Covalent Immobilization of P-Selectin Enhances Cell Rolling

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Cell rolling is an important physiological and pathological process that is used to recruit specific cells in the bloodstream to a target tissue. This process may be exploited for biomedical applications to capture and separate specific cell types. One of the most commonly studied proteins that regulate cell rolling is P-selectin. By coating surfaces with this protein, biofunctional surfaces that induce cell rolling can be prepared. Although most immobilization methods have relied on physisorption, chemical immobilization has obvious advantages, including longer functional stability and better control over ligand density and orientation. Here we describe chemical methods to immobilize P-selectin covalently on glass substrates. The chemistry was categorized on the basis of the functional groups on modified glass substrates: amine, aldehyde, and epoxy. The prepared surfaces were first tested in a flow chamber by flowing microspheres functionalized with a cell surface carbohydrate (sialyl Lewis(x)) that binds to P-selectin. Adhesion bonds between P-selectin and sialyl Lewis(x) dissociate readily under shear forces, leading to cell rolling. P-selectin immobilized on the epoxy glass surfaces exhibited enhanced long-term stability of the function and better homogeneity as compared to that for surfaces prepared by other methods and physisorbed controls. The microsphere rolling results were confirmed in vitro with isolated human neutrophils. This work is essential for the future development of devices for isolating specific cell types based on cell rolling, which may be useful for hematologic cancers and certain metastatic cancer cells that are responsive to immobilized selectins.

Introduction

Cell rolling along vascular endothelium in viscous shear flow is of primary biological importance given its role in the recruitment of leukocytes to sites of inflammation, homing of hematopoietic progenitor cells after intravenous injection, tumor cell metastasis, and other inflammatory processes.[1−3] Rolling is a receptor−ligand-mediated event that initiates an adhesion process to a target tissue through a reduction in cell velocity followed by activation, firm adhesion, and transmigration. The rolling response is primarily mediated by a family of transmembrane domain-based glycoprotein receptors called selectins that are expressed on the surface of leukocytes and activated endothelial cells.[4] Selectins bind to carbohydrates via a lectin-like extracellular domain, and the broad family of selectins is divided into L-(CD62L), E-(CD62E), and P-selectin (CD62P). L-selectin (74−100 kDa) is found on most leukocytes and can be rapidly shed from the cell surface,[5] E-selectin (100 kD) is transiently expressed on vascular endothelial cells in response to IL-1 beta and TNF alpha, and P-selectin (140 kDa) is typically stored in the secretory granules of platelets and endothelial cells.[6−8]

Because the adhesion bonds between selectins and their complementary ligands dissociate readily under shear forces, these adhesions do not firmly immobilize the cells and thus are referred to as transient adhesions. Although rolling experiments are useful for uncovering fundamental biology and for potentially building devices for cell separation, most studies to date have employed the random placement of selectins onto a 2-D substrate utilizing protein physisorption.[9,10] Furthermore, the stability of physisorbed selectins is weak because adsorbed proteins tend to desorb rapidly from the surfaces. Commonly used selectin immobilization methods do not afford a high degree of control over the presentation of selectins, which may hinder the ability to mimic relevant complexities of the in situ rolling response and to design efficient, effective separation tools.

The rolling velocity of each cell type is distinctive as a function of the local shear rate, the distribution of receptors on cell membranes, and the total number of receptors present on the cell, which may differ from one cell type to another.[1,11−13] This suggests a number of parameters to improve specificity. Through exhibiting greater control over the presentation[14] and stability of

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selectins, one may be able to model and interrogate more complex phenomena, leading to enhanced biological understanding. The covalent immobilization of proteins offers great potential for enhanced control over the presentation and stability of biomolecules on surfaces.[15] Specifically, the covalent immobilization of selectins has advantages over conventional complexation and physisorption because it would facilitate the optimization of cell-material interactions via control over density in the surface coating, spatial patterning, active site orientation, stability and shelf life, and topology as facilitated by linkers to achieve specific rolling characteristics. Although covalent immobilization procedures for peptides and enzymes have been extensively studied for decades, the covalent immobilization of large-molecular-weight biomolecules such as selectins presents significant challenges as a result of increased binding to nonspecific sites[16] and the requirement for mild processing conditions to prevent protein inactivation.

Here we investigate three kinds of conjugation chemistry based on amine-, aldehyde-, and epoxy-functionalized glass substrates using a parallel plate flow chamber that mimics physiologic flow conditions. The prepared surfaces are characterized by X-ray photoelectron scattering (XPS) and contact angle measurements. To prescreen each kind of chemistry before conducting cell-based studies, we used 10 μm microspheres conjugated with sialyl Lewis(x).[9,17,18] We found that epoxy chemistry that is stable at neutral pH in aqueous environments allowed us to achieve the longest term storage and the most bond stability without protein aggregation among the three kinds of chemistry. This chemistry led to a significant enhancement of the stability of microsphere rolling. These results were validated through in vitro cell rolling experiments.[11,12]

Experimental Section

Materials. Recombinant human P-selectin/Fc chimera (P-selectin) and mouse monoclonal antibody specific for human P-selectin (clone AK-4) were purchased from R&D systems (Minneapolis, MN). All of the functionalized glass surfaces (plain, amine, aldehyde, and epoxy) were provided by TeleChem International, Inc (Sunnyvale, CA). Heterobifunctional poly(ethylene glycol) (NH_2-PEG-COOH) was acquired from Nektar (San Carlos, CA). All other chemicals were obtained from Sigma-Aldrich (St. Louis, MO). All materials employed in this study were used without further purification unless specified.

Preparation of Surfaces. A synthetic route for surface preparation is illustrated in Figure 1. Briefly, P-selectin immobilization was performed on four different glass substrates. A glass surface with physically adsorbed P-selectin was prepared on plain glass. The plain glass substrate (SuperClean2) was washed with PBS three times for 5 min each. P-selectin (600 μL of a 5 μg/mL solution) was placed on top of the glass and incubated on a plate shaker for 18 h. For the covalent immobilization of P-selectin, the amine- (SuperAmine2), aldehyde- (SuperAldehyde2), and epoxy-functionalized (SuperEpoxy2) glass surfaces were employed. AFM analysis and other characterization results of all underlying glass substrates can be found at http://www.arrayit.com/Products/Substrates/.

