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Leukocyte Adhesion Kinetics: A Sticky Business

by
McRae Joseph Smith
Leukocyte Adhesion Kinetics: A Sticky Business

A Dissertation
Presented to
the faculty of the School of Engineering and Applied Science
University of Virginia

In Partial Fulfillment
of the requirements for the Degree
Doctor of Philosophy (Biomedical Engineering)

By

McRae Joseph Smith

January 2002
Approval Sheet

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Abstract

Leukocyte rolling during inflammation is mediated primarily by the selectin family of adhesion molecules. P-selectin glycoprotein ligand-1 (PSGL-1) is a highly characterized molecule and is the primary ligand for P-selectin. PSGL-1 is a dimeric molecule with the two identical chains linked by a disulfide bond. I have measured the contributions of dimerization and glycosylation, in the binding pocket of PSGL-1, to bonds formed during rolling and tethering. I have also used a Monte Carlo simulation to model the idealized version of leukocyte rolling, which is the rolling of microbeads with adsorbed PSGL-1 on an adsorbed P-selectin surface.

The PSGL-1 isolated from transfected cell lines had different molecular weights depending on the activity of the core-2 glucosaminytransferase. The high molecular weight species of PSGL-1 migrated a distance corresponding to ∼140 kD, consistent with PSGL-1 from a human leukemia cell line HL60, and the low molecular weight species of PSGL-1 migrated a distance corresponding to ∼120 kD under reducing conditions. The lack of core-2 activity in specific cell lines apparently led to a deficiency in PSGL-1—P-selectin bond strength. Cells with the low molecular weight species of PSGL-1 also formed tethers with a P-selectin substrate less frequently and rolled an order of magnitude faster than cells with the high molecular weight species. Cellular tethers and bonds formed with the low molecular weight species of PSGL-1 were more compliant as the dissociation rate constant increased rapidly with increased force. The high molecular weight species of PSGL-1 conferred a strengthening of bonds as the dissociation rate...
Abstract

constants increased less with force, similar to that seen with PSGL-1 tethers from neutrophils.

Dimerization of PSGL-1 did not affect rolling velocity as cells expressing both monomeric and dimeric PSGL-1 rolled 10 μm/s or less, which is similar to neutrophil rolling velocities at the same conditions. The distance between tethering events was also unaffected by dimerization. The initial tethering from flow or capture, when the cell first initiates contact with the P-selectin substrate, did increase with dimerization over the accumulation of tethering cells expressing the monomeric PSGL-1. The bond strength or variation of dissociation rate with force did not depend on PSGL-1 dimerization, implying that a tether mediated by dimeric PSGL-1 is the same as a tether mediated by monomeric PSGL-1 and they consist of similar numbers of bonds.

The Monte Carlo simulation of microbead rolling matched experimental values of microbead rolling velocity at several different wall shear stresses. The case of the model implementing multiple tethers or bonds was able to match the experimental values for all wall shear stresses, but varied in separation distance between the bead surface and the substrate. Up to two tethers could form, in the case of multiple tethers. Re-bonding, once one tether broke and the other remained, was also possible. This model matched experimentally measured bead rolling velocities at higher separation distances for higher wall shear stresses. The results from the simulation imply that multiple tethers are required for bead rolling for the conditions modeled and a hydrodynamic lift force may be affecting bead separation distance even during rolling.
### Nomenclature

<table>
<thead>
<tr>
<th>Abbreviation</th>
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<tr>
<td>300.19</td>
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<td>BJAB</td>
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<tr>
<td>C320A</td>
<td>mutant, monomeric P-selectin glycoprotein ligand-1</td>
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<td>CHO</td>
<td>Chinese hamster ovary cell line</td>
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<td>epidermal growth factor-like domain</td>
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<td>high endothelial venule</td>
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<td>PRlG</td>
<td>P-selectin recombinant IgG antibody</td>
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<tr>
<td>PSGL-1</td>
<td>P-selectin glycoprotein ligand-1</td>
</tr>
<tr>
<td>SCR</td>
<td>short consensus repeat</td>
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</table>
Nomenclature

SDS-PAGE  sodium dodecasulfate polyacrylamide gel electrophoresis
sLex       sialyl Lewis X
WT         wild-type P-selectin glycoprotein ligand-1

a          bead radius
dt          time step or interval in simulation
F_B         force on the tether
F_s         shear force
F_x         rotational force in x-direction
F^s_x       shear force in x-direction
F^t_x       translational force in x-direction
h           distance between center of bead and lower wall or substrate
h_l         height of flow chamber
k_B         Boltzmann’s constant
k_{off}     stressed dissociation rate constant
k_{off}^0   unstressed dissociation rate constant
k_{on}      unstressed association rate constant (s^{-1})
k_r         unstressed association rate constant (M^{-1}s^{-1})
n_b         number of bonds
n_f         number of free receptors
n_l         total number of ligands
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<tr>
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<td>wall shear rate</td>
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<td>$\sigma$</td>
<td>bond compliance, bond separation distance</td>
</tr>
<tr>
<td>$\tau$</td>
<td>wall shear stress</td>
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Chapter 1: Introduction

1.1 Overview of Leukocyte Adhesion

Inflammation and immune surveillance protect against trauma and infection and are also the beginning stages of wound healing. A defining characteristic in these processes is leukocyte adhesion to the endothelium. Leukocytes patrol the site of injury and mark and eliminate foreign antigens. But leukocytes are not located throughout the body and consequently are not immediately at sites of injury. Instead they are contained and travel within the vasculature and lymphatic network. The injured tissue must then signal leukocytes to home to it.

There are many overlapping steps in the process of signaling leukocytes and the migration of leukocytes to the damaged tissue. The leukocyte adhesion cascade is an established paradigm for describing this process (Fig. 1.1). Leukocytes begin adhesive interactions with the endothelium through adhesion molecules called selectins. Transient bonds formed between endothelial selectins and their ligands on the leukocyte allow for a dynamic interaction called leukocyte rolling (Lawrence and Springer, 1991). Hydrodynamic forces push the cell forward, while selectin bonds act to resist the forward movement, resulting in a rolling motion along the vessel wall. Chemoattractant molecules produced at the site of injury signal leukocytes to stop rolling and migrate towards the source of these chemoattractants. Once leukocytes receive the signal to stop, a second family of adhesion molecules, called integrins, begin to form bonds. Bonds between integrins and their ligands, immunoglobulin super family, on the endothelium are
Figure 1.1: The leukocyte adhesion cascade during inflammation. Leukocyte recruitment is a multi-step process starting with capture and rolling mediated by selectins, activation signaled by multiple, diffusing cytokines (red and blue speckles), firm adhesion mediated by integrins, and migration also mediated by integrins. (Adapted from Jung, et al., 1998.)
required for the arrest or firm adhesion of the cells. The leukocytes then search for a path between endothelial cells to migrate out of the vessel and into the damaged tissue.

1.2 Motivation

The process of leukocyte adhesion is the subject of intensive research to elucidate mechanisms to augment leukocyte function when it is lacking and to diminish leukocyte function when it is unwanted. The migration of leukocytes to tissue outside blood vessels is essential for the immune response, but can be devastating if that migration goes unchecked (Jordan et al., 1999). Much of the research has been aimed at disrupting or restoring adhesion molecule function at the stage of leukocyte—endothelium interaction. Antibodies to selectins and integrins and small molecule inhibitors have been tested for their ability to block leukocyte adhesion in vivo (Ramos-Kelly et al., 2000; Stewart et al., 2001). Chemokines have also been studied as a means for blocking leukocyte arrest (Constantin et al., 2000). In addition to leukocyte—endothelial cell adhesion, the adhesion between leukocytes, as in the immunological synapse formed between a T-cell and an antigen-presenting cell (Bromley et al., 2001; Bromley et al., 2000), is also being studied. Research in leukocyte adhesion has mainly focused on the basic science of the role and effectiveness of the molecules involved in adhesion. Research is now expanding to include bioengineering applications beyond the basic science to use molecules and cells for diagnostic and sensing devices.

Current applications in bioengineering include cellular and molecular transport within microfluidic devices for "lab-on-a-chip" technologies. Bioengineered devices will
Figure 1.2: Lab-on-a-chip device. This schematic is an example of a lab-on-a-chip device that could be used for cellular or molecular separation, molecular amplification, and other biochemical processes.
be used as biological and chemical warfare detection systems, cell and molecule sorters for isolation (Jin et al., 2001), and experimental tools to explore genomics and proteomics (Fig. 1.2). Diagnostic imaging will use contrast agents modified to target specific sites within the vasculature (Lindner et al., 2001; Lindner et al., 2000; Price et al., 1998; Villanueva et al., 1998). Similarly, delivery vehicles loaded with a drug may use adhesion molecules on their surface to target specific tissues (Fig. 1.3). The underlying principles that govern cell adhesion in these applications are basic physical processes: fluid dynamics, cell transport, diffusion, and mechanics, combined with molecular dynamics and surface chemistry (Fig. 1.4). An understanding of such fundamentals is essential to use adhesion molecules to solve clinical and research problems. In the future, new adhesion molecules may also be engineered and tailored to specific functions.

Currently, the leukocyte adhesion and molecular dynamics communities are still wrestling with the chemistry within adhesion molecules that make them kinetically and mechanically different—different from non-adhesive receptor-ligand pairs as well as different from each other. I would like to understand the differences in kinetics and mechanics between families of adhesion molecules, such as selectins and integrins, as well as differences within a family, such as P-selectin versus L-selectin or \( \beta_2 \) integrins versus \( \alpha_4 \) integrins. These differences arise from variations in the structure and chemical composition of the molecules. Since molecular function cannot be predicted by structure or chemistry alone, understanding of function is arrived at through affinity and kinetic measurements after perturbations in the structure and chemistry. The characterization of
Figure 1.3: Diagnostic contrast agent or drug delivery vehicle. Carrier vehicles may use cell adhesion molecules incorporated into their shells in order to be targeted to specific points in the vasculature. In this case, a thrombus formation, of leukocytes and platelets, has been targeted. The adhesion molecules could modulate residence times of these carrier vehicles at these sites with a long residence time and lots of adhesion (A) or short residence time with little adhesion (B).
Figure 1.4: Principles governing leukocyte adhesion and applications. Fluid dynamics, transport, and diffusion are properties important in flow. Surface chemistry, mechanics, and molecular dynamics are important properties when the approach of particles to a wall are involved.
an adhesive bond is achieved by isolating the required chemistries in the receptor and ligand and measuring the affinity and kinetics for a range of forces over which bonds occur.

There appears to be an advantage and possibly a strong dependence of leukocyte adhesion on multimeric molecules. Integrins are heterodimeric molecules. A subset of P-selectin has been reported to form homodimers through a non-covalent association (Barkalow et al., 2000). P-selectin glycoprotein ligand-1 (PSGL-1) is the primary protein ligand of selectins and is a homodimer cross-linked extracellularly near the transmembrane domain. Some of these proteins may require dimers to form bonds with their ligands, while others may have multimeric forms to increase the probability of association and decrease the probability of dissociation. The dimerization may also be important for successful or proper post-translational modifications to the proteins while being synthesized. Unexpectedly, I found suggestive evidence that post-translational modifications of PSGL-1 might depend on dimerization, which made the effect on rolling leukocyte adhesion (described below) by dimerization difficult to isolate because it might have caused additional structural differences. The dimerization of PSGL-1 is the major focus of this dissertation and the hypotheses and objectives for this subject will be discussed later in the introduction.
1.3 Leukocyte Adhesion Cascade

1.3.1 Capture and Rolling

Leukocyte capture refers to the initial binding or tether between a white blood cell, such as a neutrophil or mononuclear leukocyte, flowing at hydrodynamic velocity near the vessel or flow chamber wall and the substrate. In flow chambers, this has been used as an indicator of molecular association rate (Finger et al., 1996; Lawrence et al., 1997; Smith et al., 1999). The greater the number of tethering cells indicates that more bonds must be forming under certain hydrodynamic conditions, implying that the rate of association of molecules must be higher. For example, if in one case, there are 10 leukocytes flowing over an adhesive substrate after one minute and 3 are rolling or forming adhesive contacts, while in a second case there are 10 leukocytes flowing over an adhesive substrate and 7 are rolling; then the molecules forming the adhesive bonds in the second case must have a higher association rate.

The time necessary for a flowing leukocyte to be captured by the substrate may be on the order of milliseconds (Zhao et al., 2001). For example, the time that a selectin molecule has to find its ligand is the amount of time a leukocyte is flowing over the space occupied by a selectin molecule bound to the wall. If that distance is 40 nm, which is half the estimated length of the PSGL-1-P-selectin cross-bridge length (Ushiyama et al., 1993), and the cell is traveling at a velocity of 300 μm/s (critical velocity for 1 dyn/cm² wall shear stress in the flow chamber), then the window of opportunity for forming a bond is less than 0.2 ms. After the cell has been captured and continues to roll, the
association of new bonds in the front part of the contact area might occur under no applied force and much longer time. The bonds at the trailing edge of the contact area might be the only bonds stressed during the dynamic condition of rolling.

Leukocyte rolling is the repeated bond formation and dissociation that keeps the cell in close contact with the vessel wall or substrate. The motion of the cell during rolling is due to the forward translation of the cell as fluid forces push the cell downstream. Transient bonds are ordered in close succession and result in a peeling of the leukocyte from the substrate with the result of an apparent "rolling" of the leukocyte. The viscous drag force on the rolling cell is balanced by the force of adhesive bonds. As bonds break, the dynamic and stochastic nature of rolling becomes evident because the cell is pushed forward by the fluid. Leukocyte rolling velocity is dependent on the hydrodynamic conditions, cellular parameters like size and deformability, site density of molecules on both opposing surfaces, and rate of bond association and dissociation. These parameters can be coupled, so studying the effect of one parameter on adhesion may not be straight-forward.

Leukocyte capture and rolling are mediated by selectin molecules (Fig. 1.5). Selectin bonds are characterized by both high rates of bond association and dissociation (Smith et al., 1999), as compared to \( \beta_2 \) integrin bonds (DiVietro et al., 2001). The transient formation of bonds allows the cell to travel several hundred microns along the surface of the endothelium. This juxtaposition allows the cell to sample the vessel wall for signals in the form of chemokines, which signal the cell to arrest and migrate through
Figure 1.5: Human selectin structure. Selectins are composed of a lectin binding domain, an epidermal growth factor (EGF)-like domain, short consensus repeat (SCR) domains, a transmembrane domain, and a cytoplasmic tail domain.
the vessel wall (Campbell and Butcher, 2000; DiVietro et al., 2001; Kunkel et al., 2000). The crucial first stage of capture and rolling may not happen if there is a defect in selectins or their ligands. A lack of carbohydrate groups on selectin ligands, due to a rare genetic deficiency in fucose transport, is leukocyte adhesion deficiency type II (LAD II) (Etzioni et al., 1992; Karsan et al., 1999; Luhn et al., 2001). The carbohydrate deficiency impairs the leukocyte’s ability to form rolling interactions (selectin bonds) and leads to inadequate recruitment of leukocytes to inflamed tissue.

1.3.2 Selectins and Ligands

Selectins are highly conserved glycoproteins both across species and within the family of molecules. P-, E-, and L-selectin are all composed of an N-terminal calcium-dependent, lectin domain that recognizes sialylated, fucosylated oligosaccharides (Kansas, 1996; McEver et al., 1995). In human selectins, the lectin domain is followed by an epidermal growth factor (EGF)-like domain, short consensus repeats (SCR; 9 for P-selectin, 6 for E-selectin, and 2 for L-selectin), a transmembrane domain, and a cytoplasmic domain. The selectins have greater than 60% homology at the amino acid sequence level in the lectin and EGF domains (Bevilacqua, 1993). Co-crystalization of the lectin and EGF domains of P-selectin with a 19 amino acid peptide of the amino terminal end of PSGL-1 and sialyl Lewis X (sLex, a tetrasaccharide) and the co-crystalization of E-selectin with sLex has shown that the active site of binding is within the lectin domain and involves a calcium ion (Graves et al., 1994; Somers et al., 2000). Therefore, the EGF and SCR domains probably do not have a direct role in ligand
recognition, but may impact the structural mechanics of the molecules and can affect ligand binding indirectly (Dwir et al., 2000; Patel et al., 1995). The SCRIs likely serve as an extension away from the cell membrane surface to increase accessibility to ligands.

Human, platelet P-selectin has a molecular weight of 140 kD and has a length of ~40 nm (Ushiyama et al., 1993). P-selectin function can be altered by varying the number of consensus repeats (Patel et al., 1995), which varies the length of the molecule. This suggests that the length of each selectin may be specific to its function. P-selectin is stored in Weibel-Palade bodies in endothelial cells and α-granules in platelets (McEver et al., 1989). Mediators of inflammation and hemostasis such as histamine and thrombin activate endothelial cells to move the granular stores of P-selectin to the cell surface (Hattori et al., 1989). This mobilization process to display P-selectin on the surface of the cell takes place in minutes (McEver et al., 1989). Most early stage leukocyte rolling is P-selectin dependent because rolling is significantly reduced in exteriorized mesentery venules when P-selectin function is blocked (Ley, 1994).

P-selectin also contributes to leukocyte-platelet interactions during wound healing and hemostasis. Platelet binding to monocytes, through P-selectin, can induce the expression of tissue factor, which is an initiator of coagulation (Celi et al., 1994). Platelets within forming thrombi can recruit leukocytes through P-selectin bonds and the inclusion of leukocytes in thrombi promotes fibrin deposition (Palabria et al., 1992).

