrogel substrate. This new system provides a unique polymer-based model for the study of interactions between leukocytes and endothelium as well as a platform to develop improved scaffolds for cardiovascular tissue engineering.

**Keywords**—Poly(ethylene glycol), Hydrogel, Leukocyte adhesion, Flow chamber.

## INTRODUCTION

Leukocyte adhesion to sites of inflammation is crucial to eliminate the cause of irritation and repair the surrounding tissue. After the initiation of inflammation by cytokines such as interleukin-1 $\beta$  (IL-1 $\beta$ ), tumor necrosis factor- $\alpha$  (TNF- $\alpha$ ), lipopolysaccharide, and interleukin-3, (IL-3), leukocyte velocity slows dramatically due to leukocyte contact with the vascular wall.<sup>9,27,34</sup> Rolling, the initial transient interaction, is mediated by selectin molecules present on the activated

endothelial cell surface. E-selectin is an inducible surface glycoprotein that is known to bind the carbohydrate ligand sialyl Lewis X (SLe<sup>X</sup>), which is present on various leukocytes<sup>10,11,13,35,41,43</sup> and mediates stronger adhesions and slower rolling than P- or L-selectin.<sup>27</sup> Following this primary contact, firm adhesion takes place via activated  $\beta_2$ -integrins binding intracellular adhesion molecule-1 (ICAM-1) expressed on the endothelium, and through the most important member of the  $\beta_1$  integrin subfamily, very late antigen-4 (VLA-4;  $\alpha_4\beta_1$ ). VLA-4 binds vascular cell adhesion molecule-1 (VCAM-1), and is responsible for lymphocyte adhesion to vascular endothelium and leukocyte recruitment to the inflamed area.<sup>1,10,11,27</sup> Though well studied, the exact mechanism of adhesion to the vascular endothelium is not known. There are two suggested mechanisms of integrin activation; one proposing that activation is a result of chemokine stimulation of leukocytes<sup>2,43</sup> and another suggesting that selectin binding leads to integrin activation.<sup>12,42</sup>

Several systems have been developed to examine the dynamics of rolling and firm adhesion and to elucidate the exact mechanism of the binding cascade. Leukocytes isolated from human blood have been studied under static and flow conditions on activated endothelial cell monolayers as well as on cells engineered to express endothelial cell adhesion molecules.<sup>3,28,37,40</sup> Polystyrene microspheres coated with adhesion ligands have also been shown to interact with stimulated cell monolayers<sup>17</sup> and on substrates coated with selectins and other cell adhesion molecules.<sup>11</sup> Yeast engineered to display E-selectin has been observed rolling on substrates coated with SLeX.<sup>2</sup> Recently, poly(ethylene glycol) (PEG) has been tethered to gold surfaces to create cell resistant surfaces and spatial gradients of PEG on gold surfaces have been used to study the kinetics of static cell adhesion.<sup>32,33</sup> While each of these systems has revealed new insight into the dynamics of

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leukocyte interactions with endothelial cell adhesion molecules, the ultimate goal must be to establish a simple, cell-free system that more closely mimics the *in vivo* environment.

PEG hydrogels are crosslinked hydrophilic networks that demonstrate excellent biocompatibility, being highly resistant to protein adsorption and cell adhesion, and causing minimal inflammatory responses.<sup>20,21,22,36</sup> These highly swollen networks have similar water content and mechanical properties to soft tissues and may be engineered to contain cell adhesion peptides, growth factors, and therapeutics for localized drug delivery.<sup>5,6,26,29,30,39,44</sup> PEG diacrylate hydrogels may be covalently modified with cell adhesive peptide sequences to encourage cell adhesion, spreading, and migration and the interactions of cells with these hydrogels has been extensively studied under static conditions.<sup>15,16,24,25,29,31,38</sup> A key benefit of such materials is that the base material, PEG, is intrinsically resistant to protein adsorption, so the adhesive interactions with cells are limited to the factors that are specifically engineered into the hydrogel network. In this work we propose a novel system to study leukocyte adhesion under shear using photopolymerized PEG copolymer hydrogels. After forming thin flat PEG diacrylate base hydrogels, we are able to polymerize a layer of monoacrylate PEG-peptide (or SLe<sup>X</sup>) to the surface, and using a parallel plate flow chamber, we can observe cell adhesion using video microscopy.