To ensure effective surface modification, all reagents were used in excess quantities. According to the supplier, SuperAmine2 contains 2×10^{13} reactive groups per mm^2 whereas SuperAldehyde2 and SuperEpoxy2 have 5×10^{12} reactive groups per mm^2. Therefore, a total surface area of 10 cm^2 has approximately 2×10^{16} or 5×10^{15} reactive groups. Reagents including NH_2-PEG-COOH were used an excess molarities of 10−100×, as described below.

Detailed protocols for each surface are as follows: On the amine glass, 500 μL of a 5 mg/mL solution of NH_2-PEG-COOH (~1 mM) was preactivated by a 1:1 mixture (500 μL) of 50 mM (1.9 mg/mL) 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide (EDC)/50 mM (2.2 mg/mL) *N*-hydroxysuccinimide (NHS) in distilled water for 5 min, immediately followed by incubation on the glass at room temperature for 1 h. P-selectin (1 mL of a 5 μg/mL solution) was also preactivated in solution by EDC (19 μg) and NHS (22 μg) for 5 min, added on top of the PEGylated glass, and incubated at room temperature overnight. The glass surfaces were washed thoroughly with PBS in each step. On the aldehyde glass, 600 μL of NH_2-PEG-COOH (5 mg/mL) was added to the glass surface and incubated for 2 h. After the PBS was washed three times, some of the surfaces were treated with a 10× molar excess of sodium cyanoborohydride (5×10^{-6} mol), compared to the concentration of NH_2-PEG-COOH. This was used to reduce the unstable Schiff's bases to stable secondary amines. EDC (160 μg) and NHS (180 μg) were added to 500 μL of PBS and were used to activate the surface-bound COOH groups for 30 min. This volume was removed from the surface, and 600 μL of P-selectin (5 μg/mL) was immediately added and permitted to react at room temperature for 18 h. On the epoxy glass, NH_2-PEG-COOH was immobilized, activated by EDC/NHS, and reacted with P-selectin under the same conditions for the aldehyde glass except for the reduction reaction. (In this reaction, stabilization by a reducing agent is not necessary.)

For the stability tests, surfaces were immersed in PBS and placed on a plate shaker at room temperature. The aged surfaces were compared to freshly prepared surfaces in subsequent flow chamber experiments.

X-ray Photoelectron Spectroscopy (XPS) and Contact Angle Measurements. Surfaces at each step were characterized by XPS and contact angle measurements (Table 1). XPS measurements were performed using an Axis Ultra X-ray photoelectron spectrometer (Kratos Analytical, Manchester, U.K.) equipped with a monochromatic Al Kα source (1486.6 eV, 150 W) and a hemispherical analyzer. The mass concentration % was obtained at a takeoff angle of 20° with a pass energy of 80 eV and a step size of 0.2 eV.

Contact angles of double distilled water on surfaces were measured using VCA2000 (AST Products, Inc., Billerica, MA). Drops of 3 μL each were deposited onto the sample surface using a microsyringe attached to the system, and data were analyzed using VCA optima XE software.

Preparation of Microsphere Conjugates. SuperAvidin-coated microspheres with a diameter of 9.95 μm (Bangs Laboratories, Fishers, IN) were conjugated with multivalent biotinylated sialyl Lewis(x)-poly(acrylamide) (sLe^x-PAA-biotin, Glycotech, Gaithersburg, MD) to be used as a cell mimic for our prescreening tests according to the previous report (Figure 2).[9] Briefly, a 104.8 μL bead solution (containing 2×10^6 beads) was dissolved in 1 mL of PBS containing 1% BSA (BPBS). The mixture was washed with BPBS three times by centrifugation at 10 000 rpm for 2 min. Four microliters of 1 mg/mL sLe^x-PAA-biotin (4 μg sLe^x) was added to the mixture and incubated for 1 h at room temperature with occasional vortex mixing. The resulting solution was then washed again with BPBS three times by centrifugation at 10 000 rpm for 2 min. The final solution was resuspended in BPBS and diluted at a concentration of 1×10^5 beads/mL to be used in adhesion experiments. In control experiments, native SuperAvidin-coated microspheres (without sLe^x modification) at the same concentrations were also used to assess the velocity of noninteracting microspheres.

Flow Chamber Assay with Microsphere−Ligand Conjugates. A rectangular parallel-plate flow chamber (Glycotech) with a gasket with a thickness of 250 μm and a length of 6 cm was placed on the glass surface with P-selectin. The flow rate−shear stress relationship was calculated with the following equation[19]