E-selectin expression requires de novo gene transcription and synthesis, which is stimulated by inflammatory cytokines (Bevilacqua, 1993), in endothelial cells. Synthesis
and mobilization of E-selectin to the endothelial cell’s surface takes hours with significant contributions to leukocyte recruitment after several hours (Kunkel et al., 1996; Ley et al., 1995). Therefore E-selectin is important in the later stages or chronic inflammation. E-selectin mediated rolling is slow and contributes to the stabilization of smooth rolling in conjunction with $\beta_2$ integrins (Kunkel and Ley, 1996; Lawrence and Springer, 1993). These molecules regulate leukocyte transit times through vessels, which correlates with the efficiency of arrested cells (Jung et al., 1998).

L-selectin is constitutively expressed on the tips of microvilli of leukocytes (Picker et al., 1991). L-selectin is important in lymphocyte recirculation through peripheral lymph nodes and monocyte and neutrophil adhesion in inflamed tissue (Arbines et al., 1994; Gallatin et al., 1983; Jutila and Kurk, 1996). The location of L-selectin on the microvilli of cells is advantageous to the cell’s ability to bind immobilized mAb or ligand during flow (von Andrian et al., 1995). T and B lymphocytes travel from the vasculature to secondary lymphoid organs. High endothelial venules (HEV) are specialized endothelial cells that line the postcapillary venules of lymph nodes. Peripheral node addressin (PNAd) is a conglomerate of mucin-like sialoglycoproteins expressed on HEV that mediates L-selectin dependent lymphocyte rolling (Berg et al., 1998; Berg et al., 1991). L-selectin also mediates leukocyte rolling on endothelium in inflammation (Ley et al., 1991; Ley et al., 1993).

P-, E-, and L-selectins all recognize the carbohydrate structure sialyl Lewis X (sLex, Fig. 1.6). sLex contains fucose and sialic acid and may also be sulfated. sLex
Figure 1.6: Sialyl Lewis X (sLex) structure. sLex is a tetrasaccharide composed of a fucose (Fuc), sialic acid (Sla), galactose (Gal), and modified glucose (GlcNAc). This carbohydrate structure is located on many selectin ligands and has also been shown to support selectin mediated rolling alone.
alone can support bonds with selectins mediating cell and bead rolling interactions (Brunk et al., 1996; Greenberg et al., 2000; Lawrence et al., 1994; Rodgers et al., 2000); although, a higher avidity interaction occurs when the carbohydrate structure is displayed on a protein backbone as is the case with P-selectin glycoprotein ligand-1 (PSGL-1) (Somers et al., 2000).

PSGL-1 (Fig. 1.7), which is of central importance in this dissertation, is a 240 kD mucin-like homodimer constitutively expressed on microvilli of leukocytes (Moore et al., 1995). It is the primary ligand to P-selectin, but can also bind E- and L-selectin. The physiological significance for the E- and L-selectin binding has not been established; although, the binding to L-selectin has been shown to mediate transient (very rapid) leukocyte-leukocyte adhesion in vitro (Ramos et al., 1998; Walcheck et al., 1996). Leukocyte-leukocyte adhesion does facilitate increased accumulation of rolling cells in string-like formations on substrates in the parallel plate flow chamber (Lawrence and Springer, 1991), but has not been shown to affect the accumulation of rolling cells in an intravital microscopy preparation of a mouse model (Kunkel et al., 1997). So, secondary capture may recruit few rolling cells in vivo. Another study did show significant contributions by secondary capture to rolling cells both in arterial vessels and venules varying with the diameter of the vessel (Eriksson et al., 2001).

PSGL-1 has a disulfide bond linking its two chains at cysteine 320 near the junction of the extracellular and transmembrane regions (Moore et al., 1992). P- and L-selectin recognize the N-terminal region of PSGL-1, which includes and is dependent on
Figure 1.7: P-selectin glycoprotein ligand-1 (PSGL-1). PSGL-1 is the primary ligand for P-selectin and also binds L- and E-selectin. PSGL-1 is a 240 kD homodimer. The 19 amino acid amino terminus is important in binding P-selectin. There are three tyrosine-sulfation sites and one O-glycan site in this region.
the post-translational modifications of sulfation at tyrosines 46, 48, and 51 and sLex displayed on an O-linked glycan at threonine 57 (Goetz et al., 1997; Kansas, 1996; Pouyani and Seed, 1995; Ramachandran et al., 1999). PSGL-1 is highly glycosylated along the length of the molecule. There are many possible O-linked glycosylation sites and 3 possible N-linked glycosylation sites along the protein backbone of each chain in PSGL-1. The P- and L-selectin binding site on PSGL-1 contains one of the possible O-linked glycosylation sites and might have a branched carbohydrate structure capped by sLex (Varki et al., 1999). There are several transferases that are required for the synthesis of oligosaccharides within cells. Transferases are responsible for the proper synthesis of branched carbohydrate structures that control the molecular recognition. I have hypothesized that the carbohydrate structure also confers mechanical stability of bonds during applied, hydrodynamic forces. The mechanical stability or strength is tested by measuring tether lifetimes and calculating a dissociation rate constant, which is described in Chapter 2. While the exact structure of the carbohydrate in the binding pocket of PSGL-1 is not known, the full carbohydrate structure that extends from the PSGL-1 protein backbone may require glucosaminyltransferases (core-1 and core-2), fucosyltransferases (FT4 and FT7), and a sialyltransferase enzyme activity (Varki et al., 1999).

1.3.3 Chemokine Signal, Arrest, and Transmigration

Leukocyte rolling is followed by β2 integrin mediated arrest. The transition from rolling to arrest is still not completely understood. The signal for arrest may originate...
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from soluble factors released from the endothelium into blood flow (Lawrence et al., 1990), membrane bound molecules (Rot, 1993; Tanaka et al., 1993; Webb et al., 1993), or though adhesion (selectin) bonds themselves (Simon et al., 1999; Simon et al., 2000). A soluble chemokine released into blood flow may wash over rolling leukocytes to create a signal for up-regulation or increased β2 integrin avidity leading to the arrest of the cell (Fig. 1.8A). However, molecules secreted into the blood stream would be quickly diluted and confined to streamlines near the vessel wall and may not function as a localized signal for arrest. A molecular signal secreted into the vessel may be a whole tissue signal for arrest in the case of widespread inflammation. Chemokines that are secreted and bound by other membrane bound molecules may serve as a more specific locator for arrest (Fig. 1.8B) (Tanaka et al., 1993). There may then be several methods for controlling the localization of the signal. The expression of the display-molecule as well as the concentration of the molecule-on-display could then be regulated. Binding of the displayed chemokine could also be regulated by the selectin or selectin ligand expression, which would control the access or opportunity for the leukocyte to bind the chemokine.

1.3.4 Integrins and Ligands

Once the chemokine signal is transduced, leukocyte adhesion becomes dominated by a different set of adhesion molecules called β2 integrins. Integrins are heterodimeric molecules with both chains being transmembrane molecules (Fig. 1.9). The α and β chain associate non-covalently and have molecular weights of 90-170 kD for each chain. Integrins are grouped into families defined by their common subunits. The α chain
A convection in flow

flow

→

endothelial cell membrane

B receptor display

flow

→

Figure 1.8: Chemokine signaling. Chemokines, like IL-8 may diffuse into the lumen of the blood vessel and be swept away by convective flow (A) or be bound by held in the glycolcalyx and displayed to rolling leukocytes (B).
Figure 1.9: β₂ Integrins. Integrins are composed of an α and β subunit that noncovalently associate. Integrin binding is metal ion dependent and is required for leukocyte arrest and migration.
contains a divalent cation binding site called the metal ion dependent adhesion site (MIDAS), which may have a direct role in ligand binding (Lu et al., 2001a; Lu et al., 2001b). In fact, all integrins require divalent cations for binding.

β2 Integrins are expressed constitutively on leukocytes and excluded from microvilli (Erlandsen et al., 1993), which are sub-micron scale ruffles in the leukocyte membrane; but require an activation step for maximum avidity. The transition from rolling to arrest involves a progressive switch from selectin mediated adhesion to integrin mediated adhesion, which requires a signal to the leukocyte to make this shift (DiVietro et al., 2001; Jung et al., 1998; Kunkel et al., 2000). The avidity of integrins on the leukocyte increases and L-selectin and PSGL-1 are shed, (Davenpeck et al., 2000; Kahn et al., 1994; Lorant et al., 1995). Increased avidity could result from a conformational change in the integrins (Lu et al., 2001a; Lu et al., 2001b) or clustering of molecules (Chan et al., 1991; Dustin et al., 1996). Integrins bind immunoglobulin-like ligands, such as ICAM-1, expressed on the endothelium (Springer, 1995). There appears to be an increase in leukocyte pause times in vitro, while rolling on P-selectin (DiVietro et al., 2001) and L-selectin substrates (Fig. 1.10). The increased pause time may be due to increased β2 integrin—ICAM-1 bond lifetime, which may be a result of a conformational change or an increase in bond number due to clustering of β2 integrins. The increase in avidity of β2 integrins can be initiated and controlled by a surface bound chemokine and requires the residence time near the molecular substrate that selectin-mediated rolling affords the leukocyte to initiate the integrin and chemokine binding.
Figure 1.10: Velocity tracing of interacting neutrophils. Two IL-8 stimulated neutrophils (A, red and blue lines) interacting with L-selectin/ICAM-1 (170/50 sites/μm²) showed longer pauses than one resting neutrophil (B, green line) on L-selectin (170 sites/μm²) at 2.0 dyn/cm². The resting cell had a much higher average rolling velocity and shorter pauses. Data acquired at 40x magnification and 400 frames/s.
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The lengthening of pauses on multi-component (selectin/ICAM-1) surfaces may be due to binding between the I-domain on the integrin and the ICAM-1 (Knorr and Dustin, 1997). A pause time, kinetic analysis could be done for I-domain bonds (cells transfected with the I-domain) with ICAM-1 (substrate). A cell transfected with the I-domain and PSGL-1 could also be tested for a result in pause times for neutrophils interacting with the multi-component surface. The rolling velocity could also be compared with surfaces only containing a selectin as well.

Leukocyte activation and arrest are required for the transmigration of cells out of the vessel and into surrounding tissue. Transmigration is also linked to shear forces and chemoattractant display (Cinamon et al., 2001). The process of sensing chemoattractant gradients is a subject of intense study and is complicated by the fact that in vivo there can be several chemoattractants present throughout the inflammatory process; although, certain chemoattractant gradients appear to be preferred over others (Butcher et al., 1999; Campbell and Butcher, 2000; Foxman et al., 1997).

1.4 Molecular Kinetics and Mechanics

The kinetics and strengths of different molecular bonds may be important in development and healing processes. For instance, the time molecules stay bound and the force a bond can withstand may be vital for signal transduction and the mechanical stability of cells and tissues. Major factors that drive leukocyte adhesion are fluid dynamics, molecular kinetics and mechanics, and cell deformation. These components are the conditions that vary in the experiments designed to quantify and characterize
leukocyte adhesion. The primary component, the molecular kinetics and mechanics, controls the fundamental unit of adhesion, which is the lifetime of the molecular bond. Molecular properties such as dissociation kinetics and bond strength are important in leukocyte adhesion because they help describe the differences in the rolling kinetics of cells.

The study of molecular dissociation kinetics and mechanics of cell adhesion molecules began with the observation that rolling leukocytes tend to move or to ratchet forward, pause during bonding events, and then move again when those bonds break (Fig. 1.11) (Lawrence and Springer, 1991). The stop-and-go movement is pronounced in vitro. It also becomes pronounced at low substrate site densities, when leukocytes seem to form discrete attachments with the selectin molecules on the surface and this is evident through the motion of the cell, which seems to "pause" during bond events (Alon et al., 1995; Kaplanski et al., 1993). It was reasoned that if the substrate site density were diluted low enough, there would be very few molecules in the area for the cell to form a bond. Therefore the kinetics of the dissociation would have been the result of a single bond dissociating. Consequently, the lifetime of this event could be inferred from the motion of the cell in a shear flow. The force on the cell, estimated from the fluid dynamics, and the resulting force on the tether or bond could also be determined. This is the approach used in this dissertation to compare dissociation rate constants for mutants of PSGL-1.

There are two major assumptions in this reasoning, which are challenging to prove experimentally. One is that there really is only one molecule in the vicinity of the
Figure 1.11: Leukocyte tethering or pausing. The leukocyte travels at hydrodynamic velocity when there is no molecular interaction with the surface, but velocity lowers upon tethering or pausing due to bond formation and microvillus stretching.
cell that forms a bond, or from another viewpoint, the cell forms only one bond with the
substrate no matter how many molecules are present. The second assumption is that one
can measure the lifetime of the bond based upon the position of the cell as it moves or
pauses in shear flow.

Limitations on the spatial and temporal resolution of the leukocyte position are
the principle reasons these assumptions are difficult to prove. It is difficult to prove that
the molecules in the substrate are sufficiently or evenly distributed without an image of
the molecules attached to the surface, which has not yet been done. The time resolution
used to track the motion of the cell can affect the interpretation of the data as well. For
example, Alon and co-workers (Alon et al., 1997), measured the bond lifetimes of L-
selectin mediated adhesion and obtained a dissociation rate constant of 20 s⁻¹ for a wall
shear stress of 1 dyn/cm² and a video time resolution of 30 frames/s (fps, standard video
rate). I have used the same technique with a time resolution of 240 fps at 1 dyn/cm² and
the resulting dissociation rate constant was 90 s⁻¹ (Smith et al., 1999). The model for
bond dissociation predicts that we should both get the same value no matter what the time
resolution. So why do the two different time resolutions result in two different values for
k₀? The lower time resolution selects for longer-lived bond events because there is a
limitation in which events can be observed. Longer-lived bond events may be the result
of many bonds rather than a few or one, which is the intent of the measurement. Using a
sampling rate of 30 fps, I obtained a dissociation rate constant of 20 s⁻¹ for a wall shear
stress of 2 dyn/cm², a value similar to Alon and co-workers. But when a much higher
sampling rate of 240 fps was used, a dissociation rate constant of 100 s\(^{-1}\) was obtained, suggesting the selection for longer-lived bond events. Results of dissociation rate constants changing with sampling rate is analogous to aliasing in terms of the reconstruction of electrical signals. When the sampling rate is too low, \(e.g\). two times the true signal or less (Nyquist Sampling Theorem), an aliasing effect is possible, in which the true signal would be interpreted to be some other frequency. Therefore, it is important to measure the pause times at increasing sampling rate until the dissociation rate constant does not increase. An example of the effect of temporal resolution on measuring pause times is shown (Fig. 1.12). High temporal resolution ensures that short pauses are measured and longer pauses are measured more accurately compared to low temporal resolution. When the dissociation rate constant no longer increases, the shortest population of pause times are being measured and the dissociation rate constant from this distribution is a measure of the stochastic breaking of a small number of bonds, if not one, for a tethering or adhesive event.

Other techniques are also being used to study the kinetics of adhesion molecules. With increased interest in molecular properties of adhesion, there are a number of groups using different techniques to study the dynamic behavior of these molecules. A cantilever beam, acting as a spring, is used in atomic force microscopy (Fritz et al., 1998; Yuan et al., 2000) and the surface force apparatus (Leckband, 2001; Sivasankar et al., 2001). Atomic force microscopy uses a probe with a sharp tip mounted onto the cantilever. The sharpness of the tip dictates the number of molecules that can be coupled.
Figure 1.12: High temporal resolution is required to measure short pause times. The velocity of a bead interacting with a P-selectin substrate forming distinct pause events is shown. Critical velocity for 0.5 dyn/cm² is ~100 μm/s. Pause events may not be measured accurately (pause in green circle) or completely missed (pauses in pink circles) and not counted due to low sampling rate (red circle and dotted line, 30 fps). At higher sampling rate (blue cross and solid line, 250 fps), many short pauses are measured and longer pauses are measured more accurately. (Adapted from Eric Park, University of Virginia.)
to it. Therefore, the sharper the tip, the fewer molecules that can be coupled on it and the probe becomes a single molecular interaction force probe, in theory. Atomic force microscopy can measure intermolecular or intramolecular forces (Carl et al., 2001) ranging from 1 pN to 1 nN. The surface force apparatus is a similar device, but uses a probe with more area and thus measures the interaction of several molecules over exact distances. It can measure forces on the order of 10 nN to 1 μN and interaction distances on the order of tens of angstroms. Recently, the use of a microcantilever beam alone was used to measure recombinant E-selectin-IgG interactions with sLex (Tees et al., 2001). The E-selectin was adsorbed to the cantilever beam in a sufficiently low dilution to measure single bond kinetic parameters.

Optical tweezers use the radiation of lasers to hold beads and bring them into close association with another bead or surface. The force sustained by the interaction can then be measured as the surfaces are pulled apart and the bead moves in the optical trap. Optical tweezers can measure up to a few hundred pN of force. Optical tweezers have been used to measure the forces generated during molecular interactions, like the actin-myosin duty cycle (Guilford et al., 1997), as well as the force required to unravel single domains within molecules (Kellermayer et al., 1997).

Another technique for measuring molecular forces is micropipette aspiration. In micropipette aspiration, two cells or surfaces are brought close together so molecules can interact. Upon interactions one cell can be aspirated into the pipette and the force of association can be calculated through the deformation of the cell due to the molecular
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bonds. Here the cell acts as a spring similar to the light in optical tweezers and the cantilever in atomic force microscopy. The bioforce probe uses a red blood cell as the transducer or spring, which is coupled to a bead with the molecule of interest. This system is tapped against another cell or surface to form adhesive bonds. The bioforce probe uses reflectance interference contrast microscopy to determine distances between substrates with high temporal and spatial resolution. This technique can measure a wide range of force from 0.01 pN to 1 nN. Groups that use these techniques argue that the low frequency of adhesive bonds (1-2 for every 10 tries) measured in an experiment are statistically rare single-bond events. However, the measurement of force and lifetime of a single bond has still not been rigorously proven.

