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Probing Dynamic Cell-Substrate Interactions using Photochemically Generated Surface-Immobilized Gradients: Application to Selectin-Mediated Leukocyte Rolling

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Abstract

Model substrates presenting biochemical cues immobilized in a controlled and well-defined manner are of great interest for their applications in biointerface studies that elucidate the molecular basis of cell receptor-ligand interactions. Herein, we describe a direct, photochemical method to generate one-component surface-immobilized biomolecular gradients that are applied to the study of selectin-mediated leukocyte rolling. The technique employs benzophenone-modified glass substrates, which upon controlled exposure to UV light (350 – 365 nm) in the presence of protein-containing solutions facilitate the generation of covalently immobilized protein gradients. Conditions were optimized to generate gradient substrates presenting P-selectin and PSGL-1 (P-selectin Glycoprotein Ligand-1) immobilized at site densities over a 5- to 10-fold range (from as low as ~200 molecules/ μm^2 to as high as 6000 molecules/ μm^2). The resulting substrates were quantitatively characterized via fluorescence analysis and radioimmunoassays before their use in the leukocyte rolling assays. HL-60 promyelocytes and Jurkat T lymphocytes were assessed for their ability to tether to and roll on substrates presenting immobilized P-selectin and PSGL-1 under conditions of physiologically relevant shear stress. The results of these flow assays reveal the combined effect of immobilized protein site density and applied wall shear stress on cell rolling behavior. Two-component substrates presenting P-selectin and ICAM-1 (intercellular adhesion molecule-1) were also generated to assess the interplay between these two proteins and their effect on cell rolling and adhesion. These proof-of-principle studies verify that the described gradient generation approach yields well-defined gradient substrates that present immobilized proteins over a large range of site densities that are applicable for investigation of cell-materials interactions, including multi-parameter leukocyte flow studies. Future applications of this enabling methodology may lead to new insights into the biophysical phenomena and molecular mechanism underlying complex biological processes such as leukocyte recruitment and the inflammatory response.

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[†]Electronic Supplementary Information (ESI) available: contact angle measurements (Table S1); conditions for generation of P-selectin, E-selectin, and PSGL-1 gradients (Table S2); data from gradient linescans and approximations (Table S3); linescans from one-component gradients prepared for flow assays with HL-60 cells (Fig. S1); critical velocity values (Table S4). See DOI: 10.1039/b000000x/

Introduction

Numerous cellular processes depend on a cell's ability to sense and respond to biomolecular gradient cues. Embryonic development, neuronal guidance, angiogenesis, wound healing, inflammation, and cancer/metastasis are among the complex physiological phenomena that are regulated and/or guided by a spatial variation in the concentrations of ligands encountered either in the solution phase or presented as immobilized gradients within the cellular microenvironment (i.e. adjacent cells or the extracellular matrix).¹ In order to experimentally parse out the detailed contributions of immobilized ligand density on cell behavior, substrates that present biomolecular gradients on surfaces are of fundamental interest for *in vitro* assays enabling the investigation of cell migration and polarization in response to immobilized protein concentration.^{2–3} Of even greater interest are *in vitro* platforms that enable multi-parameter studies that systematically address the combined effects of multiple stimuli, such as the density of multiple biomolecules, topography, or shear stress, amongst others, on cell behavior.⁴

Many approaches have been developed to generate biomolecular surface gradients, including the use of microfluidics followed by surface attachment,^{2,5–6} electrochemical desorption of self-assembled monolayers on gold,⁷ photochemical surface deprotection to reveal reactive functional groups,⁸ and diffusion-guided surface adsorption.^{9–10} Each of these techniques have inherent strengths and limitations. Some require prior (bio)chemical modification of biomolecules with non-native functional groups to enable site-specific immobilization. Conversely, other attachment approaches utilize native functional groups, which offer increased biomolecular generality, but often at the cost of random orientation. Some techniques are limited to small-scale gradients (100s of microns), while others have the potential to generate gradients on the millimeter-scale. Finally, a handful of approaches have the potential to allow the controlled deposition of multiple different biomolecules onto a single surface, while others are limited to single-component substrates. (For an extensive review of the various approaches to gradient generation and surface immobilization of biomolecules, see references^{1,11}).

Our approach involves surface functionalization of glass to present a well-known photocrosslinking molecule, benzophenone (BP).^{12–26} Solutions containing the biomolecule of interest are introduced to the substrate through a flow chamber and immobilized as UV light (350 – 365 nm) induces the formation of new C-C bonds between the BP molecules on the surface and the proteins. This approach was previously shown by our lab to be useful for the generation of single- and multi-component geometric patterns and spatial concentration gradients composed of both proteins and carbohydrates.^{27–28} In choosing this particular gradient generation approach we reasoned that the overall simplicity, biomolecular generality, and capability to generate complex, multi-component substrates for biointerface studies would make this a broadly applicable platform for researchers from multiple fields of specialization possessing varying degrees of familiarity with the advanced surface chemistries and microfabrication techniques utilized in other gradient generation methodologies.

As a proof-of-principle demonstration of this technique, we were interested in applying surface-immobilized gradients to achieve a better understanding of the process of leukocyte recruitment, the first step in the inflammatory response. Over the past several decades, numerous biomolecules involved in leukocyte trafficking have been identified and characterized. Among these are a family of molecules known as selectins, which are membrane-bound glycoproteins presented on leukocytes (L-selectin) and inflamed endothelial cells (P-selectin, E-selectin).²⁹ The role of selectins is to mediate the initial flow-dependent tethering and rolling of leukocytes on vascular surfaces.^{30–33} Following selectin-mediated rolling, additional biomolecular interactions take place with other endothelial-expressed proteins and enzymes that ultimately lead to leukocyte arrest, diapedesis and migration through the tissue to the site of injury or infection.³⁴ Properly regulated inflammatory and immune responses are essential for health and survival, but if left unchecked, can result in a variety of diseases including rheumatoid arthritis, asthma, psoriasis, Crohn's disease, thrombotic disorders, and autoimmune disease.³⁵ A more complete understanding of the molecular basis of leukocyte recruitment could lead to the development of more effective therapeutics for dysregulated inflammation.