## MATERIALS AND METHODS

All chemicals were purchased from Sigma–Aldrich (St. Louis, MO) unless otherwise stated.

### *Synthesis of Polyethylene Glycol Diacrylate*

Polyethylene glycol diacrylate (PEG-DA) was synthesized by dissolving 12 g dry PEG (MW: 6000; Fluka, Milwaukee, WI) in 16 ml anhydrous dichloromethane (DCM) with an equimolar amount of triethylamine and 0.72 g acryloyl chloride (Lancaster Synthesis, Windham, NH) added dropwise. The mixture was stirred under argon for 24 h, washed with 2 M K<sub>2</sub>CO<sub>3</sub>, and separated into aqueous and DCM phases to remove HCl. The DCM phase was dried with anhydrous MgSO<sub>4</sub> (Fisher Scientific, Pittsburgh, PA), and the PEG diacrylate was then precipitated in diethyl ether, filtered, and dried under vacuum at room temperature overnight. The resulting polymer was dissolved in *N,N*-dimethylformamide-*d*<sub>7</sub> and characterized via proton NMR (Avance 400 MHz; Bruker, Billerica, MA) to determine the extent of acrylation.

### *Synthesis of PEG Derivatives Containing Cell Adhesion Molecules*

The cell adhesive peptide sequence used in this study include arginine–glycine–asparagines–serine (RGDS; American Peptide Company, Inc., Sunnyvale, CA), and a peptide containing the cell adhesive leucine–asparagines–valine sequence (glycine–proline–glutamic acid–isoleucine–leucine–asparagines–valine–serine–threonine, GPEILDVST), which was synthesized using standard fluorenylmethoxycarbonyl (Fmoc) chemistry on an Applied Biosystems 431A peptide synthesizer (Foster, CA). The high affinity 4-((*N'*-2-methylphenyl)ureido)-phenylacetyl-leucine-aspartic acid-valine-proline (Bio1211; Commonwealth Biotechnologies, Inc., Richmond, VA)<sup>5,6</sup> was also used as an alternate LDV-containing compound. The non-adhesive sequences used as negative controls were arginine–glycine–glutamic acid–serine (RGES; American Peptide Company, Inc., Sunnyvale, CA) and glycine–proline–glutamic acid–isoleucine–leucine–glutamic acid–valine–serine–threonine (GPEILEVST), also synthesized using Fmoc chemistry on a peptide synthesizer.

Peptides were conjugated to PEG monoacrylate by reaction with acryloyl-PEG-*N*-hydroxysuccinimide (PEG-NHS; MW 3400; Nektar Therapeutics, Huntsville, AL) in 50 mM sodium bicarbonate (pH 8.5) at a 1:1 molar ratio for 2 h. The mixture was then dialyzed (MWCO 1000), lyophilized, and stored at –20°C. Gel permeation chromatography with UV and evaporative light scattering detectors (Polymer Laboratories, Amherst, MA) was used to determine the coupling efficiency.

The selectin ligand sialyl Lewis X (SLe<sup>X</sup>) was conjugated to PEG using an avidin–biotin bridge. PEG-NHS (25 mg) was reacted with a lysine–biotin conjugate (biocytin) at a 1:2 molar ratio in 50 mM sodium bicarbonate (pH 8.5) for 2 h. SLe<sup>X</sup>-biotin (500 μg; Glycotech, Gaithersburg, Maryland) was reacted with avidin (10–15 units mg<sup>–1</sup>) at a ratio of 2 units avidin per mole of SLe<sup>X</sup>-biotin in 50 mM sodium bicarbonate (pH 8.5) for 2 h. The reaction mixtures were then combined to allow further conjugation of avidin and biotin, thereby linking acryloyl-PEG-NHS–lysine–biotin to avidin–biotin–SLe<sup>X</sup>.