Information about the chemical and mechanical properties of a bond is the goal of the techniques described above. In fact, this is the aim of an emerging field named dynamic force spectroscopy by Evan Evans (Evans et al., 2001; Merkel et al., 1999). It is the mapping of chemical associations within molecular bonds to specific atomic bonds at the interface. This type of information might be able to describe the structure of parts of molecules in bound states and transition states. The techniques described have already produced data correlating bond parameters to force. Force may create a unique reaction pathway for dissociation.

The Bell model is an equation (Eqn. 1) relating the dissociation of bound molecules to an applied force that is pulling the molecules apart (Bell, 1978).

\[
k_{\text{eff}} = k_{\text{eff}}^0 \exp\left(\frac{\sigma F_b}{k_b T}\right)
\]  

(1)

Leukocyte Adhesion Kinetics: A Sticky Business
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The unstressed dissociation rate, $k_{\text{off}}^0$, is the rate at which bound molecules will come apart at equilibrium conditions and zero applied force. Dissociation rate is related to the molecular affinity. The Bell model relates the unstressed dissociation to the stressed dissociation, $k_{\text{off}}$, through an energy term of the force applied, $F_B$ over a separation distance between the molecules, $\sigma$. The energy term is scaled to thermal energy by the Boltzmann’s constant, $k_B$, and absolute temperature, $T$. Two different molecular bonds can be compared by their unstressed dissociation rates and their separation distances ($\sigma$). Separation distance is a parameter used to fit the exponential relationship of stressed dissociation rates with increasing force. As force increases, the dissociation rate constant increases and a bond with a higher separation distance has dissociation rates that increase more than a bond with a lower separation distance for the same amount of force. A bond that dissociates faster at some level of force can be said to be weaker or more compliant. Bond separation distance is also referred to as bond compliance.

Bond compliance is an intrinsic property of a receptor—ligand pair that relates the chemical kinetics to the force acting on the bond. Chemical kinetics and mechanical properties of a molecular bond assembly are intimately related and coupled. The Bell model was developed from principles of polymer deformation (Zhurkov, 1965). It takes a conceptual leap from continuum mechanics to stochastic processes, by relating the force-driven polymer deformation to a probability of single bond dissociation. The dissociation mechanics are lumped into the single term separation distance or compliance. As force pulls two bound molecules apart, the higher order (tertiary,
secondary) structure can denature allowing for elongation of the molecules without deforming the bond interface. Elongation strain for the selectin lectin—EGF domain unit (0.76-2.3%) (Alon et al., 1997) is estimated to be less than, but the same order of magnitude as strain observed for globular proteins (3-5%) (Howard, 2001; Morozov and Morozova, 1993). Presumably, as the elongation strain gets large, the entire molecular structure is affected as the molecules deform or unravel (Kellermayer et al., 1997). This may include carbohydrate decorations unique to selectin ligands as well as the protein component of the binding pocket. New residues or chemical groups may be exposed as the molecules deform, possibly creating new attractive or repulsive forces, as seen in chemical modifications (aldehyde on L-selectin ligand) (Puri et al., 1998) between residues in the same molecule or between residues on different molecules across the interface of the bond. This creates an interface that is only partially effective at maintaining a bond, resulting in dissociation.

The bond separation distance or compliance is a length scale for which this dissociation is probable. In essence, this distance can be a measure of bond strain or interface deformation and correlates with bond stability. The many attractive forces between residues within and across the bond interface create the potential energy barriers to bond dissociation. Regions around the interface, both within the protein structure and carbohydrate decorations, may directly affect the deformability of the bond interface. Thus chemical groups which are capable of more atomic associations, like hydrogen bonds, may increase the overall stiffness of the domain or molecule, allowing the active
site or interface region to stay in a higher affinity state (unloaded conformation) longer before deforming and destabilizing the bond to the point of dissociation. The changes in $k_{\text{off}}$ with force can be used in the design of applications to control the amount of time surfaces with adhesion molecules interact over a range of fluid dynamic conditions. So, molecules forming a bond with a large characteristic separation distance or compliance might be used to bring surfaces together, but only for short discrete time periods; whereas, molecules forming a bond with a low compliance might be used to bring surfaces together for longer time periods as in controlled drug delivery (Fig. 1.3).

1.5 Specific Aims

The general objective of my work has been to quantify the relationship between chemical dissociation and mechanical force for selectin adhesive bonds in leukocyte adhesion. I started this work by characterizing selectin bonds by their dissociation kinetics over a range of applied force, which was published in my thesis for the Master of Science degree and the Biophysical Journal (Smith et al., 1999). I am continuing the approach of quantifying strengths of molecular bonds to dissect the important associations within a molecular bond. In my dissertation, I have studied PSGL-1 structure that is important in molecular bonds with P-selectin as well as the function of cell rolling mediated by these molecules. I have also developed a Monte Carlo simulation of microbead rolling that incorporates the stochastic nature of molecular dissociation that varies with applied force utilizing the Bell model. A simulation of bead
rolling is a simplified simulation of leukocyte rolling, which can eventually incorporate cellular features to more fully recreate leukocyte rolling.

1.5.1 PSGL-1 Structure

*Hypothesis:* Transfected cells with a mutant, monomeric form of PSGL-1 will be less efficient in forming bonds with P-selectin than cells transfected with a wild-type, dimeric PSGL-1. The bonds formed between the monomeric PSGL-1 and P-selectin will also have faster dissociation kinetics and will be more compliant (more of an increase in $k_{off}$ with force) than bonds formed between dimeric PSGL-1 and P-selectin.

*Specific Aim:* I will make several functional measurements of parameters that contribute to leukocyte rolling, to address the hypothesis that dimerization of PSGL-1 affects bonding with P-selectin. The aim is to measure rolling velocity, accumulation of rolling cells, distance between tethers, and dissociation rate constants over a range of forces for transfected cells with either monomeric or dimeric PSGL-1.

*Background:* In the first study, a transfection of FT7 and a mutant form of PSGL-1 with an alanine substituted for a cysteine (C320A), into the erythroleukemic cell line K562 (core-2 is endogenous), and could not form a dimer; showed a severe deficit in the number of interactions counted in a shear binding assay (Snapp et al., 1998a). In another study, CHO cells transfected with FTIII, C2GnT or core-2, and a mutant form of PSGL-1, in which cysteine 320 was replaced with a serine and therefore could not form a dimer, also showed no difference from a wild type transfection of PSGL-1 in a shear detachment assay and a shear binding assay (Epperson et al., 2000). A more recent publication has
shown that a monomeric 19 amino acid peptide of the critical N-terminal region of PSGL-1 with full post-translational modifications can bind the lectin-EGF domain construct of P-selectin with similar affinity as a soluble dimeric, extracellular PSGL-1 construct (Somers et al., 2000). Co-crystalization of the lectin-EGF domains of P-selectin with the critical 19 amino acid region of PSGL-1 also revealed a monomeric or 1:1 interaction; although, this may not necessarily be the prevailing stoichiometry in functional leukocyte rolling. All of these reports attempt to show the dependence of dimerization on PSGL-1 binding P-selectin. In other words, is the PSGL-1 bond with P-selectin high affinity because the PSGL-1 binding pocket is composed of both PSGL-1 chains or do the two chains act to increase the probability of binding because they are two separate binding sites (Fig. 1.13)?

These conflicting reports might be resolved by applying a flow chamber bond-kinetics assay to distinguish the mechanical compliance of the monomeric PSGL-1-P-selectin bond compared to the dimeric PSGL-1-P-selectin bond. It has been suggested and shown with a model that bond compliance, as estimated in fits to the Bell model, does increase with decreasing number of bonds (Tees et al., 2001). If leukocyte tethering and rolling mediated by PSGL-1 bonds to P-selectin were due to the two PSGL-1 chains binding two separate P-selectin molecules, then the bond compliance of this interaction might be less than if the interaction were due to both PSGL-1 chains working together to bind one P-selectin molecule. The application of hydrodynamic force in parallel plate flow chamber assays reveals strikingly different dissociation kinetics of the P-selectin-
Figure 1.13: Bonding possibilities between PSGL-1 and P-selectin. The motivation for studying PSGL-1 structure and binding kinetics lies in the molecular valency and its unique combination of carbohydrate and protein recognition elements. Each PSGL-1 chain could bind a separate P-selectin molecule (A) or both chains might form a complex to bind one P-selectin molecule (B).
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PSGL-1 bond compared to the L-selectin-PSGL-1 bond as well (Smith et al., 1999). Differences in P- and L-selectin bond stability and duration with PSGL-1 are due to differences in association points within the binding pocket and may be dimerization sensitive. Therefore, the characterization and more rigorous quantification of monomeric versus dimeric PSGL-1 bond formation and dissociation with a substrate of P-selectin might further explain how PSGL-1 structure contributes to stable leukocyte rolling.

1.5.2 Monte Carlo Simulation of Bead Rolling

Hypothesis: The rolling velocity of beads coated with adhesion molecules can be simulated using a mathematical model of fluid dynamic forces on the bead and experimental data describing the dissociation kinetics of adhesive bonds.

Specific Aim: Model bead rolling using the Goldman approximation for motion of a sphere near a wall in shear flow (Goldman et al., 1967) and the experimentally measured dissociation kinetics as a function of force from the Bell model.

Background: Recent publications have used the Bell model relationship of dissociation rate of bonds dependent on force (Chang and Hammer, 2000; Chang et al., 2000). But they include several additional parameters to model bead rolling and they only match experimental results at high site densities of molecules (several hundred and even thousands of molecules/μm²) on the substrate and on the bead. In fact, site densities higher than those used in experiments had to be used in the models to match some experimental results.
This will be the first model to use the Bell model for dissociation of P-selectin-PSGL-1 bonds under force to be incorporated into a stochastic model that is compared to experimental results. Parameters from experiments with beads will be used in the simulation wherever possible and the parameter will be varied if an experimental value is unknown to show which value would give a best fit to the experimental data.
Chapter 2: Materials and Methods

2.1 Reagents

2.1.1 Cells

K562 cells, a human erythroleukemic cell line, and BJAB cells, a human B-cell lymphoma line, were stably transfected with fucosyltransferase VII (FT7) and PSGL-1 (Snapp et al., 1998a) and endogenously express core-2 β1,6 N-acetylgalactosaminyltransferase; although the lines appeared to have differing levels of core-2 activity. Another line of K562 cells, designated K562C2, were transfected with FT7, core-2 β1,6 N-acetylgalactosaminyltransferase, and PSGL-1 cDNA to ensure high levels of core-2 activity. The wild type transfection of K562/FT7/PSGL1, BJAB/FT7/PSGL1, and K562C2/FT7/PSGL1 was designated as K562-WT, BJAB-WT, and K562C2-WT. A PSGL-1 mutant in each cell line of K562/FT7/C320A, BJAB/FT7/C320A, and K562C2/FT7/C320A was also constructed and was designated as K562-C320A, BJAB-C320A, and K562C2-C320A. In the C320A mutant, the cysteine located near the transmembrane domain was replaced with an alanine. These mutants were unable to form PSGL-1 dimers. Two additional lines of 300.19 cells were constructed. One had core-2 transfected into it along with FT7 and PSGL-1 (WT) and the other lacked core-2. These cells were designated 300.19-C2-WT and 300.19-WT respectively. All of the cell lines described in this dissertation were transfected and supplied by Dr. Karen Snapp (Northwestern University). These cells were cultured and assayed in RPMI-1640 supplemented with 10% FBS, 1% penicillin/streptomycin, 2 mM
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L-glutamine, 2% β-mercaptoethanol, and 10 μg/ml ciprofloxacin. K562, BJAB, and K562C2 transfectants were cultured and assayed in RPMI-1640 supplemented with 10% FBS, 1% penicillin/streptomycin, 2 mM L-glutamine, and 10 μg/ml ciprofloxacin.

2.1.2 Substrates

Human P-selectin was purified from platelet lysates by affinity chromatography as described previously (Lawrence et al., 1997). Human P-selectin recombinant IgG (PR1gG) was purchased from GlycoTech, Rockville, MD.

Site densities of adsorbed P-selectin and PR1gG were determined by saturation binding radioimmunoassay using mAb G1 (Ancell, Bayport, MN). The mAb was iodinated with Iodobeads (Pierce, Rockford, IL) to a specific activity of 7 μCi/μg for G1.

Polystyrene slides were cut from bacteriological petri dishes (Falcon 1058) and the diluted adhesion molecules were applied to the plates and allowed to adsorb for 2 hrs at room temperature. The slides were then blocked for non-specific adhesion with 0.5% Tween-20 in PBS overnight at 4°C.

2.1.3 Antibodies

FITC-conjugated goat, anti-mouse secondary antibody was purchased from Pharmingen (San Diego, CA) for use in flow cytometry analyses. KPL1, a function blocking monoclonal antibody (mAb) to PSGL-1, was used (Snapp et al., 1998b). PL1, another function blocking mAb to PSGL-1, was purchased from Ancell, Bayport, MN.
2.2 Flow Cytometry

Transfected cell preparations (1×10^6 cells/ml) were incubated with 1:250 of primary mAb, KPL1 (ascites fluid), in PBS containing 10% FBS for 30 minutes. The cells were washed three times and then incubated for 30 minutes with FITC-conjugated, goat anti-mouse secondary antibody specific for the Fc region of the primary mAb. Samples were washed and resuspended in 1 ml of 1% paraformaldehyde. Fluorescence was detected with a FACScan flow cytometer (Becton Dickinson, Franklin Lakes, NJ) using incorporated CellQuest software.

2.3 Western Blot

Cells were centrifuged at 1000 rpm for 5 min and washed once with PBS. Protein was extracted with lysis buffer (10^8 cells/ml) containing 1% Triton X-100 (Fisher Scientific, Pittsburgh, PA), 1 mM phenylmethyl sulfonyl fluoride, 1 mM egtazic acid, and 1% protease inhibitor cocktail (Sigma Chemical, St. Louis, MO) in PBS, pH 7.4. Cells and lysis buffer were mixed on an orbital shaker for 30 min at 4°C. The mixture was then centrifuged at 14,000 g for 30 min at 4°C and the supernatant was transferred to new tubes. Samples were then boiled for 5 min in reducing buffer (β-mercaptoethanol) and electrophoresed on an 8% polyacrylamide gel (SDS-PAGE). The protein was transferred to nitrocellulose membrane paper overnight at 25 V. The membranes were blocked with 5% nonfat milk in PBS for 1 hr at room temperature and then probed with PSGL-1 mAb KPL1 (1:2000 of ascites fluid) in 2.5% nonfat milk and 0.05% Tween-20 (Fischer Scientific) in PBS or nothing for control for 1 hr at room temperature. The
membranes were washed 3 times with 0.05% Tween-20 in PBS and then incubated with goat anti-mouse IgG conjugated to horseradish peroxidase (Pierce, Rockford, IL) for 1 hr at room temperature. The membranes were washed 6 times in 0.05% Tween-20 in PBS and then developed with enzyme chemiluminescence using a substrate kit (Pierce). The membranes were exposed to CL-X Posure X-ray film (Pierce) and developed for 5-10 min.

2.4 Flow Assay

The substrate slide was assembled as the lower wall of a parallel plate flow chamber (Fig. 2.1). The gap in the gasket is 2 cm long by 1 cm wide by 300 μm high giving a volume of 60 μl. The relationship between the chamber dimensions, flow rate, and wall shear stress is (Eqn. 2):

\[ \tau = \gamma \mu = \frac{6 \mu Q}{wh_1^2} \]  

where \( \tau \) is the wall shear stress, \( \gamma \) is the shear rate, \( \mu \) is viscosity, \( Q \) is the flow rate, \( w \) is the width of the chamber, and \( h_1 \) is the height of the chamber. The chamber was mounted over an inverted phase-contrast microscope (Diaphot-TMD; Nikon, Garden City, NY) at 10x and 20x magnification. Figure 2.2 depicts a side view for the orientation of cells and molecules upon perfusion. A KODAK MotionCorder Analyzer, Model 1000 camera (Eastman KODAK Co., Motion Analysis System Division, San Diego, CA) was used for high temporal resolution of adhesive events with the substrates. Transfectants perfused over P-selectin substrates were viewed at a frame rate of 125 fps to measure tether
Figure 2.1: Parallel plate flow chamber apparatus. The parallel plate flow chamber is composed of a polycarbonate block with an inlet and outlet sandwiched by vacuum with a gasket and slide. The gap in the gasket is 2 cm long by 1 cm wide by 300 μm high. This allows the perfusion of cells over a molecular substrate that has been immobilized on the slide.
Figure 2.2: The cellular and molecular orientation. A side view of the flow chamber would show a molecular substrate of P-selectin adsorbed to a plastic slide. Transfected cells with varying molecular properties would be perfused over the molecular substrate to observe interactions. In this case, the schematic illustrates a comparison between a monomeric and dimeric form of PSGL-1, a ligand for P-selectin.
lifetimes and distances between pauses. Standard video frame rate, 30 fps, was used for all other assays.