Previous studies have shown that selectin-mediated rolling under conditions of physiological shear stress can be studied in an *in vitro* flow assay format, yielding new insights into the biophysical phenomena underlying the first step of leukocyte recruitment.^{36–41} *In vitro* leukocyte flow assays, however, typically employ surfaces presenting non-specifically adsorbed proteins,^{42,43} which are susceptible to changes over time,^{11,44} or utilize confluent endothelial cell monolayers,⁴⁵ which express multiple relevant ligands but offer little control over ligand site density.

As schematically illustrated in Fig. 1, substrates presenting photochemically immobilized gradients of P-selectin and PSGL-1 were generated, characterized, and employed in leukocyte flow assays to determine how various selectin-ligand interactions affect the rolling behavior of two leukocyte cell lines, HL-60 promyelocytes and Jurkat T lymphocytes. Cells were perfused across substrates presenting immobilized protein under conditions of physiological wall shear stress. Videos were acquired at various positions across the gradient surface and analyzed to assess the combined effect of protein site density and wall shear stress on the number and velocity of rolling cells. By analyzing cell behavior at each combination of site density and shear stress, the interplay between immobilized protein density and shear stress were observed. This simple gradient generation approach will allow for the interrogation of the complex mechanisms involved in the biological processes that result in leukocyte rolling *in vivo* and further elucidate the interplay between substrate composition and environmental conditions.

In this study, we describe a facile, molecularly general method for generating model substrates that present covalently immobilized biomolecules at defined and varied site densities. Furthermore, it is shown that gradient substrates presenting proteins over a wide range of site densities allow data-rich information to be collected from a single substrate in a short time. This presents an additional advantage over previous approaches which utilize substrates limited to a single homogeneous presentation of a protein of interest, requiring

experiments with multiple substrates to probe the concentration dependence of leukocyte rolling.

Materials and Methods

Preparation of BP-modified substrates

All chemicals were purchased from Sigma-Aldrich, unless otherwise noted. Glass microscope slides were cleaned with Piranha solution (3:1 v/v concentrated H₂SO₄:30% H₂O₂).⁴⁶ Slides were functionalized with 4-(triethoxysilyl)butyl aldehyde (Gelest) via chemical vapor deposition under reduced pressure for 2.5 hr. Following silanization, slides were cured at 120 °C for 1 hr, soaked in absolute ethanol for 30 min, and dried under a stream of nitrogen. Slides were then incubated in a solution of 20 mM 4-benzoyl benzylamine hydrochloride (Matrix Scientific) and 200 mM NaCNBH₃ in a 4:1 DMF:MeOH solution for 4 hr. Remaining unreacted aldehyde groups were blocked by immersion in aldehyde blocking buffer (200 mM ethanolamine, 0.1 M Tris, pH 7) for 1 hr, followed by rinsing in DMF, methanol, and ethanol, and drying under a stream of nitrogen. After each step, contact angle goniometry (Ramé-Hart) was used to confirm that the chemical functionalization was successful. The resulting BP-modified substrates were stored in a dessicator until use.

Quantitative determination of immobilized protein density

A standard method for quantifying immobilized protein site density on a surface involves incubating the substrate with a saturating concentration of radiolabeled antibody followed by analysis in a scintillation counter. A calibration curve of known standards is generated and used to convert the signal to a measure of site density (the number of antibodies bound per area), assuming 1:1 binding between the antibody and antigen.^{37,47} A similar method was employed to determine the site density of proteins (P-selectin or PSGL-1) covalently attached to BP-modified substrates, as previously described.²⁸ Traditional radioimmunoassays are designed to determine the average site density of a substrate presenting a homogeneous layer of a single protein. Because our end goal was to determine site densities at various positions along immobilized protein gradient substrates, we needed a way to correlate data from fluorescence images with the site density data from the radioimmunoassays. To address this challenge, substrates presenting a homogeneous site density of protein were generated and split into two groups: one for fluorescence and one for radioactivity analysis. The results from both studies were correlated to determine the relationship between fluorescence intensity (F.I.) and site density for each protein. In this way, gradient substrates could be quantified by converting the data from fluorescence analyses to site density using the standard curves from the fluorescence-radioactivity correlation studies.

Solutions of P-selectin and PSGL-1 (R&D Systems) were freshly diluted from concentrated stocks (2 mg/mL) into PBS with Ca²⁺ and Mg²⁺ to a final concentration of 5 µg/mL. A BP-modified substrate was assembled into a rectangular parallel-plate flow chamber (GlycoTech) with a silicone gasket (127 µm thickness). The inlet and outlets tubes of the chamber were assembled to allow solution flow to be controlled by a syringe pump (Harvard

Apparatus). Protein solutions were flowed through the chamber at 3 mL/min until filled (about 200 μ L required), then flow was stopped. A UV LED (365 nm, 17 mW/cm², Clearstone Technologies) was employed to perform flood exposures of each substrate for various times (10, 30, 40, 60, 120, 180 sec) in the presence of each protein solution. Following protein photoimmobilization, the flow chamber was disassembled and substrates transferred to a solution of 1% BSA/0.5% Tween 20/PBS with Ca²⁺ and Mg²⁺ for 1 hr, and blocked in 1% BSA in PBS with Ca²⁺ and Mg²⁺ for an additional 1 hr. For each set of exposure conditions, six replicates were generated, three of which were subjected to fluorescence analysis, and three of which were analyzed for radioactivity and site density quantitation.