### *Synthesis of Bilayered PEG Copolymer Hydrogels*

Hydrogels were formed by first dissolving 0.2 g ml<sup>–1</sup> PEG diacrylate in 10 mM HEPES buffered saline (HBS, pH 7.4); the polymer solution was then filter sterilized using a 0.22 μm filter (Gelman Sciences, Ann Arbor, MI). The photoinitiator 2,2-dimethoxy-2-phenyl acetophenone in *N*-vinylpyrrolidinone (300 mg ml<sup>–1</sup>) was added at 10 μl ml<sup>–1</sup> polymer solution. This

mixture was injected between rectangular glass plates separated by 0.5 mm spacers and polymerized under UV light (365 nm,  $10 \text{ mW cm}^{-2}$ ) for 30 s. The top plate was removed and the hydrogel surface rinsed with sterile PBS. A second layer, consisting of  $5 \mu\text{mol ml}^{-1}$  of either acryloyl-PEG-peptide or acryloyl-PEG-SLe<sup>X</sup> in HBS and  $10 \mu\text{l ml}^{-1}$  2,2-dimethoxy-2-phenyl acetophenone in *N*-vinylpyrrolidinone was then layered on top of the PEG diacrylate base gel, the upper glass plate replaced, and the second layer photopolymerized by exposure to UV light (365 nm,  $10 \text{ mW cm}^{-2}$ ) for 1 min.

#### Cell Maintenance

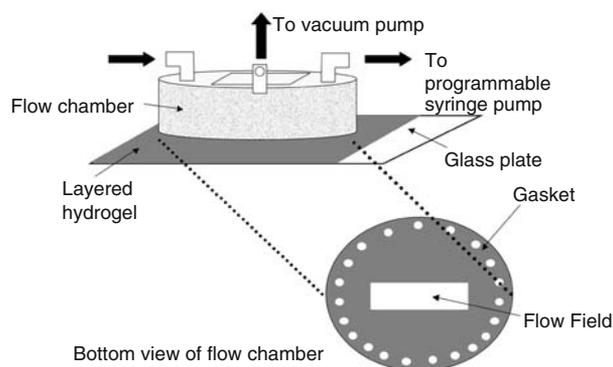
JURKAT cells (human T-lymphocytes; ATCC, Manassas, VA) were maintained in RPMI-1640 prepared with 10% fetal bovine serum (FBS; BioWhitaker, Walkersville, MD), 2 mM L-glutamine, 1 unit  $\text{ml}^{-1}$  penicillin, and 100  $\text{mg l}^{-1}$  streptomycin (GPS). 300.19/E cells (mouse pre-B lymphoblast; ATCC, Manassas, VA) were sustained in RPMI-1640 prepared with 10% FBS, 1% GPS, and 0.1 mM 2-mercaptoethanol. Cells were maintained at 37°C in a 5%  $\text{CO}_2$  environment.

#### Cell Adhesion and Rolling on Adhesive PEG Gels

Flow assays were performed using a circular parallel plate flow chamber (Glycotech, Gaithersburg, MD) mounted on the stage of a Zeiss Axiovert 135 microscope (Carl Zeiss Inc., Thornwood, NY). The chamber was placed on top of photopolymerized PEG copolymer hydrogels and vacuum sealed to the surface using a portable vacuum pump (Fisher Scientific, Pittsburgh, PA) as shown in Fig. 1. Cell suspensions were drawn through the flow field (1 cm path width, 0.01 in thickness) using a programmable syringe pump (BS-8000 Multi-Phaser™ Programmable Syringe Pump, Braintree Scientific Inc., Braintree, MA) at varying flow rates corresponding to a shear stress range of 3.5–35  $\text{dynes cm}^{-2}$ , which is comparable to *in vivo* shear rates. Cellular interactions with the hydrogels were monitored using a Nikon CoolPix 5000 camera (Nikon Inc., Melville, NY) and transferred to videotape for further analysis.