$$(\tau_s^*)_{max} = \frac{6 \times 2.95 \mu Q_{2-D}^*}{H^2}$$

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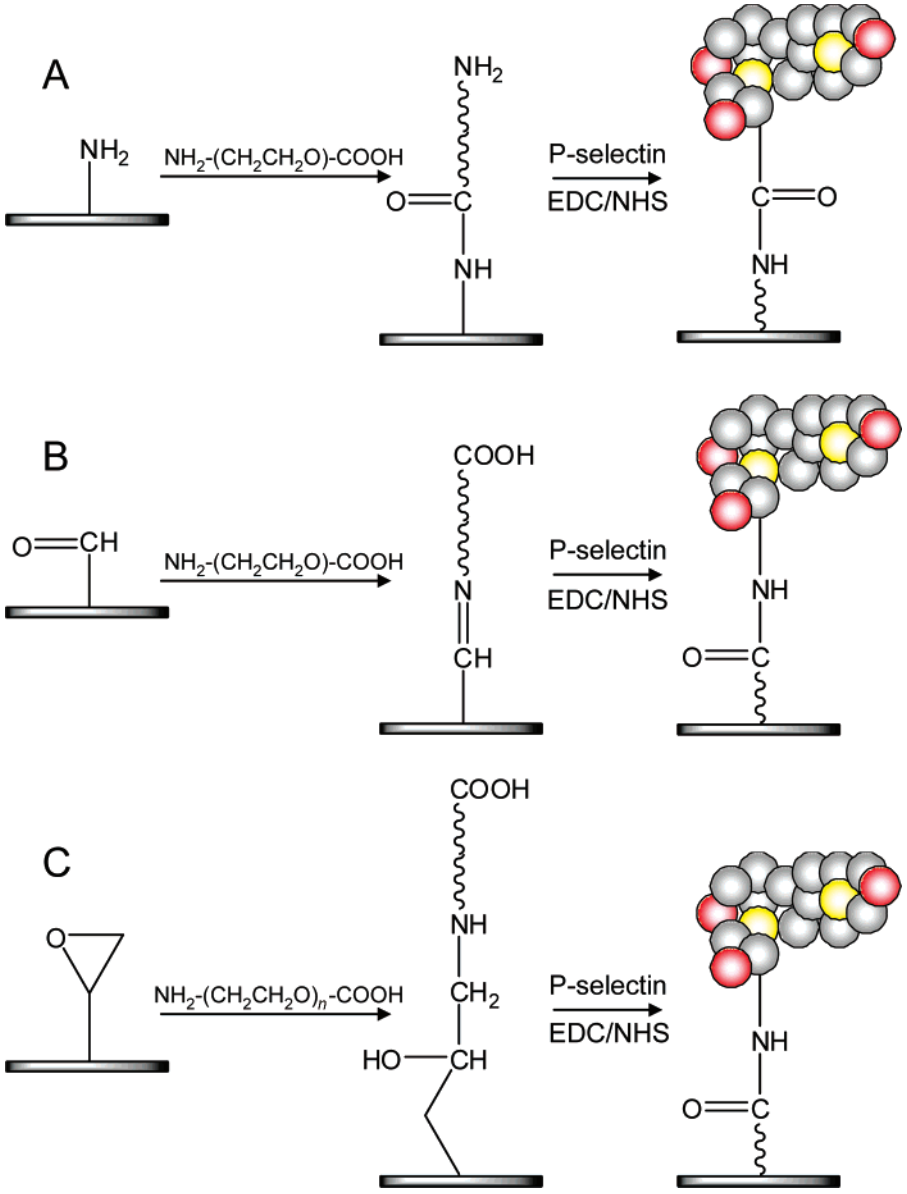

Figure 1. Surface preparation via a synthetic route. P-selectin was immobilized on (A) amine-, (B) aldehyde-, and (C) epoxy-functionalized glass surfaces through a PEG linker (NH_2-PEG-COOH). On the amine glass, NH_2-PEG-COOH and P-selectin were preactivated by EDC and NHS in solution before they were placed on the surfaces. For covalent immobilization on aldehyde and epoxy surfaces, carboxylated groups on the PEGylated surfaces were preactivated using EDC/NHS, and P-selectin was conjugated on top of the PEGylated glass surfaces. For a comparison of surface stability with the physical adsorption of P-selectin, a plain glass substrate as well as PEGylated aldehyde and epoxy surfaces were employed without preactivation (i.e., without EDC/NHS).

Table 1. Relative Surface Composition and Contact Angles of Various Surfaces

	plain glass substrate		aldehyde glass substrate			epoxy glass substrate		
	plain	P-selectin	aldehyde	PEGylated	P-selectin	epoxy	PEGylated	P-selectin
C[a]	11%	58%	20%	23%	55%	18%	19%	56%
N[a]	1%	12%		0%	9%	1%	0%	8%
O[a]	47%	23%	43%	43%	34%	43%	44%	33%
Si[a]	37%	7%	30%	33%	2%	32%	37%	3%
contact angle (deg)[b]	34 ± 2	68 ± 4	49 ± 5	45 ± 5	66 ± 4	43 ± 6	50 ± 5	65 ± 5

[a] All standard deviations for XPS data (mass concentration %) were less than ±5% (pass energy 80 eV, step size 0.2 eV) at a 20° takeoff angle as measured by XPS. [b] Contact angles of each surface were measured four times using double distilled water (mean ± SD).

where $(\tau_s^*)_{max}$ is the maximum shear stress on the surfaces, μ is the viscosity of the fluid (water = 0.01 dyn s/cm^2), Q_{2-D}^* is the flow rate per unit width in the system, and H is the height of the channel. All of the flow chamber experiments using the microspheres were performed at a flow rate of 50 μL/min, which translates into a wall shear stress of 0.24 dyn/cm^2 in this system. Note that different conditions were used for cell-based experimentation.

For the microsphere experiment, 5×10^5 mL^{-1} multivalent sLe^x-coated microspheres were prepared in PBS containing 1% BSA and perfused into a flow chamber at a shear stress of 0.24 dyn/cm^2 using