Substrate characterization via fluorescence required primary unlabeled mAbs (R&D Systems), and AlexaFluor647-labeled secondary mAbs (Invitrogen). For radioimmunoassays, an unlabeled secondary goat anti-mouse IgG/M polyclonal antibody (pAb, Amersham Biosciences), was iodinated with ¹²⁵I (Perkin Elmer) using IODO-gen reagent (Pierce). [¹²⁵I]-labeled pAb was purified using Bio-spin 6 columns (Bio-rad) to remove excess ¹²⁵I, and characterized by TLC to determine the specific activity and % free ¹²⁵I (0.69 μ Ci/ μ g, 4.42% free ¹²⁵I). For fluorescence analysis, substrates were incubated with the appropriate primary mAb (1 μ g/mL) and AlexaFluor647-labeled secondary mAb (0.05 μ g/mL) in 1% BSA/PBS overnight at room temperature. They are then rinsed in PBS and H₂O, dried under a stream of nitrogen, and analyzed using a fluorescence slide scanner (GenePix 4000B, MDS Analytical Technologies). For radioactivity analysis and quantification, substrates were incubated with their respective primary mAbs (20 μ g/mL) in BSA/PBS at room temperature overnight, followed by incubation with [¹²⁵I]-labeled secondary pAb (30 μ g/mL) in BSA/PBS at room temperature for 30 min. Slides were then rinsed with PBS and water, dried under nitrogen, and cut into 1 $\times$ 2.5 cm pieces for analysis in a scintillation counter (LS 6500 Multi-Purpose Scintillation Counter, Beckman Coulter). Standards of known amounts of [¹²⁵I]-labeled secondary pAb were used to generate a standard curve, which was utilized to convert all radioactivity measurements to the number of molecules immobilized on the surface. This correlation assumed a 1:1 binding interaction between the primary antibody and the immobilized antigen, and also between the primary and secondary antibodies. Knowing the area of each substrate analyzed, the F.I. data was plotted as a function of site density (molecules/ μ m²) and linear regression analyses were performed to determine the linear fit for each set of data. This procedure was performed separately for P-selectin and PSGL-1. The calibration curves were used to convert all F.I. data from gradient substrates to units of site density (molecules/ μ m²).

Generation of surface-immobilized gradients

For the creation of protein gradient substrates, an Ar ion laser (Coherent Innova 90-4, Laser Innovations, 350–365 nm) was employed for UV exposures and photoimmobilization. To enable large-area exposures with uniform illumination, the input Gaussian beam was converted to a flat-top output using a refractive beam shaper (π -Shaper, MT-Berlin), and expanded to a 1 $\times$ 1 cm² exposure area with a beam-expanding telescope optic configuration. The profile of the input and output beams were characterized with a laser beam profiling camera (Ophir-Spiricon) to ensure that the beam was homogeneous across the exposure

field. The laser power was adjusted to a final illumination intensity of 14 mW/cm² at the substrate.

In order to create a gradient of light exposure across the BP-modified slide, a shutter was attached to a programmable translation stage (ThorLabs Opto DC Driver) and positioned between the light source and the substrate. Illumination and linear movement of the shutter were initiated simultaneously so that the substrate was exposed to a gradient in light exposure.

The shutter was programmed to move 4.2 mm over a defined period (24–30 sec) while the substrate was exposed to the expanded and homogenized laser light (see detailed conditions for gradient generation in E.S.I.). A BP-modified substrate was assembled in the flow chamber which was positioned beneath the shutter. After protein solutions were introduced into the chamber, UV exposure and shutter movement were initiated simultaneously. Following photoattachment, the flow chamber was then immersed in a solution of 0.5% Tween 20 in PBS with Ca²⁺ and Mg²⁺, the substrate was detached, and the same rinsing and blocking steps were performed as described above.

Six replicate gradient substrates were generated for each protein; three were analyzed to determine site densities as detailed above, and the other three utilized for cell rolling studies. Conditions for incubation with fluorescently labeled binding partners and the fluorescence scanner settings were the same as described above. Fluorescence data were converted to units of site density in molecules/μm², via correlation of radioimmunoassay data. Substrates that were prepared for subsequent cell rolling studies were stored in BSA/PBS solution overnight.

Cell culture

HL-60 promyelocytes were obtained from Prof. Fei Wang (University of Illinois) and Jurkat T lymphocytes were purchased from the American Type Culture Collection (ATCC). Cells were cultured in RPMI 1640 medium supplemented with penicillin (100 U/mL), streptomycin (100 μg/mL), and 10% heat-inactivated fetal bovine serum (SCS Cell Media Facility, UIUC). Cells were passaged in order to maintain densities between 0.1×10^6 and 1.0×10^6 cells/mL.

Leukocyte flow assays

Figure 1 shows the schematic of the setup that was employed for the leukocyte flow assays. Gradient substrates were assembled into the parallel-plate flow chamber while immersed in PBS with Ca²⁺ and Mg²⁺ to prevent drying out of the substrate which could result in protein denaturation. The tubing was connected to a syringe pump to control fluid flow and the chamber was positioned on top of an optical microscope (Zeiss 40C Invertiskop, Carl Zeiss Inc.). Cells were counted, pelleted for 5 min at 500 rcf, rinsed with HBSS with Ca²⁺ and Mg²⁺, and resuspended in 0.5% human serum albumin (HSA) in HBSS with Ca²⁺ and Mg²⁺ and 10 mM HEPES at pH 7.4, to a final concentration of 0.5×10^6 cells/mL. A digital video camera was used to record cells interacting with the substrate at various positions along the gradient while being subjected to defined amounts of wall shear stress. Shear stress was

calculated using the equation $\tau = 6\mu Q/a^2b$, where μ is the apparent viscosity of the media, Q is the volumetric flow rate, and a and b are the height and width of the flow chamber, respectively. To calculate wall shear stress, the viscosity of buffer was assumed equal to water at room temperature (1.0 centipoise, 24 °C).

Phase contrast microscopy was utilized to maximize contrast between interacting cells and the substrate, which was necessary to perform analyses with ImageJ software (National Institutes of Health). Using the MTrack2 plugin, the average velocity of rolling cells was determined (reported in $\mu\text{m}/\text{sec}$). To distinguish between interacting and non-interacting cells during the analysis, the critical velocity (v_{crit}) was determined for each flow rate.⁴⁸ To determine v_{crit} , videos of non-interacting cells in shear flow near the wall of the flow chamber were acquired and analyzed (see Electronic Supplementary Information, E.S.I.).⁴⁸ Cells moving slower than the v_{crit} for at minimum of 1 sec (30 frames) were defined as rolling. To determine rolling flux, cells were manually counted in the first 2 seconds of each video (reported as # rolling cells/ mm^2). The field of view (FOV) for the microscope with a 20x objective was 0.174 mm^2 .