#### Cation Dependent Binding

JURKAT cells were treated with 2 mM magnesium ( $\text{Mg}^{2+}$ ), calcium ( $\text{Ca}^{2+}$ ), or manganese ( $\text{Mn}^{2+}$ ) or with 10 mM EDTA and then perfused through the flow chamber and allowed to settle on the gel for 5 min. Controls were exposed to standard formulations of RPMI-1640 containing 0.4 mM  $\text{Ca}^{2+}$  and 0.4 mM  $\text{Mg}^{2+}$ . Ten fields of view were scanned to get



**FIGURE 1.** Schematic of parallel plate flow chamber system. A small pump is used to create a vacuum, sealing the chamber on the surface of the gel. Cells are then perfused over the gel surface using a programmable syringe pump.

an average number of cells per field of view. Flow rates corresponding to shear stresses of 0.5, 1.0, and 10  $\text{dynes cm}^{-2}$  were used to wash away unbound cells. The number of cells remaining for each shear stress was counted and averaged over several fields of view.

#### LDV Specificity

JURKAT cells treated with 2 mM  $\text{Mg}^{2+}$  were allowed to settle on the LDV gel for 5 min and an average number of cells per field of view was determined. Specificity was demonstrated by the addition  $7 \mu\text{g ml}^{-1}$  of either a mouse anti-human monoclonal antibody that blocks VLA-4 binding to VCAM-1 (anti-CD49d, clone BU49; Ancell Corporation, Bayport, MN) or an IgG<sub>1</sub> isotype control (purified mouse myeloma IgG<sub>1</sub>; Invitrogen Corporation, Carlsbad, CA) at a shear stress of 0.5  $\text{dynes cm}^{-2}$ . The average number of cells bound per field of view was again counted to determine the amount of cells remaining bound to the surface.

Specificity was also demonstrated by the addition of a solution of either 10 mM EDTA or 150  $\mu\text{M}$  Bio1211 introduced into the flow chamber under a shear stress of 0.5  $\text{dynes cm}^{-2}$ . An average number of cells bound to the gel under flow was determined every minute for 13 min.

#### Video Analysis

Cells were allowed to settle on each gel for 5 min. An average of 10 fields of view was scanned and the number of cells settled on the peptide gel was counted. After flow began, fields of view were scanned again and the number of cells remaining (bound to the gel) was counted. After flow was established on the SLe<sup>X</sup> gels, video was paused and the number of interacting cells was counted. The numbers were averaged over 10 fields of view for each shear stress.

### Statistical Analysis

Data were compared with two-tailed, unpaired *t*-tests; *p*-values less than 0.05 were considered to be significant.

## RESULTS

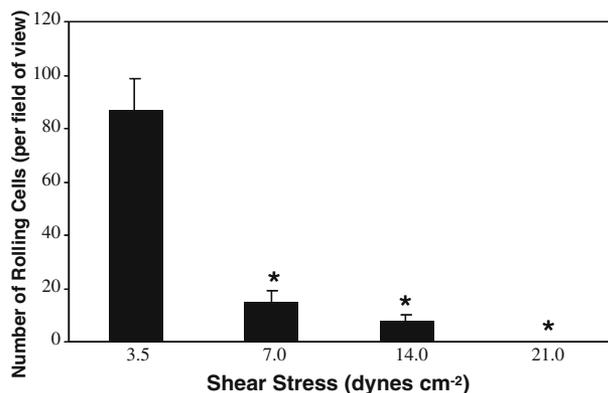
### Synthesis of PEG Hydrogels

PEG hydrogels were formed under UV light in the presence of a photoinitiator between two glass plates. Hydrogels 0.5 mm thick were formed after 30 s of exposure. The addition of an acryloyl-PEG-peptide derivative mixed with the photoinitiator to the surface of the hydrogels resulted in a covalently bound layer of cell adhesive moiety on the surface (Fig. 1).