2.5 Data Analysis

2.5.1 Determination of Rolling Cell Accumulation

Cells were perfused (0.5×10⁶/ml) through the flow chamber at a high flow rate (5 ml/min, or 3.25 dyn/cm², too high for any capture of cells by the substrate) to move the population of cells into the chamber. The flow rate was then reduced to the corresponding wall shear stress for observation of adhesive interactions. Cells were unable to interact with the substrate until the wall shear stress dropped to either 0.5 or 1 dyn/cm². The zero time point was taken at the instant in time that the pump slowed to the corresponding wall shear stress. There were no interacting cells at the zero time point because the wall shear stress was beginning the transition from 3.25 dyn/cm² down to either 0.5 or 1 dyn/cm². In essence, the population of cells was brought into contact with the surface at the zero time point. Ten fields of view (10× magnification) were then scanned at each minute for 6 min and the number of rolling cells was counted. Experiments were done in triplicate, therefore, each data point was an average of thirty fields of view. A cell was considered rolling if it displayed at least three adhesive contacts in the field of view. Rolling K562-WT transfectants were easily distinguished because they rolled at velocities well below the velocity at which a cell travels in streamlines near the wall without interacting with the substrate (critical velocity). Rolling in the K562-C320A transfectant was more difficult to distinguish as it traveled
far between adhesive bonds, but still made adhesive contacts. The site density of immobilized PRLgG was 200 sites/μm².

The accumulation of rolling cells is a measure of cellular capture from flow or the initial adhesive event by the adhesive substrate. All the transfected cells used were perfused at similar concentrations and flow rates. The cells were also similar densities and sedimented at similar rates; therefore, they were transported to the substrate at equal rates (cell flux). Furthermore, only the cells at the substrate (within 100 nm) could achieve adhesive interaction because they were within distances that allowed molecular bonding. Consequently, there were no differences in cellular transport and the measured differences in accumulation of rolling cells were due to the molecular interactions. A limitation of this measurement over time is the accumulation of slowly rolling cells because they occupy substrate space and impede new attachments by other free flowing cells. This limitation does impact this measurement at later time points of 6 min and higher for the transfectedants that rolled slowly because much of the surface is covered in rolling cells and new cells have less area to form adhesive interactions. I did statistical analyses on both the data points at each time point and the slope of the data from 1 to 6 min to show that this measurement was significant in showing the effect of dimerization, which was reported in the Results of Chapter 3. I also measured the distance between adhesive interactions to show the (lack of) effect of dimerization on the formation of tethers once the cell has begun the rolling process and has already formed the initial adhesive contact, which is described below and in the Results of Chapter 3.
2.5.2 Rolling Velocity

Movies of rolling cells or beads were captured from video tape to computer using Scion Image version 1.62 (Scion Corp., Frederick, MD). The elapsed time for each movie was 2 s and was the time window used for calculating rolling velocity. This was the maximum time for the fastest rolling cells to traverse the field of view. Again, a cell was considered rolling if it made at least three adhesive contacts in the field of view. The rolling velocity was calculated as the distance traveled over the 2 s time window. The site density of immobilized PR1gG was 200 sites/μm². At least 25 cell or bead velocities were measured for each experiment and experiments were done in triplicate. Thus each rolling velocity is an average of 75 values unless otherwise stated.

2.5.3 Data Acquisition and Tracking

Adhesive events and distances between adhesive for transfecteds interacting with P-selectin were acquired using a computer tracking program (Dr. William Walker, University of Virginia) coded in MATLAB 5 (The MathWorks, Natick, MA). This tracking program used a sum-of-absolute-difference algorithm and spline interpolation that allowed for sub-pixel resolution of changes in position to identify the cell in consecutive image frames. Video memory from the high-speed camera was played back at standard video rates for archiving on VHS tapes. Images from the VCR playback were then captured onto a computer using Scion Image v.1.62. Phase contrast microscopy was used for all experiments in this dissertation. The signal to noise ratio for a typical phase contrast image was 5. The average grayscale intensity value, where white was 1 and
black was 256, for the bright region of the cell was 73 while the dark background averaged 180. The signal intensity was taken as the difference between these values, while the noise was taken as the standard deviation of the intensity for the pixels representing the cell, which was 22. Cells were also tracked using bright field microscopy to test the accuracy of tracking. Both phase contrast and bright field microscopy resulted in similar tracking of the cells. Phase contrast microscopy has a higher signal to noise ratio as defined above, but the higher deviation of intensity from pixel to pixel within the cell in bright field microscopy was adequate to track the cell because the sum-absolute-difference algorithm resulted in an easily identifiable difference between the background and the cell. The sum-absolute-difference algorithm used a region over the whole cell in order to track it. The cell was not tracked by its centroid or edges alone. Therefore, interference fringes in phase contrast did not adversely affect the tracking. The fringes were also radially symmetric about the cell and did not result in bias or drift. The definition of the cell was taken as a rectangular region enclosing the boundaries of the cell. Therefore, any interference pattern not within 0.5 to 1 μm of the surface of the cell was not taken to be part of the cell. Video was also transferred directly to the computer to test if re-digitization from VCR tape to the computer introduced aliasing or error and the resulting movies appeared similar to movies captured from tape. Cell tracking from tape did not differ greatly from tracking completely digital movies.
2.5.4 Pause Time Definition

Temporal and spatial resolutions dictate the minimum velocity that can be measured. At high temporal resolution and low spatial resolution the minimum velocity measurable between image frames is high. So the instantaneous velocity of the cell or bead would oscillate between high values (no adhesion) and low values (adhesion). For this reason, the spatial resolution must be increased as the temporal resolution is increased in order to maintain a minimum velocity measurable that is well below critical velocity of the cell or bead being tracked.

The pause time or amount of time a cell remained bound was determined by counting the number of image frames between a drop in velocity of at least 25% and an increase in velocity of 12.5 μm/s over three frames (Fig. 2.3). The pauses measured from this example of instantaneous velocity (per frame) are circled. Fluctuations in velocity increase with flow rate, but at lower flow rates the ratio of the standard deviation of velocity to velocity is highest. The standard deviation of velocity for beads traveling near the lower wall without adhesive interactions at 0.1 ml/min (the lowest flow rate used) was 15 μm/s and the translational velocity was 100 μm/s. Therefore, the counting of a pause was initiated after the velocity decreased by 2 standard deviations. A typical, freely flowing K562 cell had a maximum fluctuation in velocity of approximately 50 μm/s at 0.5 dyn/cm², where the average free flow velocity was 275 μm/s. I wanted the
Figure 2.3: Pause time definition. Cellular pauses are circled from the instantaneous (per frame) velocity tracing. A pause is defined as the time between a drop in velocity of 25% and the next increase in velocity over 12.5 μm/s (see Methods and Materials for more explanation). There are 5 pauses circled above lasting 7, 5, 3, 6, and 12 frames. The low velocity region past 3 seconds is not counted because this pause is not a completely bounded event.
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criterion for the beginning of a pause to be well outside the range of fluctuations for a freely flowing cell, but not so strict as to ignore pauses that occurred without reaching a velocity of zero. Cells were viscoelastic and deformed during the rolling process (Park et al., 2001) and possibly during the tethering process. The velocity of the cells did not reach zero during every pause, but always decreased greatly in the first few frames during a pause. This criterion agreed well with the comparison of observed motion by viewing the digitized movie. The velocity of 12.5 μm/s was also 5 times higher than the highest fluctuation in apparent velocity of a stationary object (adherent cell) in my system. Again, because of the elasticity of the cell and stretching of microvilli during the tethering process, I wanted the definition of the end of a pause to exclude the small increases or non-zero velocity that tended to be 5-10 μm/s and corresponded to small deformations of the cell. Any increase in velocity above 12.5 μm/s was clearly indicated the end of a bonding event and a release into flow rather than the deformation of the cell. A new name for the process of tethering, such as adhesive event, should be established to fit the actual non-zero velocity definition of the adhesive interaction rather than “pause”. At least 150 adhesive events for pause time distributions and 100 distances between events were measured for each wall shear stress.

The dissociation rate constant, $k_{off}$, was calculated as the negative slope of the natural log of number of events remaining versus the duration of the events at one wall shear stress. Extreme outliers comprising the longest 5% of events were excluded from the duration distributions to minimize the effect of possible multivalent interactions.
2.5.5 Estimation of Force and Lever Arm

The force on the tether was found through the force and torque balance using the geometry of a tethered cell (Fig. 2.4). The cells used in the tether lifetime experiments, K562 and BJAB, vary in diameter, but the average was approximately two times that of neutrophils. Therefore, I used a diameter of 17 \( \mu \text{m} \) for these cells when calculating the force on the tether. The microvillus length and lever arm length were calculated by comparing \( k_{\text{off}} \) values for the cells from this study with \( k_{\text{off}} \) values previously obtained for PSGL-1 coated beads (Park et al., 2001). The true force on the microvillus tether was obtained by equating the cellular \( k_{\text{off}} \) value for a low flow rate (7.35 s\(^{-1}\) for 0.2 ml/min or 0.3 dyn/cm\(^2\)) with the \( k_{\text{off}} \) on the Bell curve for the PSGL-1 coated bead data (\( k_{\text{off}} = 1.5378 \times \exp\{0.0091 \times F_b\} \)). The force this corresponded to was taken as the best estimate of force on the cellular tether. Using the shear force that was imposed on the cell, the lever arm length and angle between the tether and substrate were then found. The shear force (\( F_s \)) was calculated for the transfected cell at the same low flow rate. The angle between the tether and the lower wall of the flow chamber was then calculated from the relationship \( F_s = F_b \cos \theta \). The procedure resulted in a \( \theta \) of 66.64°, a lever arm length of 3.67 \( \mu \text{m} \), a microvillus length of 0.76 \( \mu \text{m} \), a shear force of 220.7 pN per dyn/cm\(^2\), and a force on the tether of 555 pN per dyn/cm\(^2\). This value was used to scale the force on the tether at all wall shear stresses. When this same method was used to find the lever arm length by equating the cellular \( k_{\text{off}} \) value at the high flow rate (11.2 s\(^{-1}\) for 0.6 ml/min or 0.9 dyn/cm\(^2\)) with the bead \( k_{\text{off}} \) value, a lever arm of 16 \( \mu \text{m} \) was obtained.
Figure 2.4: Free body diagram of leukocyte paused. The free body diagram and the geometry was used to solve for the force on the tether ($F_b$) and the lever arm length ($L$). $\tau_s$ was the torque from the fluid shear, $F_r$ was the resultant shear force from the fluid, $R$ was the radius of the cell, and $\theta$ was the angle between the tether and the substrate.
A longer lever arm at a higher force suggests the stretching of the tether or microvillus. The longer lever arm calculated from this method agreed with a recent presentation from the McEver lab that suggested tethers formed from K562 and HL60 cells (through P-selectin—PSGL-1 bonds) were more elastic than neutrophils from tether pulling experiments using a micropipette.

2.6 Monte Carlo Simulation of Bead Rolling

The simulation of bead rolling used the hydrodynamics of a sphere translating near a plane under shear flow to create the movement of the bead in non-adhesive states. Simulation of the association and dissociation of bonds was coupled to the solution for the motion of a hard sphere in shear flow. Site density, flow rate, viscosity, and flow chamber gap dimensions were input parameters specified to match the experimental conditions used. The bead radius, receptor—ligand cross-bridge length, and separation distance between the bead and substrate surfaces were also input parameters. A list of independent and dependent variables along with ranges of values for each is given (Table 2.1).

The equation for translational velocity of a neutrally buoyant sphere near a plane wall in a shear field developed in the publication by Goldman and co-workers (Goldman et al., 1967):

\[ U = \frac{hS[0.5(a/h)F_xT_y' - F_x'T_y]}{F_x'T_y' - F_x'F_y'} \]  

(3)
<table>
<thead>
<tr>
<th>Independent Variables</th>
<th>Value in simulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>site density (sitedensity)</td>
<td>170 sites/μm²</td>
</tr>
<tr>
<td>viscosity (u)</td>
<td>0.01 g/cm²·s</td>
</tr>
<tr>
<td>molecular cross-bridge length (CL)</td>
<td>100 nm</td>
</tr>
<tr>
<td>separation distance (sepdist)</td>
<td>1—80 nm</td>
</tr>
<tr>
<td>radius of bead (R)</td>
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<tr>
<td>flow rate (Q)</td>
<td>0.1—0.6 ml/min</td>
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<td>total time (T)</td>
<td>5 s</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Dependent Variables</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>wall shear stress (tau)</td>
<td>0.15—0.89 dyn/cm²</td>
</tr>
<tr>
<td>reaction volume (reactvol)</td>
<td>0.05—0.14 μm³</td>
</tr>
<tr>
<td>force on tether (Fb)</td>
<td>25—160 pN</td>
</tr>
<tr>
<td>association rate constant (kon)</td>
<td>14—25 s⁻¹</td>
</tr>
<tr>
<td>dissociation rate constant (koff)</td>
<td>2—7 s⁻¹</td>
</tr>
<tr>
<td>hydrodynamic bead velocity (v)</td>
<td>23—294 μm/s</td>
</tr>
<tr>
<td>time step (dt)</td>
<td>0.52—6.6 ms</td>
</tr>
<tr>
<td>probability of tether formation, P_{form} (probbond)</td>
<td>0.72—15 %</td>
</tr>
<tr>
<td>probability of tether breakage, P_{break} (probnobond)</td>
<td>0.34—1.3 %</td>
</tr>
</tbody>
</table>

Table 2.1: Variables used in simulation. Independent and dependent variables are listed on the left and a range of values for each from the simulation is listed on the right.
was used for the motion of the model bead between adhesive bonds in the simulation (Eqn. 3). The translational velocity is \( U \), the distance from the center of the bead to the substrate surface is \( h \), the shear rate is \( S \), and the bead radius is \( a \). Forces due to the translation, rotation, and shear are designated by \( F \) with superscripts \( t \), \( r \), and \( s \) respectively. The torques due to the translation, rotation, and shear are designated by \( T \) with superscripts \( t \), \( r \), and \( s \) respectively. The method-of-reflections approximation for the case where the bead is near the wall ( \( >15 \) nm for the 10 \( \mu \)m diameter sized beads used in this work) required one set of equations for the forces and torques, while the lubrication-theory-like approximation for the case where the bead is very near the wall ( \( \leq 15 \) \( \mu \)m) required a different set of equations for the forces and torques.

Association of single bonds was calculated by converting the 3D affinity measured for the P-selectin—PSGL-1 bond (\( 4.4 \times 10^6 \) M\(^{-1}\)s\(^{-1}\)) (Mehta et al., 1998) to a per-second on rate by calculating the association based on a volume between the surfaces of the bead and the substrate and the site density of P-selectin. This volume was calculated as a cylinder and a function of the molecular cross-bridge length and a circle of reactive area on the bead (Fig. 2.5). An example calculation to achieve the per second association rate is given in the appendix. As the bead gets farther from the substrate, separation distance increases, the reaction volume decreases and results in a lower \( k_{on} \). A decreased separation distance, with the bead closer to the substrate, increases the reaction volume and \( k_{on} \). A per second association rate must be found, in order to establish the probability of bond formation described below (Chang and Hammer, 1996). Briefly, to achieve this
Figure 2.5: Geometry for simulation of bead rolling. The reaction volume, used to obtain a per second $k_{on}$ from a 3D $k_{on}$, was calculated from the molecular cross-bridge length and the separation distance. The bead in blue is closer to the substrate with a lower separation distance and larger reaction volume, which will result in a higher $k_{on}$. The bead in red is farther from the substrate with a higher separation distance, but still within the molecular cross-bridge length where adhesive bonds are possible. The lower reaction volume for the red bead will result in a lower $k_{on}$. Bead length 1 (bl1) and bead length 2 (bl2) drawn for the red bead are used to subtract out the volume of the cylinder that is occupied by the sphere (red shaded area). An example calculation of $k_{on}$ is given in the appendix.
\( k_{on} \) (s\(^{-1}\)), I multiplied the solution phase \( k_{on} \) (M\(^{-1}\)s\(^{-1}\)) by the number of substrate sites in the reactive area and divided by the reaction volume. The \( k_{on} \) in the simulation then becomes dependent on the substrate site density and the separation distance between the bead and the substrate.

The dissociation kinetics were calculated from the Bell model (Eqn. 1) (Bell, 1978), described in Chapter 1, using parameters measured from experiments of human, purified PSGL-1 coated beads tethering on human, purified P-selectin (Park et al., 2001). The fit to the Bell model produced values of 0.37 Å for bond compliance or separation distance and 1.5 s\(^{-1}\) for the zero force dissociation rate.