In this study, initial attachment assays were performed to assess the ability of cells to initiate tethering and rolling while experiencing a defined level of wall shear stress. Cells were first introduced into the flow chamber at 3 mL/min (18.6 dyn/cm^2) for 1 min; the flow rate was then dropped to achieve the desired shear stresses occurring from 10 to 500 $\mu\text{L}/\text{min}$. The predetermined shear stresses were 0.062, 0.16, 0.31, 0.47, 0.62, 0.93, 1.55 and 2.17 dyn/cm^2 . After 1 min at each flow rate, videos (10 to 30 sec) were acquired at each position along the gradient (5 to 7 positions per substrate). Between each shear stress that was investigated, the interacting cells were stripped from the surface by applying a high shear (3 mL/min, 18.6 dyn/cm^2) and the process was repeated at the next flow rate.

Statistical analysis

For leukocyte flow assays, three replicates of each gradient substrate were generated for each of the two sets of assays, which were performed on different days. Data are reported as the average with error bars representing the standard error of the mean (SEM) for $n=3$ substrates, unless otherwise noted. To determine if a set of data showed correlation between dependent and independent variables (i.e. rolling flux and site density), correlation and linear regression analyses were performed. To determine if two data points were statistically different, one-sided two-sample t -tests were performed (Microsoft Excel), assuming population variances to be equal. Two-dimensional plots were generated using OriginPro8 (OriginLab) and 3D surface plots were created with SigmaPlot (Systat Software, Inc.). Statistical significance was set at a value of $p<0.05$.

Results

Characterization of BP-modified substrates

Three batches of BP-modified substrates were generated. Following each chemical derivatization, substrates were characterized with contact angle goniometry. The contact

angle after silanization was $45.2 (\pm 2.6)^\circ$. After functionalization with BP, the contact angle increased to $58.1 (\pm 3.9)^\circ$. Detailed contact angle results are reported in the E.S.I.

Quantitative determination of immobilized protein site densities

Data from radioimmunoassays and fluorescence assays are plotted to show the relationship between F.I. and site density, as shown in Figure 2. Under the conditions used, we observed that the relationship between the F.I. and corresponding radioactivity signal (converted from cpm to site density using a standard curve) was linear for each set of immobilized protein substrates. The BP-modified substrates used herein have immobilized protein densities of up to $\sim 8,000$ molecules/ μm^2 .

The reported site densities are an estimate based on the ability of antibodies to detect the proteins. For simplicity, we assume a 1:1 binding relationship between the [^{125}I]-labeled secondary pAb and primary mAb, which likely results in an overestimate of protein loading, since each primary mAb might be recognized by multiple secondary [^{125}I]-labeled pAbs. We also assume a 1:1 binding relationship between the primary mAb and the immobilized surface proteins, although each primary mAb has two antigen binding sites. Overall, the effective site density is probably lower than that determined from the radioimmunoassay since proteins are randomly orientated on the surface and therefore not all are able to be recognized by cellular receptors. However, similar methods and assumptions have been previously made for substrates used in quantitative selectin-ligand studies assays.⁴⁷

Generation and characterization of linear one-component gradients

Our goal in preparing one-component gradient substrates for leukocyte flow assays was to incorporate biomolecules that are known to be involved in the initial steps of leukocyte recruitment. The first step of leukocyte recruitment is rolling along the endothelium, which is facilitated by selectin-ligand interactions. P- and E-selectin are expressed on the inflamed endothelium, while L-selectin is expressed on most leukocytes and binds to ligands on the endothelium as well as to PSGL-1 expressed on other rolling cells. In our studies, we generated and characterized one-component gradients of P-selectin and PSGL-1 and applied them to leukocyte flow assays. Conditions for gradient generation were optimized by adjusting protein concentration, exposure time and light intensity, to yield linear slopes encompassing a large range of protein site densities, which are detailed below. The plots in Figure 3 show linescans from three replicate substrates for each protein. The conditions utilized for P-selectin and PSGL-1 gradients were optimized for each protein. For clarity, we report the values as approximations of the precisely determined values (see E.S.I. for detailed gradient generation conditions, raw data values and assigned approximations). Gradient substrates made in parallel with those used in cell rolling experiments were characterized in a fluorescence assay and the results converted to site density using the appropriate linear relationship between F.I. and site density for each protein. The average site density at various positions along the gradient was determined for a region the size of the field of view of a 20x microscope objective.

Leukocyte flow assays

Leukocyte flow assay results are presented in Figs. 4 through 7. HL-60 cells were found to tether to and roll on P-selectin substrates over a wide range of site densities and wall shear stresses. Jurkat cells also tethered to and rolled on immobilized P-selectin, as well as PSGL-1, over a range of site densities and shear stresses. HL-60 cell rolling was not observed on PSGL-1 substrates (data not shown), which is consistent with literature reports that show low L-selectin expression on HL-60 cells.⁴⁹

In general, we found that a minimum level of wall shear stress, commonly referred to as the shear threshold,^{42,50} was required for selectin-mediated cell rolling to be initiated and sustained, and was also affected by the underlying site density of immobilized protein. A lack of cell rolling under a particular set of conditions is represented by the absence of that data point. In all cases, data from three replicate substrates is plotted as the average ($\pm$ SEM), unless otherwise noted. Alongside each 2D plot, the average rolling velocities and cell flux are plotted in 3D surface plots (without error bars for clarity) to present the combined effects of site densities and shear stress on cell rolling velocity as an interaction landscape.

Neither cell type was found to interact with BSA-blocked BP-modified substrates, confirming that leukocyte rolling on gradient substrates were indeed the result of specific interactions with the immobilized protein of interest. When Jurkat cells were incubated with a function-blocking mAb against L-selectin (10 μ g/mL) cell rolling on immobilized PSGL-1 substrates was absent under all conditions of wall shear stress investigated. Additionally, when P-selectin substrates were incubated with a function-blocking mAb (20 μ g/mL), cell rolling for both cell types was completely eliminated.

P-selectin-mediated rolling of HL-60 cells—P-selectin substrates for HL-60 flow assays presented site densities ranging from 140 to 1000 molecules/ μ m². Cell rolling was observed over the entire range of site densities and the shear threshold was met starting at a shear stress of 0.062 dyn/cm² (10 μ L/min). It was found that as site density decreased, cell rolling occurred over a smaller range of shear stresses. At site densities of 140 and 180 molecules/ μ m², cell rolling was observed from the shear threshold value up to 0.62 and 0.93 dyn/cm², respectively, while at site densities 280 molecules/ μ m², cell rolling was observed up to 2.17 dyn/cm² (Fig. 4a, left). Above 2.17 dyn/cm², HL-60 cell rolling was not observed at any of the site densities analyzed.