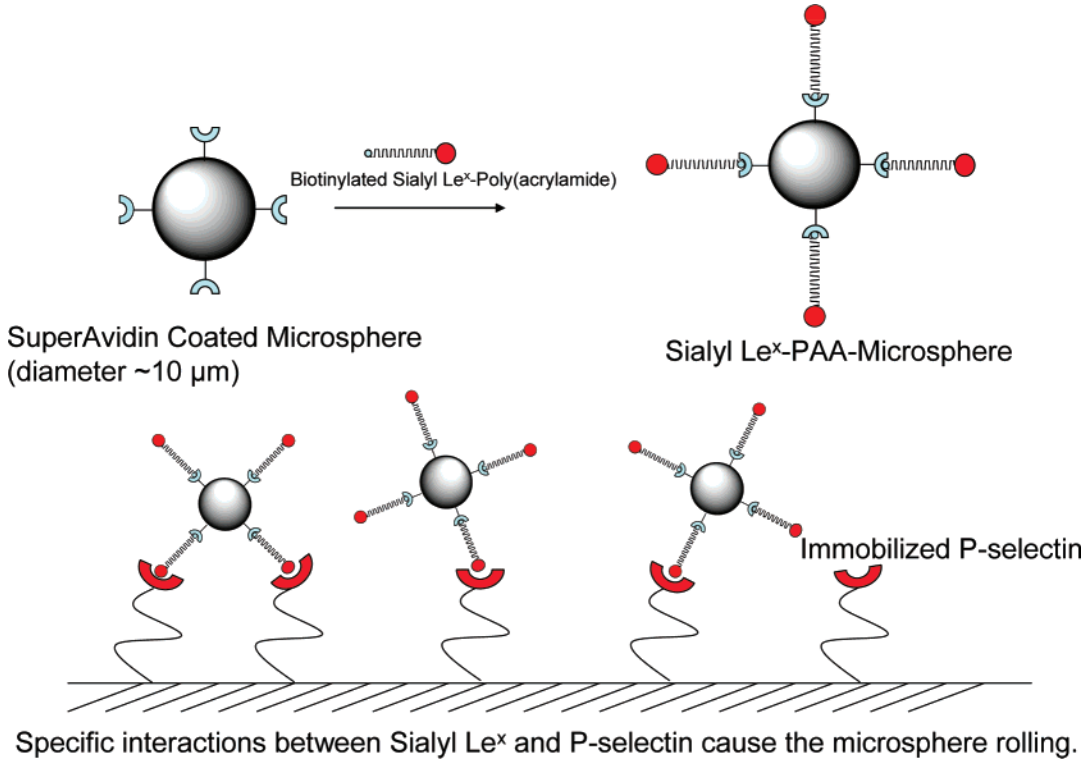

Figure 2. Schematic diagram of the preparation of microsphere conjugates and their rolling on the P-selectin-coated surface.

a syringe pump (New Era Pump Systems, Inc., Farmingdale, NY). During each microsphere experiment, flow was interrupted for 1 min, followed by image recording for 2 min. The flow was stopped to promote microsphere−surface contact via sedimentation. Images were taken every 5 s on the Axiovert 200 Zeiss microscope (Carl Zeiss, Thornwood, NY) equipped with a camera controlled by a Hamamatsu camera controller (Hamamatsu, Japan), and the velocities were calculated by measuring the displacement of each microsphere in subsequent images using Axioveision software version 3.1 (Carl Zeiss, Thornwood, NY). The average velocities were obtained by averaging values for at least 20 microspheres. For control experiments, instantaneous velocities were measured during the first 30 s (without stopping the flow) using noninteracting microspheres without sLe^x (at the same shear stress and concentration). Other control surfaces were also employed, such as BSA-treated glass and P-selectin-coated surfaces that were posttreated with P-selectin antibody (10 μg/mL) at room temperature for 2 h in order to block the specific interaction. The rolling dynamic data of microspheres was presented as mean ± SEM values.

Flow Chamber Assay with Neutrophils. Human blood was collected in a sterile tube containing sodium heparin (BD Biosciences, San Jose, CA) via venipuncture after informed consent was obtained. Neutrophils were then isolated by centrifugation (480*g* at 23 °C for 50 min) with one-step polymorphs (Accurate Chemical & Scientific Co., Westbury, NY). After isolation, neutrophils were kept in sterile Hanks' balanced salt solution containing 0.5% human serum albumin, 2 mM Ca^{2+}, and 10 mM HEPES at pH 7.4 until flow experiments were conducted. A rectangular parallel-plate flow chamber (Glycotech) with a gasket of thickness 127 μm and a length of 6 cm was placed on a P-selectin-immobilized glass surface. The assembled flow chamber was placed on an inverted microscope (Olympus IX81; Olympus America Inc., Center Valley, PA), and the neutrophil solution at a concentration of 2.5×10^5/mL was perfused into the chamber using a syringe pump (New Era Pump Systems, Inc.) at different flow rates. The perfusion pump generated laminar flow inside the flow chamber, allowing the regulation of calculated wall shear stresses from 1 to 10 dyn/cm^2.

Data Acquisition and Cell Tracking. A microscope-linked CCD camera (Hitachi, Japan) was used to monitor neutrophil rolling interactions with adhesive P-selectin substrates. Neutrophil rolling was observed using phase contrast microscopy and was recorded on high-quality DVD + RW discs for cell tracking analyses. Cell rolling videos were redigitized to 640 pixels × 480 pixels at 29.97 fps with ffmpegX software. Rolling fluxes and velocities of neutrophils interacting with immobilized P-selectin were then acquired using a computer tracking program coded in ImageJ 1.37m (NIH) and Matlab 7.3.0.267 (R2006b) (Mathworks). A cell was classified as rolling if it rolled for more than 10 s while remaining in the field of view (864×648 μm^2 using a 10× objective (NA = 0.30; type: Plan Fluorite; Olympus America Inc.)) and if it translated at an average velocity of less than 50% of the calculated free stream velocity of a noninteracting cell. (Note that this criteria was specific to the cell-based study.) The free-stream velocity was calculated using the theory of Goldman et al.[20] Rolling dynamic data was presented as mean ± SEM values of duplicate observations. Each observation was measured for 1 min under each shear stress tested. To confirm the statistical significance among the data, *p* values were calculated using a paired Student's *t* test method.