The stochastic nature of bond association and dissociation was captured by using random numbers to compare to the probability of tether or bond formation or bond breakage, which have been developed previously by Chang and Hammer (Chang and Hammer, 1996). The probabilities were developed by first assuming that the binding reaction was a reversible, bimolecular process (Eqn. 4):

\[
\frac{dn_b}{dt} = k_f n_f (n_t - n_b) - k_{off} n_b
\]

where \( n_b \), \( n_t \), and \( n_f \) are the number of bonds, total number of ligands, and number of free receptors, respectively. The dissociation rate constant is \( k_{off} \) and the association rate constant (solution phase, 3-D) is \( k_f \). If the ligand density (\( n_t \)) and the number of free receptors (\( n_f \)) is large compared to the number of bonds formed, the number of ligands can be assumed constant and the variable \( k_{on} \) can then be introduced, where \( k_{on} = k_f n_t \) and Eqn. 4 becomes:

---

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\[
\frac{dn_b}{dt} = k_{on} n_r - k_{off} n_b
\]  

(5)

For the total number of receptors \(n_r\), \(n_r = n_t + n_b\). Dividing Eqn. 5 by \(n_r\) then gives the probability distribution function, \(P_b(t) = n_b(t)/n_r\), that a specific receptor is occupied at time \(t\), resulting in:

\[
\frac{dP_b}{dt} = k_{on} (1 - P_b) - k_{off} P_b
\]  

(6)

Then by integrating Eqn. 6 and using the initial conditions of \(P_b(\text{formation}) = P_{\text{form}}(t=0) = 0\) for the conditional probability of a receptor being free at time \(t\) and becoming bound at \(t+dt\) and the initial conditions of \(P_b(\text{breakage}) = P_{\text{break}}(t=0) = 1\) for the conditional probability of a receptor being bound at \(t\) and becoming free at \(t+dt\), the following are obtained:

\[
P_{\text{form}} = k_{on} (1 - \exp(-k_{on} + k_{off} )dt))
\]  

(7)

\[
P_{\text{break}} = k_{off} (1 - \exp(-k_{on} + k_{off} )dt))
\]  

(8)

By assuming that no bonds are breaking in the period \(t+dt\) for Eqn. 7, \(k_{off}\) is very small compared to \(k_{on}\) (\(k_{off}\) goes to zero), the \(P_{\text{form}}\) can be simplified to Eqn. 9. In the same manner, assuming that no bonds are forming in the period \(t+dt\) for Eqn. 8, \(k_{on}\) is very small compared to \(k_{off}\) (\(k_{on}\) goes to zero) the \(P_{\text{break}}\) can be simplified to Eqn. 10. The probability of bond formation and probability of bond breakage become:

\[
P_{\text{form}} = 1 - \exp(-k_{on} dt)
\]  

(9)
\[ P_{\text{break}} = 1 - \exp(-k_{\text{off}}dt) \] (10)

In the assumption of one event of formation or breakage during each time step, the time step must be sufficiently small compared to the association and dissociation rate constants. Chang and Hammer used a time step ten times smaller than the inverse of the association and dissociation rate constants. I also used a time step at least ten times smaller than the inverse of the association and dissociation rate constants. Each free receptor—ligand pair had the opportunity to bind and each bond had the opportunity to break in the time interval \( dt \) according to the \( k_{\text{on}} \) or \( k_{\text{off}} \). The time interval or step, \( dt \), was dependent on the site density and the flow rate and was taken as the time required to travel between bonding opportunities (Fig. 2.6, A). During the time interval, several PSGL-1 molecules were swept over an immobilized P-selectin ligand. Therefore, a bond has the full time interval (\( dt \)) to form, rather than depending on the close proximity of an individual PSGL-1 molecule to the P-selectin molecule (Fig. 2.6, B). In order to test that the time step was sufficiently short, I decreased the time step by an order of magnitude. In decreasing the time step, each PSGL-1 molecule on the bead that passes over the P-selectin molecule on the substrate has its own probability of bond formation. The summation of the probability (cumulative probability) of each bond opportunity at the shorter time step (Fig. 2.7, B) is equal to the original probability at the longer time step (Fig. 2.7, A), indicating that the probability of bond formation was in the linear portion of the curve (Fig. 2.8). The accounting of each PSGL-1 molecule at the shorter time step is computationally challenging (due to memory and run time) and unnecessary because the
Figure 2.6: Motion of molecules on bead with respect to time interval. The bead travels at hydrodynamic or critical velocity when no adhesive bonds exist over the time interval, $dt$. A molecule of PSGL-1 (blue crescent) on the bead then travels at critical velocity between bonding opportunities (A). The bead does not form a bond at the beginning of $dt$ (dashed lines) and travels at critical velocity to the next bond opportunity (solid lines). A P-selectin molecule (red circle) on the substrate has a flux of PSGL-1 molecules on the bead to interact with during the time interval (B).
Figure 2.7: Cumulative probability of bond formation and time interval. The time interval in the original model depended on the kinematics of the bead and was $\sim 5 \times 10^{-4}$ s. This example illustrates that the probability of bond formation is equivalent if the time step is decreased, as long as $dt$ is sufficiently short to begin with. A number of PSGL-1 molecules (blue crescent) on the bead sweeps over a P-selectin molecule (red circle) during the time step, $dt$ (A). When the time interval is shortened, the cumulative probability of bond formation at the site of the P-selectin molecule remains the same if each PSGL-1 molecule is accounted for (B).

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Figure 2.8: Time interval must be sufficiently short. As long as the time step is within the linear portion of the curve (light blue region) for the probability of bond formation the cumulative probability of several opportunities of a PSGL-1 molecule forming a bond at a specific P-selectin molecule is equivalent to the probability of a bond forming for a larger time step. The longest time step used at the lowest wall shear stress of 0.1 dyn/cm² was 6.6 ms, which was within the linear region.
same probability is achieved at the larger time step and there is no need to test for bonds in regions where no P-selectin molecules exist. By allowing the time step to depend on the site density and flow rate, no time was spent on calculations between bond opportunities where no bonds could be formed. The formation of bonds decreased with increasing flow rates as the dependence on shear rate and kinematics shown experimentally would predict (Chen and Springer, 2001).

The model did test the bonding opportunity and tether formation with respect to each site in the substrate, but a P-selectin molecule had a number of opportunities to form bonds with PSGL-1 molecules as the bead surface swept by the P-selectin in a given \( dt \). I did not want to test each PSGL-1—P-selectin bond opportunity within that \( dt \), which higher time resolution required. The \( dt \) was determined by the time the bead takes to flow between P-selectin molecules because I wanted to test each opportunity with each P-selectin molecule. The \( dt \) was also limited by the amount of time the reactive area of the bead swept by a P-selectin bond opportunity. Therefore, \( dt \) could not be longer than the time for these two events, otherwise bond opportunities with P-selectin sites would have been missed. The time required to move between two P-selectin sites was the shorter of the two possibilities and was used for \( dt \).

While the probability of bond formation might not depend on the time step in a static system, the probability of bond formation for a specific receptor—ligand pair should decrease with increasing velocity of the particle or bead. The bond formation event is dependent on the kinematics of the particle over a surface or molecular substrate.
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I chose to implement this difference in bond formation by using a probability that depends on time step, which is set by the molecular spacing in the substrate and the kinematics of the bead. The $k_{on}$ in the probability of bond formation is only dependent on site density and separation distance and therefore, does not take into account the particle kinematics. But, by changing the time step with the kinematics of the particle, the probability of bond formation will also change.

Site density remained constant throughout the simulations described in this dissertation; therefore, as flow rate was increased the bead traveled faster between bond opportunities and the time interval shortened. A shorter time interval, for a higher flow rate, resulted in a lower probability of tether formation and faster rolling because more time was spent at critical velocity. The bead had the opportunity to form a bond sequentially with every molecule on the substrate. If the bead formed a bond, then the next time step and each time step thereafter the bond was tested for breakage. If the bond did not form or broke in the previous time step, the bead moved at critical velocity to the next molecule (position) on the substrate, which created the next bonding opportunity. The distance the model bead traveled over the running of the simulation, which was bound by a time, was calculated as the average rolling velocity of the bead.

The simulation was split into three different cases. One case assumed an adhesive bond existed at each bond opportunity (and time step, $P_{form} = 1$), which was dependent on substrate site density. The bond was then tested for breakage. Once the bond dissociated, the bead then moved at hydrodynamic velocity to the next bond. Another
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case incorporated a $k_{on}$ and a fractional probability of bond formation. So each bonding opportunity was tested for bond formation and then bond dissociation if a bond did form. Otherwise, the bead moved forward to the next bond opportunity at critical velocity. The third case allowed up to two bonds to form during each bond opportunity. This case assumed that there were two receptor—ligand pairs available for bond formation at each bond opportunity location (Fig. 2.9). Keeping the site density the same, the distance between bond opportunities was therefore doubled. The paired molecules could be thought of as dimers because there were two independent binding sites and opportunities, but the two molecules shared the location on the substrate. This case represented the dimerization of PSGL-1 and P-selectin (Barkalow et al., 2000) and was split into two sub-cases in which the bonds could either rebind after breaking or not rebind after breaking.

2.7 Microbead Experiments

Polystyrene microbeads (9.14 ± 0.86 μm) were purchased from Polysciences, Inc. (Warrington, PA). PSGL-1, purified from HL60 cells (Park et al., 2001), adsorption to the microbeads was carried out the day of each experiment. For adsorption of PSGL-1, the microbeads (5×10^7 beads/ml) were washed twice with alternating steps of water and ethanol, followed by incubation with PSGL-1 in HBSS, pH 7.4 for two hours at room temperature under end-to-end rotation. PSGL-1 was likely adsorbed to the polystyrene microbead through its hydrophobic transmembrane domain and retained functional activity similar to that observed for many adhesive glycoproteins. PSGL-1 that adsorbed
2 Bond Case

Figure 2.9: Flow diagram for 2 bond case of simulation. The simulation tested for the formation of 2 independent bonds and then tested each one independently for rupture. The bead moved at hydrodynamic velocity any time there were no bonds, but remained stationary if there were 1 or 2 bonds.
in other orientations may not have been functional. After washing with HBSS, the microbeads were incubated in 0.5% Tween-20 solution for 30 minutes with agitation to block the remaining surface against nonspecific adhesion. The microbeads were stored in HBSS containing 0.5% Tween-20. Prior to use in the flow chamber, the microbeads were resuspended in assay media (HBSS, 10 mM HEPES, pH 7.4, and 1 mM CaCl₂) at a concentration of $5 \times 10^5$ beads/ml. The PRIgG substrate was prepared using the same protocol described earlier. The beads were perfused over PRIgG at a site density of 170 sites/μm² and observed at 20× magnification for 5 s to calculate rolling velocity. One hundred bead rolling velocities were calculated at each wall shear stress tested.
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3.1 Specificity

PSGL-1 transfectant tethering and rolling on substrates were specific for PSGL-1 and P-selectin (Fig. 3.1). An interacting cell was taken as a cell that tethered at least three times in the field of view. Most cells rolled stably, in that once a cell attached, it remained in nearly continuous contact with the substrate. All PSGL-1 transfectants were tested for specificity by adding EDTA to the cell suspension being perfused. The EDTA chelated the Ca\(^{2+}\) ions in the assay media and no interactions with the substrate were observed. The anti-PSGL-1 mAb, KPL1, also blocked adhesion, while the non-blocking antibody KPL2 did not. Slow, stable rolling of transfectants was reduced to no interactions or very sparse adhesive events when KPL1 was present in the assay media. All adhesion was completely blocked by the mAb G1 to P-selectin. The difference in the number of tethering K562 cells compared to the BJAB and K562C2 cells might have been due to differing expression levels of PSGL-1. The incomplete blocking of the K562 adhesion with KPL1 might have been due to degradation of the antibody from an old stock, whereas a new stock of KPL1 was used for blocking experiments for the BJAB and K562C2 cells.

3.2 Western Blot

Cell lysates prepared from the transfectants were analyzed by SDS-PAGE and Western blotting (Fig. 3.2). Under reducing conditions, the prominent bands identified by KPL1 had molecular weights from 120-140 kD. The strong bands in the lanes for
Figure 3.1: Tethering of transfected cells. Transfected cells were perfused for 1 min at 0.5 dyn/cm² wall shear stress and tethering cells were counted for ten fields of view for 2 experiments with a native P-selectin site density of 200 sites/µm². Cells perfused alone (blue bar) had varying tethering rates dependent on the cell line. KPL1, an anti-PSGL-1 mAb, greatly reduced tethering cells (purple bar), while KPL2, a non-blocking mAb to PSGL-1, did not affect tethering rate (yellow bar). G1 (green bar), an anit-P-selectin mAb, and EDTA (red bar), a divalent cation chelator, also greatly reduced tethering cells.
Figure 3.2: Western blot of PSGL-1. SDS-PAGE was run under reducing conditions. Bands at the very top of the blot are unreduced PSGL-1 at 240 kD. Low molecular weight species of PSGL-1, from K562 and 300.19-WT transfected cells, migrated farther at 120 kD compared to the high molecular weight species of PSGL-1, from K562C2, BJAB, 300.19-C2-WT, and HL60 cells, which migrated less at 135-140 kD. The high molecular weight species of PSGL-1 conferred the function of slow rolling and the low molecular weight species of PSGL-1 conferred fast rolling.
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K562 lysates migrated closer to 120 kD, while the BJAB lysates had strong bands at the higher molecular weight of 140 kD, which was similar to the mobility of monomeric PSGL-1 from HL-60 lysates. The band of reduced PSGL-1 from HL-60 cells migrated to a molecular weight of 135 kD. HL-60 cells have been reported to have 15 decameric repeats in their PSGL-1 chains (Veldman et al., 1995), which might account for the difference in molecular weight between the PSGL-1 of BJAB cells and all the other cell lines (Afshar-Kharghan et al., 2001). Cell lines are typically transfected with PSGL-1 cDNA purchased from a commercial source that has been pooled from donors. In one population tested, there were three human alleles for tandem repeats in PSGL-1, with 81% having 16 repeats, 17% having 15 repeats, and 2% having 14 repeats (Afshar-Kharghan et al., 2001). Therefore, the PSGL-1 synthesized from purchased cDNA could represent one of the alleles described.

The difference in migration pattern between the K562 and BJAB transfectants was similar to that observed in core-2 glucosaminyltransferase—deficient mice (Sperandio et al., 2001). There was also a faint band of a higher molecular weight species in the K562-WT cell lysate that migrated ~135 kD, suggesting that there was some core-2 activity in these cells. The higher molecular weight species was totally absent in the K562-C320A. This small fraction of higher molecular weight species of PSGL-1 in K562-WT cells might have been responsible for the similarities in functional data for BJAB and K562C2 cells described later. The dimerization of PSGL-1 in the
K562-WT cells could have amplified the minimal level of core-2 activity that could not be observed in the monomeric K562-C320A cells.

I then hypothesized that the K562 cell line had a similar core-2 glucosaminyltransferase deficiency as found in the core-2 deficient mice. By transfecting the K562 cell line with human core-2 β1,6 N-acetylglucosaminyltransferase, the new K562 cell line, designated K562C2, expressed core-2 at a higher level and the resulting Western showed a less mobile monomeric PSGL-1 band at 135 kD. The 300.19-C2-WT cells also showed a higher molecular weight PSGL-1 at 135 kD contrasting with the 300.19-WT PSGL-1, which migrated a distance consistent with a molecular weight of 120 kD. These results suggested that the increased mobility of PSGL-1 in the original K562 cell line was due to a glycosylation deficiency likely due to weak core-2 activity. The increased mobility in the 300.19-WT cell line, used as a host cell, was also due to an apparent glycosylation defect from an absence of core-2 activity. Even small amounts of the higher molecular weight PSGL-1 conferred slow rolling on the cells. But only having the lower molecular weight PSGL-1, which the K562-C320A and 300.19-WT cells expressed, conferred unstable, fast rolling.

As the differences in glycosylation were being tested, I also tested the relative contributions of dimerization and glycosylation to cell adhesion in flow by measuring rolling velocity and then breaking rolling velocity down into cellular association and dissociation parameters.
3.3 Rolling Velocity

Cells were perfused through the flow chamber, flow was stopped, and cells were allowed to settle before starting flow again. Rolling cells interacted with the substrate in several, sequential molecular adhesive events, which were detected by significant decreases in velocity below a defined critical velocity. Critical velocity is the velocity that cells translate near the wall of the flow chamber without adhesive interactions. Cells rolled similar velocities on comparable site densities of native and recombinant P-selectin and the interaction was completely abolished by EDTA or function blocking mAbs G1, against P-selectin, and KPL1, against PSGL-1 (Fig. 3.1).

For both BJAB and K562C2 transfectants, C320A (monomeric PSGL-1) cells rolled at the same velocity as the WT (dimeric PSGL-1) cells on PR1gG (1 μg/ml, 200 sites/μm², Fig. 3.3B, C). Cells rolled between 5-11 μm/s (with standard deviations on the same order of magnitude) over a range of wall shear stress from 0.4–1.9 dyn/cm², suggesting dimerization of PSGL-1 did not affect rolling velocity. None of the average rolling velocity values were statistically significant between the C320A cells and the WT cells for the BJAB and K562C2 cell lines. The K562-WT cells also rolled 5 μm/s over this range of wall shear stress; however, K562-C320A cells rolled much faster (Fig. 3.3A). The K562-C320A rolled an order of magnitude faster at approximately 40 μm/s (with standard deviations similar to the mean). The average rolling velocity for K562-C320A cells was statistically different (p<0.05, Student’s T-test) from the average rolling velocity for K562-WT cells at all wall shear stresses tested. The 300.19-C2-WT cells
Figure 3.3: Transfected cell rolling velocity. Rolling velocity (mean ± SD) was not dependent on dimerization at the PR1gG site density of 200 sites/μm² tested. BJAB transfectants (C), K562C2 transfectants (B), and the K562-WT transfectant (A) all had rolling velocities between 4 and 12 μm/s over the range of wall shear stresses tested. But rolling velocity was dependent on core-2 activity as the K562-C320A transfectants (A) rolled at much higher velocities (~40 μm/s). *K562-C320A and 300.19-WT significantly different (p<0.05) from all other transfectants at each wall shear stress tested.
also rolled at slow velocities of approximately 4 μm/s over the same wall shear stresses, while the 300.19-WT (lacking core-2) rolled at much higher velocities of approximately 30 μm/s, suggesting that core-2 glycosylation added stability and strengthened rolling interactions.

I fixed cells to rule out improper or lack of PSGL-1 cytoskeletal anchorage causing the higher rolling velocity in K562-C320A cells. The C320A chains might have been uprooted creating a shorter apparent bond lifetime and faster rolling velocity. The K562 cells (WT and C320A) were fixed with 0.4% paraformaldehyde. Anchoring the PSGL-1 chains by fixation did not decrease the rolling velocity. In fact, the rolling velocity increased slightly for both fixed K562-C320A and K562-WT cells. The average rolling velocity for fixed K562-C320A and K562-WT at 0.5 dyn/cm² were 53 and 14 μm/s, respectively. At 1 dyn/cm², the average rolling velocities for fixed K562-C320A and K562-WT were 64 and 15 μm/s, respectively. Cells were perfused over a surface of native P-selectin at a site density of 200 sites/μm². The faster rolling velocities of fixed cells might have been due to the increased rigidity of fixed cells keeping the cells in streamlines farther from the surface, which flow at a faster rate (Park et al., 2001; Tempelman et al., 1994). Rigid, fixed cells have rigid microvilli or protrusions, which keep the cell body farther from the surface compared to an elastic microvilli, which can be compressed to allow the cell body closer to the surface.