Rolling velocity data for HL-60 cells on P-selectin is represented in a 3D surface plot (Fig. 4a, right), showing the combined effect of site density and shear stress on rolling velocity. It is seen that decreasing site density from 220 to 140 molecules/ μ m² resulted in an increase in the average rolling velocity as a lesser number of receptor-ligand interactions led to faster rolling. We speculate that saturation of cell surface-expressed PSGL-1 occurs above 220 molecules/ μ m², resulting in a cell rolling velocity that is not significantly affected by shear stress. Overall, HL-60 cell rolling velocity increased as shear stress increased, and the effect was most pronounced at lower site densities.

Fig. 4b shows the effect of shear stress and site density on cell rolling flux. Consistent with cell rolling velocity data presented in Fig. 4a, increasing the site density above 220

molecules/ μm^2 at a given shear stress did not significantly affect rolling flux. The effect of shear stress at a given site density, however, exhibited the following trend: At site densities of 220 molecules/ μm^2 and higher, rolling flux remained constant from 0.062 dyn/ cm^2 to 0.93 dyn/ cm^2 , above which it dropped until cells were no longer observed to roll. Conversely, at the lowest site densities analyzed (140 and 180 molecules/ μm^2), rolling flux was unaffected by increasing shear stress up to 0.93 and 1.55 dyn/ cm^2 , respectively, above which cells were no longer observed to roll. Overall, fewer cells (~3-fold less) were found to roll on the lowest site densities analyzed (140 and 180 molecules/ μm^2), compared to at site densities 280 molecules/ μm^2 .

P-selectin-mediated rolling of Jurkat T cells—P-selectin-mediated cell rolling was also assessed for Jurkat T cells. Many trends were similar to those observed with HL-60 cells, including an overall positive correlation between cell rolling velocity and shear stress (Fig. 5a), and an inverse correlation between shear stress and rolling flux (Fig. 5b). However, at a given shear stress, rolling velocity was largely unaffected by increasing site density, except at 0.47 dyn/ cm^2 , at which cells rolled at a significantly higher rolling velocity at 650 molecules/ μm^2 compared to at 1800 or 2200 molecules/ μm^2 as illustrated in Fig. 5 ($p < 0.05$). Notably, the range of average rolling velocities for Jurkats on P-selectin is smaller and lower than that of HL-60 cells (approximately 3 – 12 $\mu\text{m}/\text{sec}$ for Jurkat cells, compared to 1 – 50 $\mu\text{m}/\text{sec}$ for HL-60 cells).

Similar to HL-60 cells, the number of rolling Jurkat cells on immobilized P-selectin decreased with increasing shear stress and, under certain shear stress conditions, increased with site density. More specifically, the number of rolling cells on a P-selectin site density of 1800 molecules/ μm^2 was significantly higher than on 650 molecules/ μm^2 in the range of 0.16 to 0.47 dyn/ cm^2 ($p < 0.05$). At shear stresses greater than 0.47 dyn/ cm^2 , site density had no effect on rolling flux (Fig. 5b).

PSGL-1-mediated Jurkat cell rolling—To probe L-selectin-mediated rolling of Jurkat cells, substrates presenting immobilized PSGL-1 ranging from 800 to 6000 molecules/ μm^2 were utilized in leukocyte flow assays with Jurkat cells. On PSGL-1, the shear threshold for rolling was found to be 0.31 dyn/ cm^2 , compared to 0.16 dyn/ cm^2 on P-selectin. Overall, average Jurkat cell rolling velocities on PSGL-1 were approximately 20-fold higher than on immobilized P-selectin (98.11–227.94 $\mu\text{m}/\text{sec}$ on PSGL-1, compared to 2.40–11.91 $\mu\text{m}/\text{sec}$ on P-selectin). The average rolling velocity decreased with increasing site density at a given wall shear stress, and increasing shear stress above 0.47 dyn/ cm^2 had no significant effect on rolling velocity at a given site density (Fig. 6a). On positions presenting PSGL-1 at site densities from 2500 to 6000 molecules/ μm^2 , cell rolling was observed from 0.31 to 1.55 dyn/ cm^2 , while at 1800 molecules/ μm^2 rolling was observed from 0.16 to 0.93 dyn/ cm^2 . Comparatively, P-selectin-mediated Jurkat cell rolling occurred over a smaller range of shear stresses: Cell rolling was observed from 0.16 to 0.62 dyn/ cm^2 on site densities ranging from 1000 to 2200 molecules/ μm^2 , and from 0.31 to 0.62 dyn/ cm^2 on a site density of 650 molecules/ μm^2 (Fig. 5).

Data presented in Fig. 6b Jurkat cell rolling flux immobilized PSGL-1 substrates increased linearly as a function of site density in a manner which was dependent on the applied shear

stress. This is evidenced by the slopes of the linear regression analyses of rolling flux vs. PSGL-1 site density. In all cases, a positive correlation was found ($p < 0.05$, $r^2 = 0.95, 0.98$, and 0.86 for $0.47, 0.62$, and 0.93 dyn/cm^2 , respectively), revealing that Jurkat cell rolling flux on PSGL-1 increased with increasing site density at a given shear stress. Interestingly, the slope of the linear fit was highest for data collected at 0.62 dyn/cm^2 , revealing that at this level of shear stress, site density had a greater effect on rolling flux compared to higher or lower shear stresses (0.47 or 0.93 dyn/cm^2).