Results and Discussion

An early example of cell rolling studies was performed in Springer's laboratory in 1991 using selectins within lipid bilayers.[21] This model system was used to reproduce early leukocyte interactions with vascular endothelium, and the subsequent interest in this area is evident in the nearly 4500 references ascribed to the original report.[4] In addition to using in vitro model systems to study leukocyte rolling, the rolling phenomenon may be applied as a tool for cell separations.[22,23] Specifically, selectin molecules coated onto a solid surface can be used as an affinity chromatography mechanism to capture a

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number of cell types from blood, including hematopoietic stem cells, leukocytes, platelets, and some metastatic cancer cell lines.[1] To date, most of these studies have employed the physisorption of selectins to affect cell rolling. Because physisorbed proteins adhere mainly through weak intermolecular forces (i.e., van der Waals interactions) with an equilibrium established between the adsorbed and free proteins, these surfaces have limited stability and are typically useful only for immediate or short-term use. Here we explore the covalent immobilization of selectins using a variety of substrate chemistries to enhance stability and offer the potential of controlling spatial orientation.

Covalent Immobilization of P-Selectin−Amine Substrate Chemistry Does Not Improve P-Selectin Presentation. As illustrated in Figure 1, surfaces for the covalent immobilization of P-selectin were precoated with NH_2-PEG-COOH. The heterobifunctional PEG was used to provide reactive sites for P-selectin and to produce nonfouling surfaces.[24] On top of the PEGylated surfaces, P-selectin was immobilized, forming amide bonds between the carboxylate groups and primary amine groups.

The availability of primary amines and carboxylics on the surface of proteins makes amine coupling commonplace. Amine reactive groups on a solid substrate (silanized glass) bind to the carboxyl groups of the PEG linker, and the amine groups of the linker react with the carboxyl termini of proteins. This chemistry was initially thought to be useful for the enhanced orientation of P-selectin because the active site of the protein is known to be near the amine termini (the opposite end of the carboxyl termini). However, given the relatively low reactivity of amine groups, carboxyl termini on P-selectin must be activated by EDC and NHS for the reaction to occur. We found that the EDC/NHS activation of P-selectin in solution led to the aggregation of P-selectin, which resulted in the undesirable formation of micrometer-sized particles as observed by fluorescence microscopy with an antibody−FITC conjugate (Supplementary Information, Figure 1). Because we were unable to curb this aggregation substantially by changing the reaction conditions, we rationalized that this strategy was not suitable for enhancing control over P-selectin presentation.

We next investigated aldehyde chemistry given that it does not require the activation of P-selectin or the PEG linker in solution because aldehyde groups on silanized glass possess high reactivity toward amine groups. Aldehydes bind through Schiff base aldehyde−amine chemistry to amines on the PEG. After activation of the PEG with EDC/NHS, the carboxylate terminus on the PEG reacts with amine groups within lysine residues of proteins or via the primary amine terminus. For an additional strategy, P-selectin was immobilized on the PEGylated epoxy-coated glass substrate that has been widely used for protein conjugation, particularly in microarrys.[25] Epoxy-coated slides are derivatized with epoxysilane where proteins are covalently attached through an epoxide ring-opening reaction primarily with surface amino groups on proteins. In comparison to the amine chemistry, the common advantage of epoxy-based and aldehyde-based chemistry is that EDC/NHS activation can be performed on the surfaces, which does not cause protein aggregation due to intramolecular loop formation and/or intermolecular interactions. In addition, the epoxy-based chemistry has an added advantage over aldehyde chemistry because the reaction between epoxy and amine results in very stable bond formation. A stable bond can be also formed using the aldehyde-based chemistry if the bond is reduced by a reducing agent such as sodium cyanoborohydride.[26] However, this requires an additional step and in our experiments reduced the functionality of immobilized P-selectin.

Surface modifications were confirmed by XPS and contact angle measurements, as shown in Table 1. The aldehyde and epoxy functionalities were evidenced by the increased carbon/oxygen ratios as compared to that of the plain glass substrate. P-selectin immobilization (both physisorbed and chemically bound) was evidenced from the increase in nitrogen composition, decreased visibility of silicon in the underlying glass substrate, and increased contact angle. All surfaces treated with P-selectin had a high degree of coverage, as evidenced by the lack of visible underlying silicon. Furthermore, the microspheres and cells encountered similar substrate properties given the consistency in elemental composition and surface energy (contact angle values). Higher relative oxygen concentrations were observed on the chemically immobilized P-selectin surfaces, likely because of the presence of underlying PEG in comparison to the physisorbed substrates.

Aldehyde-Based Chemistry Exhibits a Lack of Long-Term Stability. To test the adhesion properties of the prepared surfaces, microspheres conjugated with the sLe^x-PAA-biotin ligand were employed prior to testing with human cells. The adhesion properties of the P-selectin immobilized surfaces were tested with the sLe^x microspheres using a flow chamber as previously described.[9] To confirm that the observed response was due to specific interaction between sLe^x and P-selectin, a series of control experiments were carried out. Microspheres without ligands (sLe^x) demonstrated no rolling behavior (average velocities of 32−40 μm/s on the P-selectin coated surfaces), and surfaces without P-selectin did not reduce the velocity of flowing microsphere conjugates (Supporting Information, Figure 2A). In addition, to block the specific interaction between P-selectin and sLe^x on the microspheres, P-selectin-coated surfaces were posttreated using the P-selectin antibody, followed by the perfusion of microsphere conjugates into the flow chamber. After antibody treatment, the microsphere average velocities on the P-selectin-coated surfaces were increased from 0.4 to 31.6 μm/s and from 3.4 to 29.2 μm/s on P-selectin immobilized epoxy and aldehyde surfaces, respectively (Supporting Information, Figure 2B). These results indicate that the observed velocity reduction on P-selectin-coated surfaces is solely due to a P-selectin-mediated interaction.

All of the freshly made surfaces, including P-selectin-adsorbed plain glass and P-selectin-immobilized aldehyde (Figure 3A) and epoxy (Figure 3B) glass substrates, significantly reduced the microsphere velocities. The microsphere conjugates traveled on the PEGylated aldehyde and PEGylated epoxy surfaces without P-selectin at average velocities of 25−30 and 25−40 μm/s, respectively. Note that the calculated velocity of a microsphere with a diameter of 9.95 μm is 57.5 μm/s at a wall shear stress of 0.24 dyn/cm^2 according to the Goldman calculation.[20] The velocities of sLe^x-bound microspheres on control surfaces without P-selectin were examined each day and were used to standardize the day-to-day variation in data as plotted in Figure 3B,D.