The higher rolling velocity of the K562-C320A and the 300.19-WT cells might have been due to less frequent tethering or shorter bond lifetimes or a combination of
both. I attempted to quantify the relative contributions of bond association and bond
dissociation to rolling velocity. The number of rolling cells accumulating over time and
the distance traveled between bonds or tethering events were then measured as an
indication of the ability to form bonds. I also measured tether lifetimes over a range of
forces to obtain dissociation rate constants and bond compliance.

3.4 Accumulation of Rolling Cells

More cells tethered and rolled on PR1gG (200 sites/μm²) at the 0.5 dyn/cm² wall
shear stress than 1 dyn/cm² for all cell types. There were 71 rolling K562-WT/mm²
versus 24 rolling K562-C320A/mm² after 6 min of perfusion at 0.5 dyn/cm² wall shear
stress (Fig. 3.4A). At the higher wall shear stress of 1 dyn/cm², there were 37 rolling
K562-WT/mm² compared to 21 rolling K562-C320A/mm² (Fig. 3.4D).

The difference between C320A and WT in accumulation persisted even after
core-2 activity was increased in the K562 cells (Fig. 3.4B and E). There were 313 rolling
K562C2-WT/mm² and 228 rolling K562C2-C320A/mm² after 6 min at 0.5 dyn/cm². At
1 dyn/cm², there were 151 rolling K562C2-WT/mm² and 120 rolling K562C2-
C320A/mm² after 6 min.

The BJAB cell line showed a similar difference in the accumulation of rolling
cells for the monomeric BJAB-C320A compared to the dimeric BJAB-WT (Fig. 3.4C
and F). There were 120 rolling BJAB-WT/mm² compared to 56 rolling BJAB-
C320A/mm² after 6 min at 0.5 dyn/cm². This was also true at 1 dyn/cm², where 22 BJAB-
WT/mm² rolled compared to 6 BJAB-C320A/mm².
Figure 3.4: Accumulation of rolling cells. The dynamic accumulation of rolling cells (mean ± SD) was dependent on dimerization, but not on core-2 activity. Rolling WT transfected cells accumulated at a higher rate on P-selectin than C320A transfected cells at both wall shear stresses of 0.5 dyn/cm² (A, B, C) and 1 dyn/cm² (D, E, F). K562 transfectants (WT—black diamond and C320A—white diamond) are shown in panels A and D. K562C2 transfectants (WT—black square and C320A—white square) are shown in panels B and E. BJAB transfectants (WT—black triangle and C320A—white triangle) are shown in panels C and F. *WT significantly different (p<0.05) from C320A at each min from 1-6 min. The slopes of the data from 1-6 min are also significantly different (p<0.05) between WT and C320A.
The difference between the number of WT cells per area and the number of C320A cells per area was statistically significant (t-test, P<0.05) at each time point from 1 min to 6 min for all of the transfectants. The slopes of the accumulation of cells from 1 min to 6 min were also significantly different (t-test, P<0.05) between the WT and C320A cells for all cell lines. The t-test statistic was generated from the difference in slopes and the standard error of slopes. These data suggest that dimerization increased the probability of capture from free flow. Dimerization appeared to dominate cell capture from flow and conversion to rolling over the difference in glycosylation from core-2 activity.

The delivery of cells to the interactive substrate has been reported in several ways. The number of rolling cells after some elapsed time (5 min) or the number of tethering or rolling cells per area per time has been reported. The number of transient events (tethers) per cell per time has also been reported. The measure of transient events per cell is an indication of the frequency of formation of bonds (similar and related to the distance between tethers, which I have also measured) once the cell is near the substrate and is not an indication of the initial tethering event that converts the cell to a rolling cell. Here, in the accumulation of rolling cells, I have measured the number of rolling cells per area per time. The number of rolling cells is an indication of the cells that are sufficiently near the substrate and have converted from cells in the free flow to cells that are consistently forming bonds with the substrate through the formation of an initial tether. Only the cells near the substrate and in the focal plane of the microscope have the opportunity to form
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Figure 3.5: Distance between tethering events. The distance between tethering events (mean ± SD) at 100 sites/μm² and 0.5 dyn/cm² was dependent on core-2 activity as the K562-C320A cells traveled much farther than the rest of the cells. For the transfectants with sufficient core-2 activity (BJAB and K562C2), the distance traveled between tethers was similar for both the WT (black square) and the C320A (white square). *K562-C320A mean distance is significantly different (p<0.05) from all other cell line distances.
difference in the distance traveled between tethering events was observed with the 300.19-WT and 300.19-C2-WT cell lines, again suggesting that core-2 glycosylation contributed to more stable, slower rolling. The tethering frequency and possibly the PSGL-1—P-selectin association rate constant (k_{on}) were much lower for the K562-C320A and 300.19-WT.

No difference in the distance between tethering events existed in the monomeric or dimeric BJAB cells or K562C2 cells. These cell lines traveled 1-2 μm between tethers regardless of dimerization. Even at a 10-fold lower site density of 10 sites/μm^2, no significant difference could be observed in the distance traveled between tethers for these cell lines. Both monomeric and dimeric cells traveled approximately 1 ± 1 μm (mean ± SD) between adhesive events. Therefore, dimerization did not decrease the distance between tethers by increasing the tethering efficiency. Tethering efficiency remained the same. The cellular expression of PSGL-1 chains was sufficiently high in both the monomeric and dimeric cells to drive binding to the substrate even at the low site density examined. Experiments at substantially lower site density might be necessary to observe a difference between the monomer and dimer. But the core-2 glycosylation did affect tethering because the K562-C320A and 300.19-WT, which carried only the low molecular weight species of PSGL-1, traveled the longest distance between the formation of bonds.
3.6 Dissociation Rate Constants

The dissociation rate constants ($k_{\text{off}}$) are calculated from the slope of the distribution of pauses as in Fig. 3.6. The distributions plotted in this figure were K562-WT and K562-C320A bond events with PR1 IgG at 0.5 dyn/cm$^2$. The K562-C320A bond events had a $k_{\text{off}}$ of 26 s$^{-1}$ compared to 12 s$^{-1}$ for K562-WT bond events. The pauses for K562-C320A lasted a much shorter time, which gave a higher $k_{\text{off}}$, compared to K562-WT, a difference initially attributed to the mutation of PSGL-1 into a monomer.

Dissociation rate constant ($k_{\text{off}}$) values plotted over a range of forces gives the relationship between the chemical dissociation and the applied mechanical force on the bond (Fig. 3.7). The $k_{\text{off}}$ values for the BJAB cells and the K562C2 were similar to previously published values (2-10 s$^{-1}$) for neutrophil PSGL-1 bonds with P-selectin over a similar range of forces (Alon et al., 1995; Ramachandran et al., 1999; Smith et al., 1999). The BJAB and K562C2 had similar $k_{\text{off}}$ values over the range of force measured regardless of dimerization, giving rise to the similar bond compliances. The $k_{\text{off}}$ values for the K562C2-WT and K562C2-C320A were 12 and 11 s$^{-1}$ at a wall shear stress of 0.9 dyn/cm$^2$ or an estimated force of 500 pN per tether or bond cluster. The $k_{\text{off}}$ values for the BJAB-WT and BJAB-C320A were 11 and 8 s$^{-1}$ at the same force. As force was increased, the $k_{\text{off}}$ increased more rapidly for K562-C320A than K562-WT bonds with PR1 IgG. The $k_{\text{off}}$ at the higher force measured was increased 3.5 times for K562-C320A. The dissociation rate constant for K562-C320A at 0.9 dyn/cm$^2$ was 43 s$^{-1}$, while the dissociation rate constant for K562-WT was 12 s$^{-1}$. The 300.19-WT also had a higher $k_{\text{off}}$
Figure 3.6: Dissociation rate constants. The dissociation rate constant, \( k_{off} \), is the slope of a cumulative distribution of pause times. The difference between the K562-WT (black square, solid line) and the K562-C320A (white circle, dotted line) kinetics is plotted for 0.5 dyn/cm\(^2\). The tether lifetimes follow a first order kinetics distribution.
Figure 3.7: Bond mechanics or compliance. The $k_{off}$ over a range of forces on the tether displays the strength of bonds. Each data point on this graph is a slope of a distribution (as in the previous figure). Proper glycosylation from core-2 activity strengthens bonding decreasing the compliance. The K562-C320A (white circle, solid line) has $k_{off}$ values that increase greatly with force indicating weak bonds, with $k_{off} = 8.2$ s$^{-1}$ and $\sigma = 0.14$ Å. All other cell lines have sufficient core-2 activity and proper glycosylation to strengthen bonds with P-selectin. The BJAB-WT (black triangle, dotted line) curve, with $k_{off} = 6.1$ s$^{-1}$ and $\sigma = 0.05$ Å is plotted for comparison. The $k_{off}$ values for the 300.19 cells were calculated at 0.9 dyn/cm$^2$, but this corresponded to ~300 pN of force because of the smaller diameter of the 300.19 cells compared to K562 cells.
of 21 s\(^{-1}\) than the 300.19-C2-WT, with a \(k_{\text{off}}\) of 10 s\(^{-1}\), at this level of wall shear stress, which corresponded to approximately 300 pN of force due to the smaller diameter of 300.19 cells.

The low site density of P-selectin, required to form tethers with all the wild-type PSGL-1 transfectants, resulted in very few bonds or tethers with K562-C320A because the bond formation frequency was correspondingly low. The adhesive events appeared to be dominated or limited by the K562-C320A on the transfectant rather than the selectin on the substrate. In order to increase the tethering frequency of K562-C320A to have more tether lifetimes to measure, I used higher site densities of P-selectin. The increased site density and resulting increase in tethering frequency allowed measurement of tether lifetimes over a wider range of forces because the probability of forming bonds was increased. This experimental modification was crucial in order to measure enough adhesive events (at the higher forces) to give a distribution, which would have been difficult at lower site densities. But a higher site density could potentially increase the probability of higher numbers of bonds in the tether as well. However, the dissociation rate constants did increase greatly with force on the tether, even with a higher site density.

The measurements of \(k_{\text{off}}\) and bond compliance were not greatly affected by dimerization of PSGL-1 evident in the BJAB and K562C2 measurements. The \(k_{\text{off}}^0\) and \(\sigma\) values for the BJAB-WT interactions, which were representative of all of the cells except the K562-C320A, were 6.1 s\(^{-1}\) and 0.05 Å, respectively. This implied that the
tethers and bonds formed in these experiments were the result of single chains of PSGL-1 forming bonds with single P-selectin molecules even when both PSGL-1 chains were present. Another interpretation is that the number of bonds made on the tethers of monomeric PSGL-1 cells were similar to the number of bonds made on the tethers of dimeric PSGL-1 cells, resulting in similar tether dissociation. So, dimerization of PSGL-1 chains did not lower $k_{off}$ values or decrease the bond compliance, which would be expected if the dimeric PSGL-1 were forming more bonds and consequently distributing the force more (Evans, 2001; Tees et al., 2001).

However, the glycosylation defect in the K562-C320A and the 300.19-WT cells did impact the $k_{off}$ values and bond compliance. These cells were inefficient at forming new bonds once rolling was initiated and the bonds that they did form had a significantly higher $k_{off}$ at higher forces indicating a higher compliance or weaker bonds. The $k_{off}$ and $\sigma$ values for the K562-C320A interactions were 8.2 s$^{-1}$ and 0.14 Å, respectively. Differences in bond compliance have been observed previously, when glycosylation of CD34 was altered by periodate treatment (Puri et al., 1998). The periodate treatment altered the chemical ring structure in the sLex moiety and increased the stability of bonds through increased pause times and lower dissociation rate constants, which decreased the bond compliance. In this study, there was likely a considerable glycosylation defect, which destabilized the bonds that did form between K562-C320A or 300.19-WT PSGL-1 and P-selectin.
3.7 Monte Carlo Simulation

3.7.1 Experimental Bead Rolling Velocity

Experimental rolling velocities were measured for PSGL-1 coated beads interacting with a PRIgG substrate. The rolling velocity for PSGL-1 coated beads followed an exponential curve for the range of wall shear stresses tested (Fig. 3.8). The increase in rolling velocity corresponded to an exponential increase in dissociation rate for increasing force in the Bell model. The average rolling velocity remained low, only 2 \( \mu m/s \) or less, up to 0.6 dyn/cm\(^2\). The average rolling velocity ranged from 0.3 \( \mu m/s \) at 0.15 dyn/cm\(^2\) to 13 \( \mu m/s \) at 0.9 dyn/cm\(^2\). The standard deviation of rolling velocity also increased with increasing flow rate or wall shear stress. For comparison, neutrophils rolling on the same surface at 1 dyn/cm\(^2\) have a velocity of 6 \( \mu m/s \). The simulation of bead rolling was then compared to these values of experimentally observed bead rolling velocity.

3.7.2 Hydrodynamic Velocity of Bead Without Molecular Interactions

The simulation of bead rolling included the approximation by Goldman et al. (1967) for the hydrodynamic motion of a sphere in a shear field. The distance traveled between bonding events depended on the distance of the bead from the wall and the hydrodynamics. The theoretical velocity of a sphere near a wall in a shear field (without molecular interactions) is shown in Figure 3.9 for varying wall shear stress and varying separation distance. Separation distance is the distance between surface of the bead and the lower wall of the flow chamber. The lubrication-theory-like approximation was used
Figure 3.8: Experimental bead rolling velocity. The bead rolling velocity (mean ± SD) increased with increasing wall shear stress. The standard deviation also increased with increasing wall shear stress. The site density of PR1gG was 170 sites/μm².
Wall Shear Stress (dyn/cm²)

Figure 3.9: Theoretical hydrodynamic velocity of bead. The theoretical hydrodynamic velocity of a bead (without molecular interactions) increases with increasing wall shear stress for a given separation distance (Goldman et al., 1967). The velocity also increases with increasing separation distance for a given wall shear stress. The lubrication-theory-like approximation was used for separation distances of 15 nm or less, while the method-of-reflections approximation was used for separation distances greater than 15 nm.
for separation distances of 15 nm or less. The wall effect had a large influence on the free flow velocity of the sphere and small increases in the separation distance produced large increases in velocity. A three-fold increase in separation distance from 5 to 15 nm produced a 15% increase in velocity from 455 to 520 μm/s at 2.5 dyn/cm². For separation distances greater than 15 nm, the sphere velocity is approximated by the method-of-reflections as wall effects were less and the velocity was characterized more by bulk flow. Once the separation distance was increased beyond 15 nm, the velocity was affected less by separation distance. Increasing separation distance five-fold from 20 to 100 nm only produced a 4% increase in velocity from 810 to 840 μm/s at 2.5 dyn/cm².

The Goldman approximation was then tested experimentally. Beads blocked for non-specific interactions were perfused over a plate also blocked for non-specific interactions. The beads were allowed to settle and then a shear field was applied at the indicated flow rate (Fig. 3.10). The Goldman approximation for the hydrodynamic or critical velocity of a bead at a 20 nm separation distance is shown as a line. The data points are experimentally measured hydrodynamic velocities for 15-20 beads at each flow rate. The experimental velocities match the approximation very closely up to 0.4 ml/min, but then increase over the theoretical predictions above 0.4 ml/min. The difference between the experimental velocity and the theoretical velocity increased with increasing flow rate. The syringe pump precision increased at higher flow rates and longer time points for the range tested, suggesting that the deviation from theoretical velocities was real.
Figure 3.10: Comparison of experimental and theoretical hydrodynamic velocity of bead. The experimental bead velocity (mean ± SD) was measured over a range of wall shear stress (black diamond). The theoretical or model bead velocity (black line) is also plotted for this range of wall shear stress at a constant separation distance of 20 nm. The experimental velocities match the model velocities below 1 dyn/cm², but begin to increase over the model velocities above 1 dyn/cm², suggesting a hydrodynamic lift.
The bead velocity data suggest that the separation distance increased with increasing flow rate. Increases in separation distance or hydrodynamic lift between colloidal particles and a wall has been experimentally observed before for both Poiselle flow in pipes and parallel plate chambers at low Reynolds number (Leal, 1980; Tempelman et al., 1994). Numerical simulations of the lift of a single particle in a Newtonian fluid have been performed recently (Patankar et al., 2001). While the simulations used length scales on the order of centimeters, lift-off was a function of the Reynolds number based on the sedimentation velocity scale and would apply to the micron length scale used in my flow chamber system. The simulation showed that the critical shear Reynolds number for lift-off to occur was approximately 0.5, which corresponded to a wall shear rate of 50 s$^{-1}$ or 0.5 dyn/cm$^2$ wall shear stress for values of my system. The equilibrium height to which the simulated sphere traveled increased with increasing flow rate and shear Reynolds number as well. An increased equilibrium distance from the wall with increased flow rate might be expected for the microbeads in my system also. The deviation between experimentally measured velocities and the theoretical predictions becomes important in the comparison of experimentally measured bead rolling velocity and the simulation, with respect to separation distance. Simulated bead rolling velocities increasingly lagged experimentally measured rolling velocities with increasing flow rate. An increasing separation distance with increasing flow rate could explain higher rolling velocity as the bead spends a greater time on average in higher streamlines with higher velocities (between bonding events) and with lower
probability of bond formation. Separation distance was the only input variable not known from experimental data; therefore, separation distance was varied to find the best fit with the experimental measurements.