HL-60 cell rolling and adhesion on two-component substrates presenting a gradient of ICAM-1 and P-selectin—BP-modified substrates were tailored to present two biomolecules, intercellular adhesion molecule-1 (ICAM-1, R&D Systems) and P-selectin, and utilized to investigate the effect of ICAM-1 density on the transition from cell rolling to adhesion (Fig. 7). Briefly, a BP-modified substrate as assembled into the flow chamber and solution of ICAM-1 ($5 \text{ } \mu\text{g/mL}$) was introduced into the chamber. A relief mask with an array of circles ($800 \text{ } \mu\text{m}$ diameter, $200 \text{ } \mu\text{m}$ spacing) was placed on the substrate, and a 5 mm gradient was generated over the array mask by programming the shutter to move a total of 5 mm over the duration of the UV exposure (12 sec , 14 mW/cm^2 , as discussed above). PBS was flowed through the chamber to rinse out residual protein solution ($400 \text{ } \mu\text{L/min}$ for 3 min), then a solution of P-selectin ($1.25 \text{ } \mu\text{g/mL}$) was introduced into the chamber and was photopatterned through the array mask for 5 sec (14 mW/cm^2).

In this manner, substrates were tailored to present a gradient of an adhesive protein, ICAM-1, amidst a uniform density of P-selectin. Substrates were characterized in a fluorescence immunoassay (Fig. 7a,b) and were utilized in leukocyte flow assays. HL-60 cells were flowed over the substrate at a shear stress of 0.23 dyn/cm^2 , and the total number of rolling and adhering cells were determined as a function of ICAM-1 ligand density. At low levels of ICAM-1, the majority of cells are found to roll on the substrate, with less than 30% of interacting cells leading to firm adhesion. However, at high levels of ICAM-1, the majority of interacting cells are found to firmly adhere. On substrates presenting comparable levels of ICAM-1 in the absence of P-selectin, no cells were found to interact with the substrate (data not shown), which is consistent with previous literature reports that demonstrate that selectin-mediated rolling is a prerequisite for ICAM-1-mediated cell adhesion.⁵¹

Discussion

In this proof-of-principle study, we demonstrate that one-component gradients generated using BP-modified substrates can be tailored to present protein site densities in a controlled manner and can be applied to leukocyte flow assays. Previous reports of leukocyte flow assays have typically employed substrates that present adsorbed proteins at a uniform site density. The advantages of utilizing photochemically immobilized protein gradients over adsorbed protein substrates are increased biomolecule attachment stability (due to a covalent linkage of proteins to the surface) and the ability to present ligands at controllably varied site densities on a single substrate. This provides an more direct route to conducting comprehensive investigations to assess the effects of various combinations of shear stress and site density on cell behavior using a single substrate, as opposed to many different

substrates that each present only a single immobilized density. In this way, large amounts of data can be acquired from a single experiment, minimizing the amount of materials and time that are required for a multi-parameter analysis of selectin-mediated rolling.

It is well-established that interactions between the selectins (P-, E-, and L-selectin) and their respective ligands facilitate leukocyte rolling, both *in vitro* and *in vivo*.³³ P-selectin is responsible for initiating tethering and rolling of leukocytes on the blood vessel endothelium,⁵² while L-selectin is expressed on most leukocytes and is able to bind to ligands on the endothelium⁵³ as well as PSGL-1 presented on other cells.^{54–55} This latter process is known as secondary tethering and enables rolling leukocytes to recruit additional leukocytes to the inflamed endothelium. In our studies, we generated substrates presenting varied site densities of P-selectin and PSGL-1 and applied them to leukocyte flow assays. We found that both HL-60 and Jurkat cells rolled on immobilized P-selectin gradients, while only Jurkat cells rolled on immobilized PSGL-1, consistent with literature reports of low L-selectin expression on HL-60 cells.⁵⁰

In our studies, gradient generation was performed on two separate days for flow assays involving HL-60 cells and Jurkat cells. The range of protein site densities presented on our substrates is comparable to the values that have been previously reported in the literature for applications in leukocyte flow assays. Specifically, Brunk and Hammer⁴⁷ report microsphere rolling on E-selectin substrates ranging from 1400 to 3500 molecules/ μm^2 and Alon, *et al.*⁵⁶ used P- and E-selectin substrates of 500 and 650 molecules/ μm^2 for investigating T cell rolling. Important insights have been gleaned from these previous studies conducted at densities comparable to those generated in this paper.

The trends we observed in cell rolling velocity and rolling flux are consistent with previous reports of leukocyte rolling. For example, Lawrence, *et al.* studies HL-60 cell rolling on immobilized P-selectin⁵⁰ and observed, similar to our findings, that rolling flux is enhanced by increasing fluid shear up to a certain level, above which it decreases until cell rolling ceases to occur. In another study, Alon, *et al.* analyzed HL-60 cell rolling on an uncharacterized substrate of immobilized P-selectin up to wall shear stress levels of approximately 3 dyn/cm², observing a plateau in rolling velocity as shear stress was increased.⁵⁶ This is similar to our results for HL-60 cells rolling at P-selectin site densities of 280 molecules/ μm^2 . Additionally, for each selectin-ligand interaction probed, we found that a minimum shear threshold level was required in order for cells to roll on the surface, and that the threshold level was different for each protein.

As noted in the literature,³³ an interesting characteristic of selectin-mediated rolling, as opposed to rolling mediated by immobilized antibodies, is the ability to maintain near constant cell rolling velocities even as cells are subjected to increasing levels of shear stress. Observed in both *in vivo*^{57–58} and *in vitro*⁵⁹ studies, this is understood to be the result of an “automatic braking system”, which comes from a combination of cellular features (i.e. deformability and extrusion of membrane tethers) and certain molecular features of selectins and their corresponding ligands.³³ When a cell comes into contact with a surface, there are repulsive forces that may be overcome by increasing shear stresses, enabling increased bond formation. In our data, this trend is most clearly seen in initial attachment assays for HL-60

cells on P-selectin (at site densities ~ 280 molecules/ μm^2). Rolling velocities were seen to plateau instead of increasing linearly in proportion to increasing shear stress (see Fig. 4a). Although rolling velocity was not affected by increasing shear stress from 0.93 to 2.17 dyn/cm², the number of rolling cells decreased over this range of shear stresses (Fig. 4b). It appears, then, that shear stress affects the ability of HL-60 cells to tether to P-selectin in order to initiate rolling. However, once cells are rolling, shear stress does not affect the velocity at which they will roll. Initial attachment assays assessed the ability of cells to tether to the surface and initiate rolling in the presence of a given shear stress. Our data suggests that the velocity of cells rolling on P-selectin remains constant over a wide range of wall shear stresses when the cells initiate rolling at each level of shear evaluated (Fig. 4a).