After 20 days in PBS at room temperature, P-selectin-immobilized surfaces prepared using the aldehyde glass lost their adhesion property, leading to a loss in rolling behavior (Figure 3A,B). Moreover, there was no significant difference between preactivated (EDC/NHS) and untreated surfaces in terms of sustained adhesive function. This result can be attributed to the

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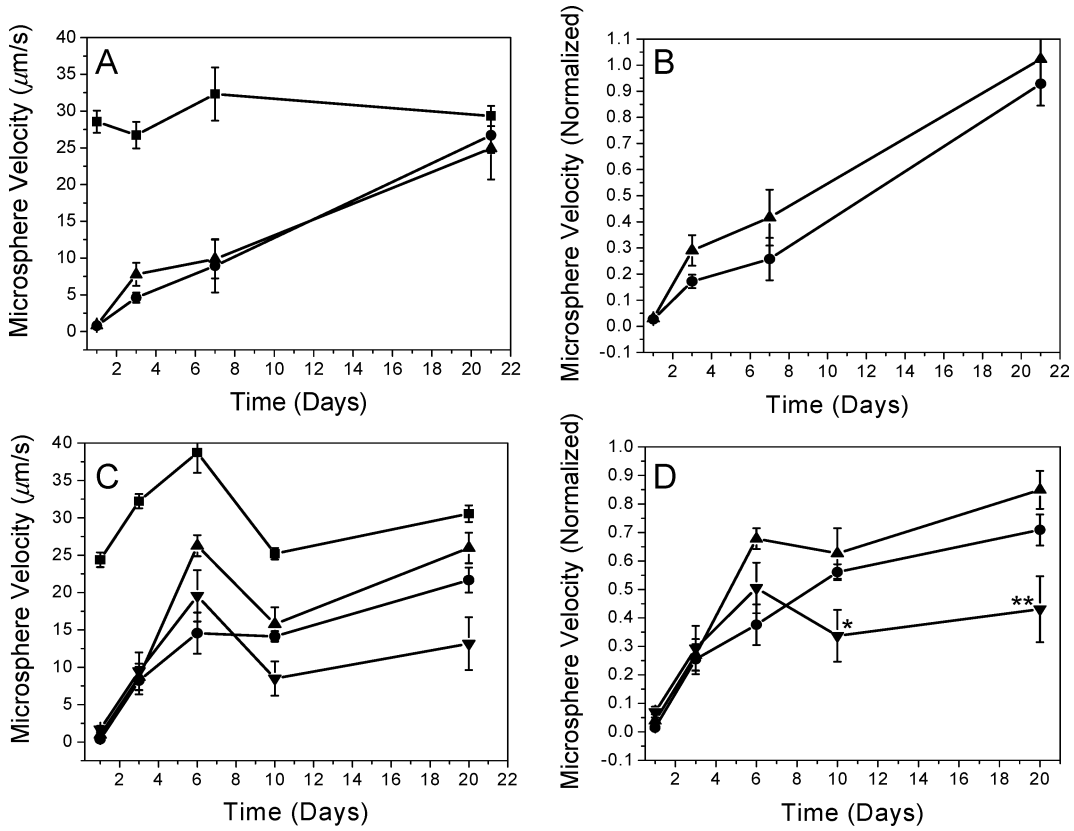

Figure 3. Comparison of surface stability detected through microsphere rolling. A solution of 1.0×10^5 microspheres/mL was perfused at 0.24 dyn/cm^2 of shear stress. (A) Aldehyde surface microsphere velocities and (B) normalized velocities (with respect to PEGylated physisorbed surface controls). (C) Epoxy surface microsphere velocities and (D) normalized velocities. (A and B) Microsphere velocities on PEGylated aldehyde glass (■) and P-selectin immobilized on the PEGylated aldehyde surface without EDC/NHS preactivation (●) and with EDC/NHS preactivation (▲). (C and D) Microsphere velocities on PEGylated epoxy glass (■), P-selectin adsorbed on plain glass (●), and P-selectin immobilized on the PEGylated epoxy surface without EDC/NHS preactivation (▲) and with EDC/NHS preactivation (▼). Although surfaces prepared on aldehyde glass do not show any enhanced stability regardless of EDC/NHS preactivation, P-selectin-immobilized surfaces prepared on the PEGlyated epoxy glass preactivated using EDC/NHS exhibit significantly enhanced stability. All of the rolling dynamic data is represented as mean ± SEM values. (D) *$p < 0.05$ (both ▼ vs ● and ▼ vs ▲), **$p < 0.05$ (▼ vs ●), and $p < 0.005$ (▼ vs ▲).

unstable chemical bond (Schiff base) between aldehydes and PEG leading to P-selectin detachment from the surface over time.

Epoxy-Based Chemistry Exhibits Enhanced Stability of P-Selectin Observed via Microsphere Rolling. In comparison to the aldehyde chemistry, P-selectin covalently immobilized onto epoxy glass exhibited a significant enhancement in long-term stability compared to both physisorbed P-selectin and unactivated surfaces (without EDC/NHS), as shown in Figure 3C,D. Specifically, after 20 days in PBS at room temperature, the P-selectin-immobilized surface (preactivated) exhibited the highest reduction in microsphere velocity (∼40% compared to the PEGylated epoxy surface without P-selectin) whereas the P-selectin-immobilized epoxy glass untreated with EDC/NHS (∼85%) and P-selectin-adsorbed plain glass (∼70%) allowed the conjugates to travel relatively faster. After 21 days, the average microsphere velocity was 13.1 μm/s on the P-selectin-immobilized surfaces compared to 30.6 μm/s on the PEGylated surfaces without P-selectin. This result may be important for the design of devices for separating or isolating cells based on rolling behavior where one would need specific functionality for extended periods of time.