### Quantification of Cell Adhesion and Rolling

Cell rolling on SLeX hydrogels was quantified over a range of shear stresses, and  $86.8 \pm 11.6$  cells per field of view rolled on the gel surface at  $3.5 \text{ dynes cm}^{-2}$  with an average rolling velocity of  $141.3 \mu\text{m s}^{-1}$  (Fig. 2). Rolling decreased with increasing shear stress ( $7.0 \text{ dynes cm}^{-2}$ :  $15.2 \pm 3.8$  cells, average rolling velocity  $232.7 \mu\text{m s}^{-1}$ ;  $14 \text{ dynes cm}^{-2}$ :  $8.2 \pm 1.9$  cells, average rolling velocity  $486.8 \mu\text{m s}^{-1}$ ;  $21 \text{ dynes cm}^{-2}$ : no rolling observed).

Approximately  $98.5 \pm 18.6\%$  of cells contacting hydrogel surfaces modified with Bio1211 adhered to the gel surface. In comparison,  $41.5 \pm 13.4\%$  of cells in contact with the surface of PEG-LDV, an interaction specific for the  $\alpha_4\beta_1$  integrin expressed on JURKAT cells, adhered to the hydrogels and  $23 \pm 8.5\%$  of cells contacting PEG-RGDS hydrogels adhered to the gel

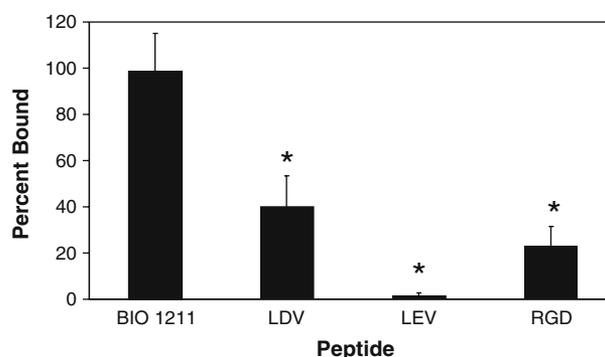


**FIGURE 2.** 300.19/E cells rolling on SLeX modified gel. 300.19 cells transfected with human E-selectin was perfused over a SLeX modified gel. The number of rolling cells were counted per field of view. SLeX ability to support rolling decreased as shear stress increased ( $*p < 0.02$  as compared to shear rate of  $3.5 \text{ dyne cm}^{-2}$ ).

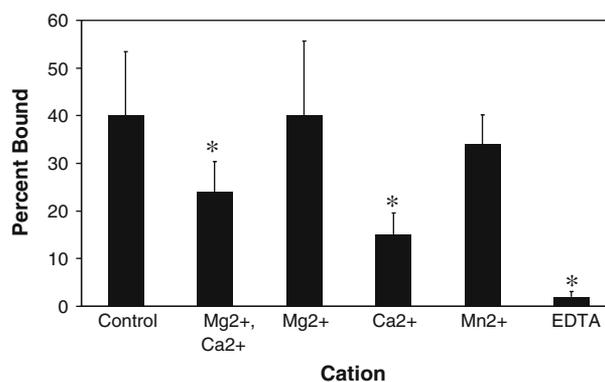
surface (Fig. 3). No cells adhesion to PEG-DA or PEG-RGES hydrogels was observed; however the control LEV peptide-modified gels had  $1.6 \pm 1.2\%$  of cells contacting the surface adhere.