For P-selectin-mediated Jurkat rolling, only at higher site densities (~ 1400 molecules/ μm^2) did the rolling velocity of cells plateau with increasing shear stress (Fig. 5). PSGL-1-mediated Jurkat cell rolling velocities (Fig. 6a) show this same trend at site densities ~ 3500 molecules/ μm^2 .

We also found that HL-60 and Jurkat cells show differences in cell rolling behavior on immobilized P-selectin substrates. Comparing data in Fig. 5b to Fig. 4b, it is seen that Jurkat rolling flux on P-selectin was lower than that of HL-60 cells (maximum of 75 rolling cells/mm² for Jurkat cells, compared to 250 rolling cells/mm² for HL-60 cells). Additionally, Jurkat cell rolling on immobilized P-selectin was limited to a lower range of shear stresses compared to HL-60 cells. The differences in the trends between HL-60 cells and Jurkat cells are likely due to the fact that Jurkat cells express an improperly glycosylated form of PSGL-1⁶⁰ that results in a weaker receptor-ligand interaction that is correspondingly less stable when challenged with increasing levels of shear stress, as compared to HL-60 cells that have fully functional PSGL-1.

Data from initial attachment assays of Jurkat cells on immobilized PSGL-1 suggest that there are optimal levels of shear stress that enable cells to more sensitively respond to differences in protein site density (Fig. 6b). This is demonstrated by the fact that the dependence of rolling flux on site density at a shear stress of 0.62 dyn/cm² was greater than at 0.47 or 0.93 dyn/cm². This demonstrates that there is a dynamic relationship between the combination of shear stress and site density on the observed cell rolling flux.

There are numerous other reports that utilize both leukocytes and leukocyte models (i.e. bioconjugated microspheres) in flow assays to better understand the biophysical phenomena underlying leukocyte recruitment. Various selectin-ligand interactions have been studied using ligand-coated microspheres in flow assays.^{42,61–62} Selectin- or ligand-transfected cell lines,⁵⁰ and various leukocyte subsets isolated from blood samples including T cells^{56,63} and neutrophils^{51,64–66} have been assessed for their ability to roll on selectin-coated substrates. These *in vitro* approaches are complementary to *in vivo* studies and have the unique ability to provide a quantitative assessment of leukocyte recruitment (mediated by numerous different receptor-ligand interactions) in a well-defined experimental environment. In this way, *in vitro* approaches have contributed to a clearer understanding of the complex physiological processes underlying the inflammatory response.

In addition to probing leukocyte interactions on substrates presenting one biomolecule at a time, we preliminarily demonstrated the potential for this gradient methodology platform to develop substrates presenting two biomolecules, and the subsequent application of these substrates to leukocyte flow assays (Fig. 7). Substrates were tailored to present a gradient of ICAM-1 amidst a uniform background of P-selectin. Leukocyte flow assays that probed the ability of HL-60 cells to interact with the substrates through rolling and firm adhesion revealed that higher levels of ICAM-1 resulted in a greater percentage of adhered cells.

It is widely accepted that selectin-mediated rolling is a necessary first step to integrin-mediated adhesion through interactions with intercellular adhesion molecules.⁵¹ Immobilized chemokines are often involved in this process, as they serve to activate expression of integrins on the cell surface,^{51,67} but some reports show that selectin-ligand interactions are sufficient for integrin activation.^{68–70} Our findings are consistent with these reports, since cells failed to roll or adhere on substrates presenting ICAM-1 alone (data not shown), yet are able to roll and adhere upon substrate presenting both P-selectin and ICAM-1. While more detailed experiments are beyond the scope of this manuscript, we feel as though substrates of this type, which present multiple relevant ligands at variable densities, will be extremely valuable for probing the interplay between multiple biomolecular interactions that corroborate to determine cellular responses *in vivo*.^{62,71–72}

Our gradient generation approach offers a means of streamlining the investigation of the interplay between ligand site density and applied shear stress, and their combined effects on rolling behavior by enabling the creation of high-information-content substrates that present many different underlying ligand conditions that can be assayed in a single experiment. Large-scale continuous gradients, such as those in Fig. 3, have potential for applications in high-throughput, multi-parameter analyses of cell-substrate interactions which would otherwise be more time- and labor-intensive due to the need to create multiple substrates, each presenting a different, uniform densities of the ligand(s) of interest. On the other hand, gradient arrays, such as those in Fig. 7, can be tailored to present adhesive ligands in a manner that mimics an inflammatory site in a blood vessel wall, which can be on the order of 100s of μm s in size,⁷³ allowing the combined effects of biomolecule ligand density and feature size on the complete leukocyte recruitment process to be investigated.

This is the first demonstration of the generation and quantitative characterization of biomolecular surface gradients presenting a wide range of surface-immobilized protein site densities, and their subsequent application in a comparative study of rolling behavior of leukocyte cell lines. We have assessed the combined effects of immobilized site density and shear stress on the resulting velocity and flux of rolling cells, demonstrating that this methodology is capable of generating gradient substrates for applications in rapid multi-parameter leukocyte flow investigations. Finally, although this manuscript is focused solely on leukocyte rolling assays, the general methodology of photochemical gradient generation will likely be of utility to a wide number of researchers interested in understanding biochemically complex cell-substrate interactions.

Conclusions

We present a simple gradient generation approach that produces rationally controlled and quantifiable substrates, requiring only BP-modified glass substrates, protein solutions, and UV light. Immobilized proteins are shown to retain their function, as demonstrated by the ability of leukocytes to engage them in biologically relevant behaviors.