Although covalently bound P-selectin on the epoxy surfaces appeared to be more stable than physisorbed P-selectin, it is curious why all of the surfaces tested in this study exhibit an increase in microsphere velocity over time, particularly during the first 3 days. This implies that P-selectin immobilization on the surfaces occurs through both covalent binding and physisorption or P-selectin forms multilayers on the surfaces. In other words, P-selectin molecules that are adsorbed on top of other P-selectin and/or directly onto the surfaces can be readily desorbed from the surfaces for the first few days. This is not surprising because proteins can be spontaneously adsorbed on surfaces by enthalpic contributions such as van der Waals, electrical double layer, and hydrophobic interactions.[27,28] After all of the additionally presented P-selectin is desorbed, the observed difference in terms of functional stability can then be attributed to the difference between covalent immobilization and physisorption. Therefore, actual stability should be compared after aging the surfaces for at least 3 days because the adhesive function of covalently bound P-selectin was constant after that period of time. This issue can be resolved by employing a flow system for P-selectin immobilization so that the additional P-selectin on the surfaces can be rapidly removed by shear force. A study to examine this issue is now being conducted and will be the subject of future publications.

Covalent Immobilization Enhances Neutrophil Cell Rolling. To confirm that the microsphere results are consistent with live human leukocytes, we investigated the neutrophil rolling interaction with immobilized P-selectin using a parallel-plate chamber

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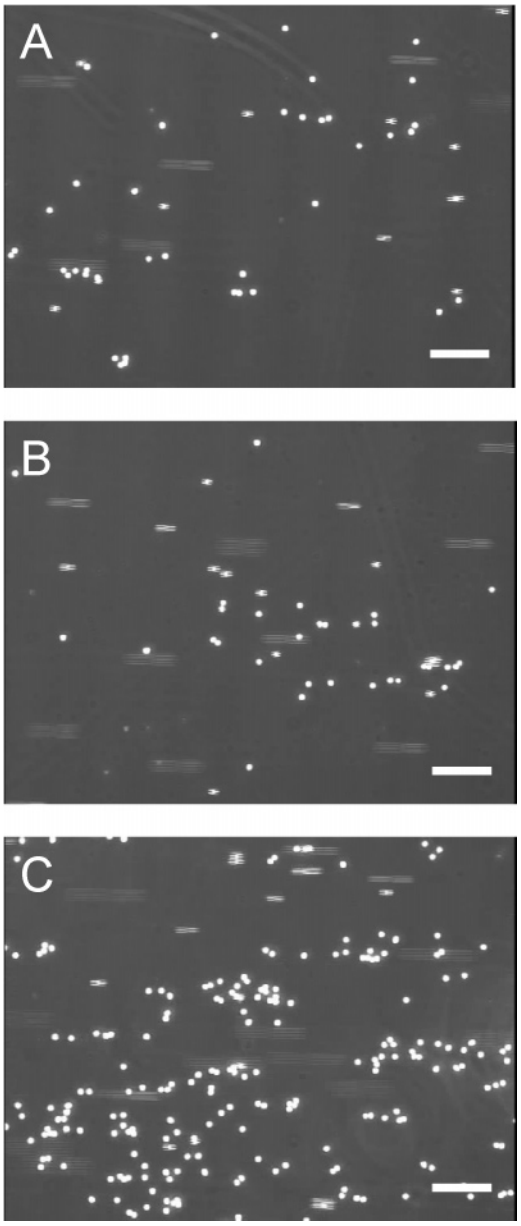

Figure 4. Representative phase contrast micrographs of neutrophil rolling adhesion on P-selectin-adsorbed substrates. (A) Still image of rolling adhesion of neutrophils on a P-selectin-adsorbed surface on plain glass and a PEGylated epoxy glass slide without (B) or with (C) preactivation using EDC/NHS. A 2.5×10^5/mL neutrophil solution was perfused on a 28-day-old P-selectin-surface under 1 dyn/cm^2 of shear stress. A total magnification of 100× was applied, and all scale bars indicate 100 μm.