### Cation Dependant Binding

Cation sensitivity was assessed by the addition of cations or EDTA to cell cultures prior to their exposure to PEG-LDV hydrogels. The presence of 10 mM EDTA had the greatest inhibitory effect on binding, followed by 2 mM  $\text{Ca}^{2+}$ .  $\text{Mg}^{2+}$  and  $\text{Mn}^{2+}$  had little effect on the ability of JURKAT cells to bind LDV, and a higher number of cells exposed to both calcium and magnesium were able to bind to the gel surface than those exposed to calcium alone (Fig. 4).



**FIGURE 3.** Peptide ability to bind VLA-4. JURKAT cells were allowed to settle on PEG-DA gels conjugated with different peptides at similar densities. After 5 min, cells were subjected to a shear stress of  $0.5 \text{ dynes cm}^{-2}$  to remove unbound cells and the remaining cells were counted ( $*p < 0.02$  compared to Bio1211).



**FIGURE 4.** Cation dependent binding of VLA-4 to LDV peptide. JURKAT cells were incubated with 2 mM cations or 10 mM EDTA prior to settling on the LDV gel; controls were exposed to standard formulations of RPMI-1640 containing  $0.4 \text{ mM Ca}^{2+}$  and  $0.4 \text{ mM Mg}^{2+}$ . The percentage of cells on the gel was determined based on the total number of cells before initiating a shear stress of  $0.5 \text{ dynes cm}^{-2}$  ( $*p < 0.05$  compared to Control).

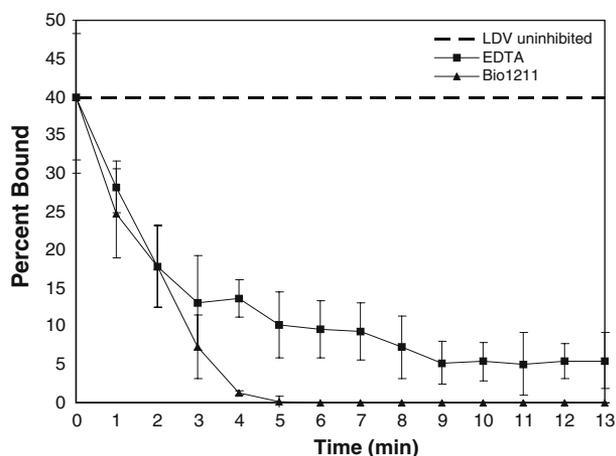
### LDV Specificity

JURKAT cell binding to PEG-LDV hydrogels was reversed upon the addition of a monoclonal anti-VLA-4 ( $3.4 \pm 2.1\%$  bound). Cells exposed to an isotype-matched control antibody were still able to adhere to the hydrogel surface ( $88.8 \pm 33.4\%$  bound), demonstrating specificity of LDV for the VLA-4 integrin. The higher affinity of Bio1211 compared to the LDV peptide was also observed by the addition of unbound Bio1211 after cells were allowed to settle on PEG-LDV hydrogels (Fig. 5). Bio1211 was able to remove cells from the gel surface better than EDTA through competitive binding of VLA-4.

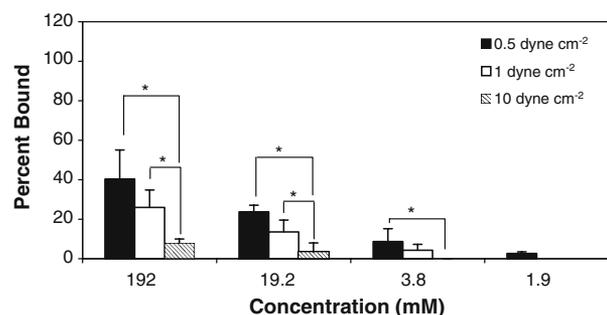
The influence of peptide concentration on cell adhesion was determined by varying the amounts of LDV bound to the hydrogel surface (Fig. 6). The number of cells bound to the gel increased with LDV concentration, and LDV was better able to support adhesion at lower shear stresses for all concentrations when compared to higher rates of shear.