3.7.3 Single Bond, $k_{off}$ Only

In this case, a bond was assumed to exist at the beginning of each time step (no $k_{on}$ and probability of forming bond was 1) and the bond was then tested for dissociation at the end of each time step ($k_{off}$ only). This assumption resulted in very low rolling velocities with simulated beads barely moving in comparison to the experimental data. The bead rolling velocity did not get above 0.5 μm/s, even for the highest flow rate of 0.6 ml/min and highest separation distance of 80 nm tested, because the bead remained bound most of the time. The data is not shown for this reason. But this case did prove that, during bead rolling, the bead is not continuously forming bonds at each molecular opportunity.

A $k_{on}$ and probability of bond formation was required to better model the lack of molecular interaction occurring during the translation at hydrodynamic velocity between bonds. Although this “single bond, $k_{off}$ only” model did not result in rolling velocities similar to those obtained for the experimental conditions used here, it may be useful for simulating the condition of very high site density in which the formation of many sequential bonds would be expected. The algorithms for each case of single versus multiple bond and $k_{on}$ and $k_{off}$ are shown with commenting in the appendix. The
algorithms are very similar with minor adjustments made to accommodate both an association rate and dissociation rate as well as the number of bonds.

3.7.4 Single Bond, $k_{on}$ and $k_{off}$

The next case tested was also a single bond model, but an association rate constant, $k_{on}$, was included. In this case, the probability of bond formation was compared to a random number to test for a successful bonding event. Then, if a bond did form, the bond was tested for breakage at the end of each time step until it dissociated and the process for testing for bond formation started again. If a bond did not exist, the bead then traveled at the velocity approximated by Goldman, termed hydrodynamic or critical velocity, until the next bond was formed. The effect of separation distance on $k_{on}$ is described in the Chapter 2 and the appendix.

The dissociation rate constant obtained from simulated pauses during rolling was checked against the input dissociation rate constant (Fig. 3.11). The $k_{off}$ calculated from the simulation was always very similar to the input $k_{off}$. At 0.9 dyn/cm² wall shear stress, the input $k_{off}$ was 6.5 s⁻¹, calculated from the force on the bonds and the Bell model, and the resulting $k_{off}$ from the simulated pauses was 7.7 s⁻¹.

The number of simulated rolling beads was also increased (200) until a Gaussian-like distribution of rolling velocities, similar to what has been observed with cells (Jung et al., 1996), was reached (Fig. 3.12). This required two hundred simulated rolling beads, which was used to obtain the average rolling velocities for all simulated bead rolling.
Figure 3.11: Dissociation rate constant from simulation of bead rolling. The calculated $k_{\text{off}}$ was 6.5 s$^{-1}$ for one set of specified parameters, which was set as the input in the simulation. The $k_{\text{off}}$ calculated from pause times taken from the output of the rolling simulation case of single bonds for several beads (with a $k_{\text{on}}$) was 7.7 s$^{-1}$ indicating that there is some variance between input and output, but the dissociation of molecular tethers is faithfully recreated in the simulation. The variation of the simulation calculated $k_{\text{off}}$ values for different runs of the simulation varied about the input $k_{\text{off}}$ of 6.5 s$^{-1}$.
Figure 3.12: Histogram of bead rolling velocity from simulation. A Gaussian-like distribution (similar to what is observed in experiments) of rolling velocities is obtained by simulating the rolling of 200 individual beads. The average rolling velocity was 7.4 μm/s with a standard deviation of 3.8 μm/s for beads rolling at a separation distance of 20 nm at 0.9 dyn/cm² for the multi-bonding (2 bond) case with rebonding possible.
The average rolling velocity was 7.4 μm/s with a standard deviation of 3.8 μm/s at 0.9 dyn/cm².

A range of separation distances was tested for each wall shear stress. The simulated bead rolling velocity increased with increasing separation distance (single bond case, blue diamond, Fig. 3.13). The rolling velocity also increased with increasing wall shear stress for a given separation distance. There was also a discontinuity at 15 nm because of the change in equations depending on the lubrication-theory-like or method-of-reflections approximation. The simulated rolling velocities never correlated with the experimental rolling velocities for any of the separation distances at any of the wall shear stresses tested. The single bond or tether simulation always rolled faster than the experimental velocity; suggesting that, for the 170 sites/μm² site density used in the simulation and the experiment, more bonds or tethers were required to slow down the rolling bead. So multiple bonds or tethers were added to the simulation to create the two-bond case described next.

3.7.5 Multiple Bonds, k_{on} and k_{off}

The multiple bond case was created because the single bond case could not adequately describe the rolling velocity measured from experiments. But the multiple bond case may also reflect the molecular distribution on the beads and the substrate more accurately because both PSGL-1 and P-selectin have been shown to be dimeric molecules (Barkalow et al., 2000; Moore et al., 1995). The distribution of PSGL-1 chains on the
Figure 3.13: Simulation of bead rolling. Three cases of the simulation are plotted. The single bond case (blue diamond) always resulted in rolling velocities much higher than the experimental velocity (green line) at every separation distance. The multi-bonding (2 bond) case with no re-bonding (multi-no-re, black square) resulted in some rolling velocities that matched the experimental velocities, but this occurred at very small separation distances (<5 nm). The multi-bonding (2 bond) case with re-bonding (multi-re, red triangle) also resulted in rolling velocities that matched the experimental velocities. These rolling velocity values matched at separation distances of ~20 nm or higher.
bead is likely in dimeric form because the dimers are cross-linked by the disulfide bond. The P-selectin dimer is a non-covalent association and may exist as the dimer or a multimer rosette due to hydrophobic interactions in the transmembrane domain and other parts of the molecule in the substrate of the flow chamber. P-selectin recombinant IgG may also act as a dimer because the P-selectin domains are displayed on both F\textsubscript{ab} arms of the molecule. The multiple bond case, which allowed up to two bonds to form at each bonding opportunity (but sometimes one or none formed), was split into two sub-cases that were similar to the single bond case in most other respects. The two sub-cases were re-bonding (multi-re) and no re-bonding (multi-no-re). The possibility of forming two bonds at each bond opportunity had the additional opportunity in the re-bonding sub-case for a bond that broke to reform so long as the other bond still held. In order to keep the two molecules in close proximity. This additional opportunity was removed in the no re-bonding sub-case.

Another method for lowering the time interval (dt) in the simulation, to test its validity, was to increase the site density, since the time step in the model depended on molecular spacing of the substrate. By doubling the site density to 340 sites/\mu m^2, the time step in the simulation was shortened to $3 \times 10^{-4}$ s from $6 \times 10^{-4}$ s and the rolling velocity decreased to 1.6 \mu m/s from 4.6 \mu m/s for 0.9 dyn/cm\textsuperscript{2} and 20 nm separation distance, indicating that the time step did not artificially result in rolling velocities matching the experiment. While I did not experimentally measure rolling velocity at this site density, I would expect beads to roll slower at higher site densities and will do this
experiment in the future. This is a second and independent verification that the time step was sufficiently short in the model.

Both the multi-re and multi-no-re cases resulted in rolling velocities that increased with increasing wall shear stress as well as separation distance similar to the single bond case (multi-re, red triangle, multi-no-re, black square, Fig. 3.13). But the rolling velocities were consistently decreased compared to the single bond case as expected. The multi-re case resulted in the lowest rolling velocities because the opportunity for longer-lived bonding events or pauses existed because of the re-bonding possibility. The multi-no-re case resulted in intermediate rolling velocity values between the single bond case and the multi-re case.

The experimental rolling velocity at each wall shear stress is shown as a green line (because the separation distance was unknown). The multi-no-re case rolling velocity matched the experimental rolling velocity at separation distances of 1 or 2 nm for most wall shear stresses. This separation distance would be hard to reach physically because both P-selectin and PSGL-1 have been estimated to be extended 40-60 nm above the cellular membrane by rotary shadowing (Li et al., 1996; Ushiyama et al., 1993). Even if these molecules are bent over and pressed flat on each surface, a 2 nm separation distance between the substrate surface and the bead surface would be nearly impossible.

In contrast, the multi-re case matched experimental rolling velocity at separation distances ~20 nm and higher. The match between simulation and experiment occurred at higher separation distances for higher wall shear stresses. The rolling velocities obtained
with the multi-re simulation matched the experimental rolling velocity at separation distances of approximately 20, 20, 20, 25, 40, and 60 nm for wall shear stresses of 0.15, 0.3, 0.45, 0.6, 0.75, and 0.9 dyn/cm², respectively. The correlation at increased separation distances suggested that the average separation distance between bond events or pauses is increased for increasing wall shear stress experimentally. Higher separation distances during rolling could be related to the increased hydrodynamic velocity over theoretical predictions for bead motion in a shear field described earlier. But during rolling, the molecular bonds keep the bead sufficiently close to the wall to maintain rolling even though the bead spends the time farther from the wall on average between bond events.

The multi-re simulation, set at a 20 nm separation distance, of rolling velocity agreed well with the experimentally measured rolling velocity below 0.6 dyn/cm² (Fig. 3.14). But the experimentally measured rolling velocity was higher than the simulated velocity above 0.6 dyn/cm², indicating that the separation distance might be increased. The experimental values for rolling velocity higher than 0.6 dyn/cm², were matched by the simulation if the separation was increased to 45 and 60 nm. But the standard deviation of rolling velocity tended to be less in the simulation than the experiment at higher wall shear stresses. This may be due to the lack of dynamic changes in separation distance during the simulation of rolling because the separation distance was held constant. In other words, the bead may spend more time at higher separation distances than what was simulated due to the lift force between bonds during the experiment.
Figure 3.14: Comparison of experimental and simulated bead rolling velocity. The simulation case of multi-bonding with rebonding (white circle) at a separation distance of 20 nm matched experimental bead rolling velocity (black diamond) below 0.6 dyn/cm², but the experimental velocities were greater above 0.6 dyn/cm², again suggesting that separation distance is increasing with increasing wall shear stress due to a hydrodynamic lift force.
bead will begin to experience the lift force as soon as the bond breaks and the bead is freely flowing again.
Chapter 4: Discussion

4.1 Dimerization

Previous reports have been contradictory regarding the necessity of dimerization of PSGL-1 chains to support adhesion to P-selectin. A static assay (Pouyani and Seed, 1995) and a shear detachment and rolling interaction assay (Epperson et al., 2000) showed no decrease in binding of mutant, monomeric PSGL-1 transfected cells to P-selectin. But another rolling interaction assay (Snapp et al., 1998a) showed severely decreased binding by the mutant, monomeric PSGL-1 transfected cells to P-selectin.

In the static assay, transmembrane and cytoplasmic domains of PSGL-1 were replaced with CD43 antigen and expressed in COS cells along with FT7. The resulting molecule had no cysteine residues and could not form disulfide bonds. The PSGL-1 on these transfected cells did bind adsorbed PRIgG. But COS cells do not appear to express core-2 enzyme activity however (Skrincosky et al., 1997), so the mutant PSGL-1 may not have had the proper post-translational glycosylation and binding of PSGL-1 from these cells to P-selectin may not be comparable to PSGL-1 from other cells.

The Epperson et al. (2000) study of the effect of PSGL-1 dimerization used CHO cells transfected with core-2, FT3, and monomeric or dimeric PSGL-1. The mutation in the monomeric PSGL-1 consisted of a serine replacing the cysteine, again eliminating the disulfide bond. Both monomeric and dimeric transfectants did bind (at 0.5 dyn/cm²) and detach (over a range of wall shear stress) from a P-selectin substrate in similar quantities.
Dimerization has been studied in another adhesion molecule. An L-selectin dimer was previously created in 300.19 cells (Li et al., 1998). The L-selectin dimer was cross-linked either by mAb or coumermycin. The coumermycin induced cross-linking by binding two GyrB subunits that had been fused to the L-selectin cytoplasmic domains. L-selectin dimerization resulted in 3-7 times as many cells rolling and a 35% lower median rolling velocity compared to monomeric L-selectin transfecteds on a HUVEC monolayer at 1.85 dyn/cm².

Similar to the L-selectin dimer, I also observed an increase of 3-5 fold in the number of rolling, dimeric PSGL-1 transfecteds over the number of rolling, monomeric PSGL-1 transfecteds on P-selectin. My results for the accumulation of rolling cells and the efficiency of the initial tether agree with those previously seen in L-selectin dimeric transfecteds. However, I did not observe a difference in rolling velocity between the monomeric and dimeric transfecteds on P-selectin. I would not expect to observe a difference in rolling velocity because the tether lifetime for the PSGL-1—P-selectin bond is probably much longer than the L-selectin—HUVEC ligand bond (as much as an order of magnitude) at higher wall shear stresses or forces (Smith et al., 1999). There is more time for a new bond to form in the contact area before the trailing edge bonds break and the cell ratchets forward. A difference in rolling velocity may yet be observed with PSGL-1—P-selectin at much lower site densities when the formation of new bonds becomes less frequent. The difference in rolling velocity may only be observable in the
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Discussion

case of (initially) fast dissociation kinetics where the probability for a new bond to form must be increased a great deal in order to obtain the effect of slower rolling velocity.

Non-covalent dimerization of P-selectin in platelets and HUVECs has been shown to occur using a chemical cross-linking approach during the lysis procedure before SDS-PAGE and Western blotting (Barkalow et al., 2000), although the physiological relevance has yet to be elucidated. But as seen in the Results section, the covalent dimerization of PSGL-1 greatly increased the likelihood of cells interacting with a substrate of P-selectin. Therefore, the dimerization of P-selectin might also significantly increase the probability of initial capture of leukocytes and continued interaction in rolling. The volume swept or sampled by a dimer might be greater than that for a monomeric molecule making the microenvironment of molecules crucial to consider. The dimerization of both PSGL-1 and P-selectin and potentially forming two independent bonds greatly increases the probability of successful bond formation in the local microenvironment when these molecules become close to each other.

I measured tether lifetimes for PSGL-1—P-selectin bonds with a low site density of P-selectin on the substrate to ensure measurement of a fundamental component of tether adhesion. The results indicate that there is no difference in dissociation rate or bond compliance whether there is monomeric or dimeric PSGL-1 on the cell. Low numbers of bonds appear to form due to the low site density of the substrate. If there were several bonds forming due to a non-uniform distribution of P-selectin molecules on
the substrate (oligomers), then I would expect the dissociation rate and the compliance of
dimeric PSGL-1 tethers to be lower than monomeric PSGL-1 tethers.

The influence of multiple bonds on compliance or separation distance and
dissociation rate at zero force was simulated using a Monte Carlo process (Tees et al.,
2001). The bond compliance and dissociation rate at zero force only decreased ~10% for
the case of 35% multiple bonds. These results suggest that dissociation rates and
compliance should be less for multiple bonds versus single bonds, but this difference may
be very small. However, I calculated a similar compliance for tethers with monomeric
PSGL-1 as tethers with dimeric PSGL-1, suggesting that in both cases I am measuring a
fundamental unit of dissociation between a tether and the substrate that probably involves
very few bonds, if not one. In other words, the dissociation kinetics suggest that in both
cases of monomeric and dimeric PSGL-1, I am measuring similar numbers of single
chain PSGL-1 bonds with single P-selectin molecules.

4.2 Comparison with a Recent Publication

A very recent publication compared the kinetics of monomeric and dimeric
PSGL-1 bonds with soluble (monomeric, implied) and membrane-derived (multimeric,
implied) P-selectin (Ramachandran et al., 2001). The soluble form of the molecule has
no transmembrane and cytoplasmic domain and is assumed to associate less in solution
because of fewer hydrophobic interactions. There was no difference in the number of
rolling cells (CHO and K562), transfected with either monomeric or dimeric PSGL-1, on
membrane-derived P-selectin, after 4 min of perfusion over a range of wall shear stresses,
an observation consistent with their data indicating that PSGL-1 spontaneously forms dimmers. But fewer cells with monomeric PSGL-1 than dimeric PSGL-1 remained bound in a shear detachment assay. The accumulation of rolling CHO transfectants with dimeric PSGL-1 was greater than transfectants with monomeric PSGL-1, agreeing with my data. No differences in rolling velocity between the monomeric and dimeric PSGL-1 transfectants were observed, which also agreed with my data. The kinetics of tethers of monomeric and dimeric PSGL-1 transfectants with soluble P-selectin for a range of forces were similar and this was consistent with my data again (except for an increased bond compliance overall, which could be due to differences in cellular deformability).

The kinetic data from Ramachandran et al. (2001) was difficult to compare with my data as there were no site densities given. The kinetic behavior of the cells on membrane-derived P-selectin was not reconstituted with double the site density of soluble P-selectin to show the equivalent nature of the two different substrates. Soluble P-selectin may adsorb in such a way, which may include a shorter molecular cross-bridge (bond) length, as to limit the formation of a strong bond with PSGL-1 (similar to the membrane-derived P-selectin). The use of standard video frame rate (30 frames/s, 0.033 s between frames) to calculate the kinetics of tethers at higher forces resulting in dissociation rate constants as high as 60 s⁻¹ is also a concern. Taking 60 s⁻¹ to be true, tethers are then breaking at 0.017 s; much faster than they are physically able to measure because of their time resolution. Therefore, there might be an aliasing effect by
neglecting to measure a population of short tethering events as well as measuring longer events inaccurately.