Gradient substrates have an advantage over homogeneous substrates for applications in leukocyte flow assays due to their ability to yield high-content data by probing numerous combinations of shear stress and immobilized site density in a single set of experiments. This approach is more efficient compared to generating numerous separate sets of substrates for each combination of ligands and parameters under investigation. Additionally, covalent immobilization of biomolecules eliminates the risk of protein desorption due to disrupted non-covalent interactions. This methodology will be used to perform more comprehensive studies of selectin-mediated rolling of primary neutrophils and neutrophil models. Future work will also involve the generation of more complex substrates that simultaneously present multiple ligands in order to probe synergistic biomolecular interactions that control leukocyte rolling. These studies will be part of an effort to fully illuminate the cooperative effects of biomolecular interactions that facilitate leukocyte tethering, rolling, and adhesion to the endothelium during an inflammatory response. Finally, in addition to clear applications in leukocyte biology, this work represents a key extension of a simple and straightforward surface derivatization technique that is well-suited to construct multi-component models for many cell-substrate interfaces that are characterized by multiple modes of biomolecular interaction.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Insight, innovation, integration

We describe a simple, direct and molecularly general approach to generate surface-immobilized biomolecular gradients. The resulting gradient substrates are quantifiable and facilitate multi-parameter investigations of cell-substrate interactions, as shown in this proof-of-principle investigation of selectin-mediated leukocyte rolling. In addition to clear applications in leukocyte biology, this technique is well-suited to construct models for many cell-biomaterial interfaces that are defined by multiple modes of biomolecular interaction. Similarly, this facile gradient generation methodology will enable new biological insights to be gleaned about the molecular basis of other complex biological processes including inflammation, and cell development and migration in response to immobilized cues.

Fig. 1.

A schematic representation of the process by which surface-immobilized gradients on benzophenone (BP)-modified substrates are generated, characterized, and applied to multi-parameter leukocyte flow investigations.

Fig. 2.

The relationship between fluorescence intensity and site density was determined for photoimmobilized P-selectin (red) and PSGL-1 (green) on BP-modified substrates. Substrates were generated in triplicate via flood UV exposures (four different exposure times ranging from 10 to 180 sec) in the presence of protein solution, resulting in homogeneous substrates presenting a wide range protein site density levels. Fluorescence measurements were acquired after incubating substrates with fluorescently labeled antibodies. For site density determination, substrates were incubated with saturating concentrations of primary monoclonal antibody (mAb) and [¹²⁵I]-labeled secondary polyclonal antibody (pAb). Control substrates were used to account for non-specific mAb and pAb binding. Data is plotted as the average ($\pm$ 95% C.I.) for $n=3$ substrates.

Fig. 3.

One-component gradients of (a) P-selectin and (b) PSGL-1 on BP-modified substrates were generated and characterized by converting data from fluorescence assays to site density using the results from fluorescence-radioactivity correlation studies (Fig. 2). For substrates used in Jurkat flow assays, immobilized protein site densities ranged from approximately 180 to 2400 and 800 to 6000 molecules/ μm^2 for P-selectin and PSGL-1, respectively.

Fig. 4.

Results from initial attachment assays of HL-60 cells on immobilized P-selectin. HL-60 cells were perfused over one-component P-selectin gradient substrates at shear stresses ranging from 0.062 to 2.17 dyn/cm². 2D data plots and 3D surface plots show the average cell rolling velocity (a) and rolling flux (b) plotted as a function of shear stress and P-selectin site density. At low site densities (140 and 180 molecules/μm²), rolling velocity increased sharply as a function of shear stress, while at all higher site densities analyzed, the rolling velocity leveled off beyond 0.93 dyn/cm². At site densities of 140 and 180 molecules/μm², significantly fewer rolling cells were observed compared to at higher site densities ($p < 0.05$), while increasing the site density from 280 to 1000 molecules/μm² had no significant effect on rolling flux at shear stresses > 0.16 dyn/cm². The mean rolling velocity or rolling flux was determined for each substrate, and the means from three substrates were averaged together and plotted as the average ($\pm$ SEM). Open data points represent the average ($\pm$ range) of $n=2$ substrates.

Fig. 5.

Results from initial attachment assays of Jurkat cells on immobilized P-selectin. (a) A 3D surface plot of rolling velocity as a function of shear stress and P-selectin site density reveals that increasing shear stress resulted in an overall increase in rolling velocity. However, the velocity of rolling cells at a given shear stress was largely unaffected by increasing site density, except at 0.47 dyn/cm^2 , in which case rolling velocity was higher at a site density of 650 $\text{molecules}/\mu\text{m}^2$ compared to 1800 or 2200 $\text{molecules}/\mu\text{m}^2$ (*, $p < 0.05$). (b) Rolling flux was greatest at higher site densities and lower shear stresses and decreased with increasing shear stress, except at the lowest site densities analyzed (650 and 1000 $\text{molecules}/\mu\text{m}^2$) at which rolling flux was steady over the range of shear stresses under which cell rolling was observed. The mean rolling velocity or rolling flux was determined for each substrate, and the means from three substrates were averaged together and plotted as the average ($\pm$ SEM). Open data points represent the average ($\pm$ range) of $n=2$ substrates.

Fig. 6.

Results from initial attachment assays of Jurkat cells on immobilized PSGL-1. (a) Overall, cell rolling velocity was highest at lower site densities. At site densities 3500 molecules/μm², rolling velocity increased with increasing shear stress up to 1.55 dyn/cm². As site density decreased below 3500 molecules/μm², the range of shear stresses at which cell rolling occurred got smaller, and increasing the shear stress had no effect on the rolling velocity. (b) The rolling flux of cells was affected by site density in a manner that was dependent on the shear stress. Linear regression and correlation analyses reveal that at 0.62 dyn/cm², the rolling flux increased most sharply as site density increased compared to at all other shear stresses analyzed. At all site densities analyzed, rolling flux increased as shear stress increased from 0.47 to 0.62 dyn/cm², and decreased thereafter. The mean rolling velocity or rolling flux was determined for each substrate, and the means from three substrates were averaged together and plotted as the average (±SEM). Open data points represent the average (±range) of *n*=2 substrates.

Fig 7.

Substrates presenting ICAM-1 and P-selectin in an 800-μm circle array were characterized and utilized in leukocyte flow assays to assess the interplay of rolling and adhesion. (a) A fluorescence image of the two-component gradient substrate (top: ICAM-1, Cy5, red; middle: P-selectin, Cy3, green; bottom: overlay), and (b) the corresponding fluorescence linescans. (c) A greater percentage of cells are found to adhere to the substrate in areas with high levels of ICAM-1, under conditions of shear stress (0.23 dyn/cm²). Data points represent the total percentage of rolling and adhered cells in three different rows of circles on a representative substrate. Scale bar, 800 μm.