## DISCUSSION

The system of hydrophilic hydrogels modified with cell adhesion peptides implemented in this study exhibits the ability to mimic the cell adhesion cascade that occurs during the onset of inflammation in the



**FIGURE 5.** LDV Specificity for VLA-4. 10 mM EDTA or 1.5  $\mu$ M Bio1211 was introduced into the flow chamber at a wall shear stress of 0.5 dynes  $\text{cm}^{-2}$  after JURKAT cells were allowed to settle on the LDV gel for 5 min. Bio1211, which has a higher affinity for VLA-4 was able to compete the cells off the LDV modified gel surface. EDTA was not as effective as Bio1211 as cells detached more slowly and not completely. The dashed line represents the uninhibited controls, in which  $40 \pm 15.2\%$  of the cells initially exposed to the hydrogel remained bound through the duration of the experiment. Each data point represents the average percent of bound cells per 10 fields of view and error bars represent the standard deviation of the percent of bound cells within those 10 fields of view.



**FIGURE 6.** Dilution effect of LDV. The number of JURKAT cells bound to the gel decreased as the LDV peptide concentration was lowered. LDV was better able to support adhesion at lower shear stresses for all concentrations (\* $p < 0.05$  comparing shear rates at each concentration).

vascular system. The capacity to modify hydrogels with cell adhesion molecules for the study of cellular interactions with biomaterials for drug delivery and tissue engineering is well documented.<sup>4,7,15,16,18,24–26,29–31,38,39,44</sup> This study implements the use of these materials under physiological flow conditions to examine the mechanisms of leukocyte rolling and firm adhesion.

The formation of thin flat PEG diacrylate hydrogels for use with a parallel plate flow chamber improves upon earlier systems that utilize hydrophobic surfaces with adsorbed cell adhesion molecules. The highly crosslinked structure of swollen PEG networks have water content similar to native vessels, and covalent incorporation of cell adhesive peptides guarantees control of concentration and allows for patterning of one or more adhesion sequences on the gel surface.<sup>23</sup> This system is based on a flexible substrate with tunable stiffness, the properties of which can be exploited to examine the responses of different cell types in microenvironments that mimic native tissues in various states of development, remodeling, regeneration, and disease. It has been suggested that the differentiation of cellular function and response could depend significantly on matrix elasticity,<sup>8,14,45</sup> and varying the polymer composition or concentration in the hydrogel can alter the permeability and mechanical properties,<sup>36</sup> simulating a range of biologically relevant conditions. In addition, the local mobility of adhesive ligands in the solvated hydrogel system should be considerably better than on solid substrates, allowing more variable orientations which could contribute to a greater fraction of accessible ligands available to receptors on the cell surface.

Leukocyte cell adhesion to the surfaces of modified PEG-DA hydrogels was highly specific, reversible, and sensitive to ligand site density and affinity, demonstrating the efficacy of the system to mimic the events leading to the firm adhesion of immune cells on

activated endothelium. The slow rolling of 300.19/E cells on the surfaces of PEG-SLe<sup>X</sup> hydrogels confirms the integration of SLe<sup>X</sup> into the system through the use of an avidin–biotin linkage, which did not disrupt the active binding site of the carbohydrate. Incorporation of the RGD and LDV peptides encouraged firm cell adhesion to the materials surface under shear in a concentration dependant manner, and cells remained adherent throughout the duration of the flow experiment. These results illustrate that this method of studying leukocyte adhesion succeeds in mimicking the interactions seen on the *in vivo* vascular wall under shear. With further optimization, such as incorporation of signaling molecules, this system using of PEG gels can improve insight into the mechanisms of rolling and firm cell adhesion at sites of active vascular disease.

## CONCLUSIONS

The covalent modification of PEG hydrogel surfaces with cell adhesive peptides was accomplished in continuous layers of adhesive regions. These materials encouraged vascular cell adhesion through both transient interactions with selectin molecules and firm binding via integrins. The system presented here to expose adhesive hydrogels to cells under flow conditions represents a method to study cell–material interactions in environments that closely mimic *in vivo* environments.

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