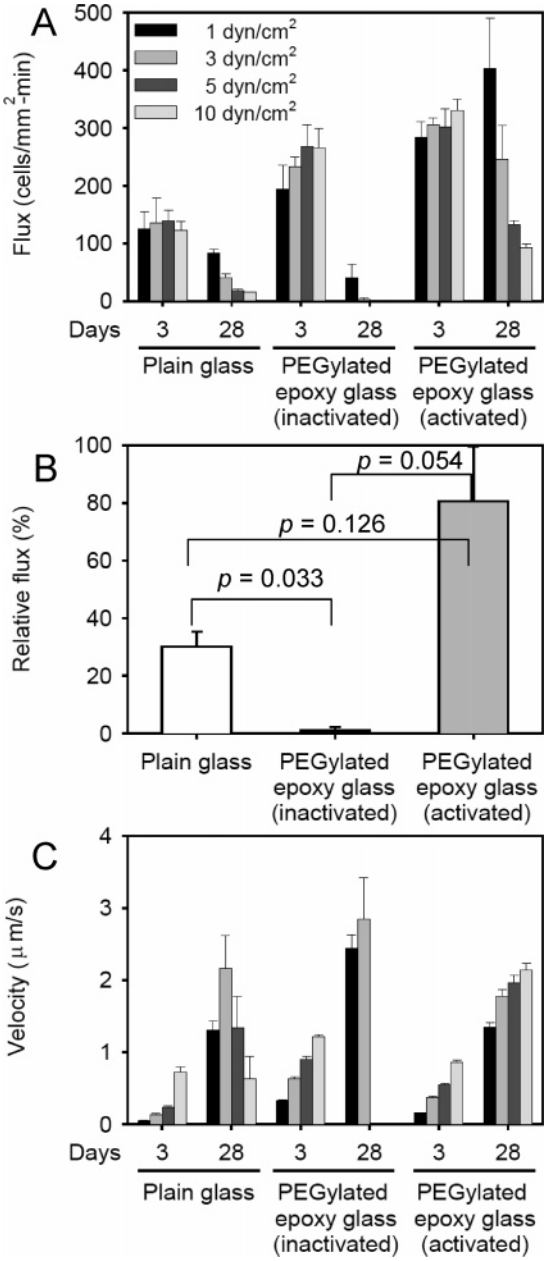

Figure 5. Rolling dynamics of neutrophils on P-selectin-immobilized surfaces under shear flow. A 2.5×10^5/mL neutrophil solution was perfused on a 3- or 28-day-old P-selectin-surface under wall shear stresses from 1 to 10 dyn/cm^2. Rolling fluxes (A) and rolling velocities (C) of neutrophils were measured for each condition. (B) Plot of relative rolling fluxes on the 28-day-old P-selectin-surfaces at 3 dyn/cm^2. The mean values of fluxes from the 3-day-old surface are set to 100%, and data from the 28-day-old surface are expressed as the mean ± SEM (%). All p values in A are listed in Supporting Information, Table 1. All p values in C were <0.0005 except for the plain glass slide at 10 dyn/cm^2, which had $p = 0.319$ between the 3- and 28-day-old surfaces.

under flow. First, control surfaces that did not have P-selectin (i.e., plain glass and a PEGylated epoxy glass slides) showed no cell adhesion (data not shown). From this in vitro cell rolling assay at four different wall shear stresses (1, 3, 5, and 10 dyn/cm^2), the number of rolling cells was significantly greater on the P-selectin-immobilized surface with preactivation of EDC/NHS than on the rest of the P-selectin surfaces 28 days after preparation (Figure 4). In contrast, the rolling fluxes dramatically decreased on the older P-selectin-adsorbed surfaces on plain glass and on a PEGylated epoxy glass slide without having EDC/NHS activation compared with those on a newer (3-day-old) surface under the same condition, as shown in Figure 5A. Specifically, at 3 dyn/cm^2, the rolling flux on the older P-selectin immobilized on the epoxy surface (preactivated with EDC/NHS) did not significantly decrease (80.6 ± 19.1% (mean ± SEM) of that on the new surface), but fluxes on the older P-selectin-adsorbed glass and on the older P-selectin immobilized on an epoxy surface without EDC/NHS preactivation dropped to 30.1 ± 5.2 and 1.1 ± 1.1%, respectively (Figure 5B).

Cell rolling velocity analysis indicates that a large number of neutrophils on the aged P-selectin-immobilized epoxy surface sustained continuous rolling as the shear stress increased while most of cells on the other two surfaces detached and rejoined the free stream (Figure 5C). It is noteworthy that the observed rolling velocities of cells were significantly lower than those of

microspheres, especially given that the shear stresses were higher for the cells by an order of magnitude. This is due to two main differences: (1) the microvilli on the neutrophil surface extend to reconcile the dissociation force applied on the P-selectin−ligand bond and (2) neutrophils possess the stronger-binding PSGL-1 selectin ligand , whereas the microspheres were coated with the weaker-binding sLe^x group.[29,30] Note that the glycoprotein PSGL-1 possesses not only the sLe^x carbohydrate but also two sulfated tyrosines that strengthen the bond with P-selectin relative to sLe^x alone.[31] In addition, the contact area of a neutrophil with a ligand-bearing surface is known to flatten and increase during cell rolling, making additional receptors available for binding.[32] As shown in Figures 4 and 5, the average rolling velocities of neutrophils on all P-selectin-coated surfaces were lower than those of sLe^x-microspheres, although the microspheres traveled at a reduced wall shear stress of 0.24 dyn/cm^2. Although most previous studies of carbohydrate-coated microspheres and neutrophils rolling on P-selectin did not test wall shear stresses below 0.5 dyn/cm^2, Yago et al. did test the entire range of shear stresses from 0.25 to 32.5 dyn/cm^2 (both neutrophils and microspheres on P-selectin), and our results are in good qualitative agreement with that work.[33]

In addition, the small number of rolling cells that rolled slower on the older P-selectin plain surface at 5 and 10 dyn/cm^2 is likely to be from small patches of P-selectin retaining their adhesive activity. These data are consistent with data obtained using microspheres, indicating that our prescreening tests are reliable for quickly testing prepared surfaces in terms of adhesive function.

Here we have demonstrated that the covalent immobilization of P-selectin enhances cell rolling interactions through improved long-term stability (Figure 3−and 5) and homogeneity (Figure 5) compared to typical adsorption protocols. Given the difficulty in cost-effectively isolating large quantities of P-selectin, it is important to note that the immobilization conditions presented here utilized the same amounts of P-selectin that were used for the adsorbed controls, thus indicating the practical utility of these methods. The improved stability is a requirement for developing implantable devices that capture specific target cell types based on cell rolling. Through further optimization of the presentation of active P-selectin binding sites, the efficiency of these surfaces may be significantly improved. Additionally, orientation and density control through chemical immobilization should be useful for performing controlled studies required to uncover the mechanisms of physiological and pathological cell rolling.

Conclusions

We have achieved the stable immobilization of P-selectin using epoxy-based conjugation chemistry without compromising its biological adhesive function. The immobilized surfaces were demonstrated to be superior to the conventional physisorbed controls, and the observed rolling response was dependent on a P-selectin-mediated interaction. These results are directly applicable to the design of therapeutic or diagnostic devices for capturing specific cells. Covalent immobilization techniques can be extended to enhance control over the presentation of P-selectin, including surface density and patterning. We are presently translating this result to animal studies to capture hematopoetic stem cells and circulating cancer cells from whole blood.

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Supporting Information Available: Fluorescence microscopy of the P-selectin antibody−FITC conjugate. Confirmation of the specific interaction between P-selectin and the surface-bound ligand on microspheres. Statistical analysis for the comparison between samples examined 3 and 28 days after preparation. This material is available free of charge via the Internet at http://pubs.acs.org.

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