To compare between labs or between cell lines, three options exist. The kinetic measurements from cellular-tethering could first be compared to bead tethering kinetics in order to subtract out cellular contributions, such as microvillus deformation, to the strength of tethers. Differences in molecular strength will then be apparent. Another option when comparing cells is to compare the kinetic data at the same level of shear force on the cell. Resolving the force on the cell onto the tether adds the complication of estimating the angle between the tether and the surface (see Chapter 2, Fig. 2.4). The geometry involving the tether length, angle, and lever arm must be known to compare across cell lines and across labs. For example, Ramachandran et al. (2001) decided on a seemingly arbitrary angle of 50° between the tether and the surface. If that angle is 67°, similar to what I calculated, the force on the tether becomes larger and the bond compliance will decrease, dramatically altering their conclusions. Therefore, the bond compliance is sensitive to the geometry of the tethering cell and should be normalized for comparison. Finally and ideally, a bead system with adsorbed molecules would work best when measuring the strength of molecular tethers because the cellular component is taken away and only molecular interactions remain.

4.3 Bond Numbers on Microvillus Tethers

Tether lifetimes are measured by using low site densities so that the distance between tethers is large and the number of bonds formed between tether and substrate is
small and hopefully one. The contact area is also very small depending on the fluid forces and might be a single microvillus. Electron micrographs of neutrophils fixed while rolling also show that a single microvillus can be bearing part if not much of the load as cells ratchet in vitro (Park et al., 2001). These micrographs were taken with even higher site densities than those used in this dissertation. The transfected cells have microvilli with lengths of ~0.6 μm. The diameter of the tip of the microvilli is approximately 1/5-1/10 that length. If the microvillus is a cylinder with a radius of 0.06 μm, it has a flat area on the tip of 0.011 μm². An estimation of L-selectin molecules on the tip of microvilli is 10 (Bruehl et al., 1996) and PSGL-1 may have a similar distribution with fewer molecules per tip on average (100,000 L-selectin per cell versus 30,000 PSGL-1 per cell). But the tip is rounded and depending on the geometry of the microvillus as it contacts the substrate and is drawn out, molecules along the cylinder, near the tip might also be able to form bonds. Therefore, if I assume that half the microvillus is deformed as it is forced into the wall (Zhao et al., 2001), the area covered is 0.124 μm². For this area of coverage and even with multiple molecules on the microvillus, the number of P-selectin molecules on the substrate is estimated to be 1 for the for the low site density of 10 sites/μm² used in tethering experiments. Microvillus tethers that form bonds with the substrate in these experiments are likely to form very few bonds, especially at lower forces when the force and torque pushing the cell into the wall is smaller.
4.4 PSGL-1 Glycosylation

The glycosylation of PSGL-1 plays an important role in the functional binding of P-selectin during rolling. In experiments using PSGL-1 variants attached to microbeads, the beads with a PSGL-1 peptide that included a carbohydrate branch displaying a sLex group, without any possibility of tyrosine-sulfation, rolled slower and at higher wall shear stress on P-selectin than beads with PSGL-1 that had only the tyrosine-sulfation and no carbohydrate (Rodgers et al., 2001). I have observed similar results with other K562 transfectants. One transfectant had PSGL-1, but no FT7 (FT7-, deficient carbohydrate in binding pocket), another had PSGL-1 in which the threonine was mutated to an alanine (T16A, no carbohydrate in binding pocket), and the third had no tyrosine-sulfation (FFF). The T16A mutant did not interact with a P-selectin substrate at all suggesting that there was some carbohydrate structure at this site in the K562-C320A and 300.19-WT because I observed some adhesive interactions with these cells. The FT7- transfectant did roll on P-selectin, but at much reduced numbers and a high rolling velocity of 50 μm/s. The FFF mutant also rolled on P-selectin in high numbers at a rolling velocity of 10 μm/s. The carbohydrate group on PSGL-1 appears to be more important than the tyrosine-sulfation for rolling on P-selectin (Rodgers et al., 2001).

I used the same cells used in the earlier study by Snapp et al. (Snapp et al., 1998a) that were transfected with a mutant, monomeric PSGL-1 in which the cysteine was replaced by an alanine. Either the wild-type (WT) or mutant (C320A) PSGL-1 was transfected into K562 cells with FT7. I observed a major deficit in the K562-C320A
binding and rolling mediated by P-selectin. The Snapp et al. study used a monolayer of P-selectin expressing CHO cells, while in this dissertation P-selectin was adsorbed to the lower wall of the flow chamber. The original K562 cell line had low amounts of core-2 activity.

In this dissertation, other cell lines were transfected with the same cDNAs. These other cell lines were BJAB and K562C2 and both had high levels of core-2 activity. The decreased rolling velocity of K562-C320A was eliminated upon the additional transfection of core-2; although, a decreased accumulation of rolling cells over time remained. There was also no difference in tether lifetime between the monomeric and dimeric transfectants when core-2 activity was high (both BJAB and K562C2). This suggests that dimerization is not required for binding of PSGL-1 to P-selectin during rolling. However, the probability of forming an initial tether may be increased when two chains are in close proximity at the microenvironment level as they are in the case of wild-type PSGL-1. The proper or fully elongated glycosylation appears to be important in strengthening the interaction by increasing the tether lifetime for a given force.

Wild-type PSGL-1 was also transfected into 300.19 cells with FT7 and with and without the additional transfection of core-2. The core-2 again restored similar function to the cells by decreasing rolling velocity, decreasing the distance between tethers, and increasing tether lifetimes. The cells without core-2 had similar function to the K562-C320A cells, with high rolling velocity and decreased tether lifetimes.
The monomeric PSGL-1 in the K562 transfectant and the dimeric PSGL-1 in the 300.19 transfectant had a lower molecular weight and appeared to be indicative of a lack in core-2 activity (Sperandio et al., 2001). These cells had decreased function but not a complete loss of function. Other studies have shown a complete loss of function in PSGL-1 when the threonine in the active site has been mutated (Ramachandran et al., 1999). Therefore, the K562-C320A and 300.19-WT probably have some form of carbohydrate at threonine 16 that was capped by sLex. The carbohydrate at this position may not have been as elongated or highly branched as that which would be synthesized if core-2 activity were high (Varki et al., 1999). While a glycosulfopopeptide with sLex at the end of a short core-1 based O-glycan did not bind immobilized P-selectin by affinity chromatography (Leppanen et al., 1999), the extension of other core-1 oligosaccharides that include sLex have promoted L-selectin-mediated adhesion (Yeh et al., 2001), implying that the carbohydrate on PSGL-1 may be more important for binding P-selectin and the tyrosine-sulfation may be more important for binding L-selectin. The carbohydrate chain that was anchored at threonine 16 on the K562-C320A and 300.19-WT cells must have been sufficient to form PSGL-1 bonds with P-selectin, but not a strong or stable association because the $k_{off}$ increased rapidly with force. A shorter carbohydrate chain might explain the decrease in tether formation and decrease in tether lifetime that resulted in a higher rolling velocity in the K562-C320A and 300.19-WT cell. Using a chain-link analogy, a longer carbohydrate structure may allow more deformation in and around the binding site and creating a more forgiving bond in terms of stresses.
because other links in the chain would bear some load. With fewer links, the binding site will bear more of the load.

4.5 Cellular Carbohydrate Synthesis

The post-translational modifications of N-linked glycosylation are initiated in the endoplasmic reticulum and continued in the Golgi apparatus, with many carbohydrate transfers building and breaking down the structure until the final glycosylation is accomplished (Helenius and Aebl, 2001; Verbert and Cacan, 1999). The localization and synthesis of O-linked glycosylation is still unknown, but presumably follows a similar pathway, with most if not all of the processing completed in the Golgi apparatus (Piller et al., 1990; Piller et al., 1989; Rottger et al., 1998). Glycosylation enzymes can be cell and tissue-specific as well as cellular compartment-specific. Some glycosyltransferases only glycosylate specific substrates when located in specific sites of the Golgi (Rottger et al., 1998). In addition, glycosyltransferases (core-2, core-3, core-4, sialyltransferases, fucosyltransferases, etc.) that overlap in the Golgi compartments, can compete for the glycoprotein substrate indicating that subcellular localization is critical in determining the final carbohydrate structure. Glycosyltransferase expression may also be dynamically regulated depending on the dynamic state of the cell (Dalziel et al., 2001). Specifically, a decrease in core-2 efficiency was shown when co-localized with a sialyltransferase in the Golgi (Dalziel et al., 2001; Skrincosky et al., 1997). In particular, the addition of a sialic acid will terminate chain elongation and the possibility of a stunted carbohydrate structure is likely, which could have a direct effect on the O-glycan branch in the terminal
region of PSGL-1 and its corresponding ability to form bonds. A lack of core-2 activity in the proper compartment of the Golgi could lead to enhanced activity of other competing glycosyltransferases, which could contribute to a deficient carbohydrate structure that does not add to bond stability in the K562-C320A and 300.19-WT cells. The deficient carbohydrate structures may or may not include sLex capping as other glycoproteins have shown carbohydrate structures terminating in sialic acid or fucose, but not sLex (Easton et al., 2000). High affinity binding to P-selectin in biochemical assays occurs when sLex capping occurs on core-2 based PSGL-1 glycosulfopeptides, but not on an extended core-1 based O-glycan (Leppanen et al., 1999), suggesting that even if the PSGL-1 on the K562-C320A and 300.19-WT cells did have sLex capping, a high affinity interaction may still not occur.

4.6 Simulation

The simulation of bead rolling developed here differs from previous simulations (Chang and Hammer, 2000; Hammer and Apte, 1992; Tozeren and Ley, 1992; Zhao et al., 1995) in the physical model of rolling. The fundamental unit of pauses, either spaced far apart or close together, drive the rolling behavior of beads and cells. The Tozeren and Ley model (Tozeren and Ley, 1992) is similar to the simulation I have developed, except that I have added the biochemical data from the kinetics of pauses at varying forces and an association rate calculated from the solution phase affinity. In my simulation, the only molecular parameters are the association rate and dissociation rate. Any elasticity of the molecules or bond used in the spring model of dissociation is assumed small and lumped.
into the dissociation by the bond compliance. Because the model of rolling developed from molecular pause events, in which a low number of bonds can actually hold the bead or cell without forward motion, high concentrations or site densities of receptors and ligands (>500 sites/μm²) as well as high molecular association rates (>100 s⁻¹), as was necessary in previous work, were not necessary to simulate rolling in this work. Other models of rolling have been developed on the assumption that bonds are always stretching while they exist (Chang and Hammer, 2000), creating rolling behavior that almost never includes zero velocity.

Bead rolling can be simulated using only bond kinetics and mechanics to oppose the downstream motion in a shear field and allowing for varying separation distances leading to hydrodynamics between adhesive molecular interactions. The experimentally determined 3-D association rate (M⁻¹s⁻¹) converted to a per second association rate (s⁻¹) and varied for the volume between the bead surface and substrate was sufficient to maintain the continual formation of bonds as the bead rolled. The per second association rate dropped two orders of magnitude with increasing separation distance the length of the molecular cross-bridge (100 nm) because the molecules on the opposing surfaces are less likely to interact as they are pulled farther apart. The per second association rate constant increased from 7 to 35 s⁻¹ for decreasing separation distance from 80 to 20 nm. The dissociation rate from simulated pauses closely followed the input dissociation rate, when single bond kinetics were applied. The output or apparent dissociation rate dropped substantially upon the application of multiple bond kinetics that mimicked the possible
distribution of P-selectin on the lower wall and PSGL-1 on the bead. The apparent
dissociation rate constant (output) was 1.5 s\(^{-1}\) for bead pauses at 0.9 dyn/cm\(^2\) compared to
the 6.5 s\(^{-1}\) input dissociation rate constant. The reduced dissociation rate constant was
due to the possibility of two bonds forming and the possibility of rebinding.

The association and dissociation of receptor-ligand pairs was kept completely
separate in the case of multiple bonds. This assumption may be incorrect as the
dimerization and structure of the molecules (like elongation and stiffness) may influence
the association and dissociation of both chains. In other words, the binding of the two
chains might be coupled through their structure.

The simulated bead rolling at 170 sites/\(\mu\)m\(^2\) required multiple bonds to match
comparable experimental conditions. This implies that PSGL-1 bead rolling on P-
selectin, derived from single bonds or tethers, breaks down at a site density at or below
170 sites/\(\mu\)m\(^2\). The threshold site density that produces multiple tethers could be
ascertained using the simulation with experimental confirmation.

The simulation could be used to study the apparent lift force and the resulting
increased separation distance by increasing the wall shear stress until bead rolling
becomes unlikely. Eventually the beads will reach a separation distance greater than the
distance required to form molecular cross-bridges. The simulation rolling velocity at a
high separation distance \(\sim 80\) nm will match the experimental rolling velocity at the wall
shear stress just below this limit.

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The experimental rolling velocity matched the simulated rolling velocity for the multi-re case at a separation distance of 20 nm for low wall shear stress. At these low wall shear stresses, the hydrodynamic lift may be sufficiently small and the time for the sedimentation of the beads between bonding events sufficiently long that the beads are at a minimum separation distance. This separation distance may be the minimum physical distance between surfaces due to their protein coatings.

Many additional parameters describing bead and cellular rolling motion are possible to implement. Cellular deformability parameters such as microvillus stretching could be added to expand the model to neutrophil rolling. The hydrodynamic lift force and a sedimentation force could also be added, which would then create the possibility of migration across streamlines or motion normal to the surface. The position with respect to the substrate would then be known through each time step of the simulation. The bead or cell motion could then be described beginning with the hydrodynamic motion to initial binding through the pivot about the tether, with the separation distance varying throughout the motion. The tether then might stretch and release.

4.7 Conclusion

A high level of core-2 activity is crucial for the tether lifetimes, bond compliance, and rolling velocity that is observed with leukocytes interacting with P-selectin through PSGL-1. Although the exact carbohydrate structure in the binding pocket of the core-2 deficient PSGL-1 is not known, I have shown that the resulting carbohydrate structure is insufficient to form strong bonds and long-lived tethers at higher wall shear stresses.
required for the slow rolling observed in cells with core-2 and a higher molecular weight PSGL-1. The proposed insufficient core-2 activity in the original K562-WT cell may have been compensated for by the dimerization of the PSGL-1 providing a higher transport and more substrate for the enzyme to work on. Without the proper core-2 activity and branched carbohydrate structures on PSGL-1 in transfected cells, the adhesive interaction is reduced to tethers with shorter lifetimes, especially at higher forces. Cells with weak to no core-2 activity do not tether as often. This combination of effects results in a high rolling velocity with cells unable to maintain a smooth, continuous rolling interaction. With high expression of core-2, these results are reversed and tether lifetimes increase, rolling velocity decreases, and distance between tethers decreases. But the number of rolling cells over time does not increase back to the higher level of the dimeric transfectants. Dimeric PSGL-1 is responsible for the increased number of interacting cells caught from free flow by the initial adhesive event, but once the cells form interactions and roll on the P-selectin surface, the monomeric transfectants form bonds and tethers just as well as the dimeric transfectants and these tethers have similar strengths of adhesion. In other words, dimerization increased cellular capture, but did not affect rolling velocity.

The simulation of bead rolling was developed with few parameters, obtained from experimental measurements, to keep the model as simple as possible and modular for future expansion. However, the simplicity of the simulation resulted in limitations to bead rolling, which could be overcome by extending the model in the future to include
more parameters to simulate cell rolling. The current simulation of bead rolling is limited
to one dimension along the path of bond opportunities. The number of tethers or bonds is
limited to two and could be expanded to include all of the bonds in the reaction area with
the addition of a second, space dimension for the bead and substrate. Site density is also
an important parameter to vary, which was not done here. Varying the site density will
validate the application of the dissociation rate constants derived from single tethering
experiments to rolling over the correct range of site densities. These additions to the
simulation could then begin to test the number of bonds and number of tethers important
in rolling on a range of site densities. The simulation is also limited to bead rolling
because the velocity and $k_{\text{on}}$ and $k_{\text{off}}$ are developed for a hard sphere. Cellular features
could be incorporated to begin to simulate leukocyte rolling by adding cellular
parameters like shape index, microvillus stretching, and contact area.

The current model of bead rolling, mediated by adsorbed, purified PSGL-1, on P-
selectin is dependent on multiple molecular tethers at the 170 sites/μm$^2$ studied. The
average separation distance between the bead surface and the substrate increases with
increasing wall shear stress possibly because of a hydrodynamic lift force, which works
to lift the bead away from the surface during the time that it spends at hydrodynamic
velocities without molecular interactions. The bead is still able to form adhesive bonds at
the wall shear stresses I tested and simulated because the separation distance did not
increase over the limit of the molecular cross-bridge length (100 nm). But the higher
separation distance due to lift means the bead travels at higher hydrodynamic velocity on
average when not bound. Sedimentation and hydrodynamic lift of the beads could be
worked into the simulation in the future, which would add a dimension to include a
dynamic separation distance. The higher force on the bonds at higher wall shear stresses
along with higher hydrodynamic velocity pushes the bead and higher rolling velocity
results. Bead rolling velocity follows the exponential relationship between dissociation
rate of tethers and force because the tethers are the only force opposing the downstream
forces and motion of the bead.
Appendix

Appendix

Calculation for $k_{\text{on}}$ (s$^{-1}$)

First, convert $k_{\text{on}}^{3D}$ (M$^{-1}$s$^{-1}$) to $\mu$m$^3$/molecules·s. Refer to figure 2.5 for variables and visualization.

$$k_{\text{on}}^{3D} = 4.4 \times 10^6 \frac{1}{M \cdot s} = 4.4 \times 10^6 \frac{L}{\text{mole} \cdot s} \cdot \frac{1000 \text{ml}}{1 \text{L}} \cdot \frac{(10,000 \mu \text{m})^3}{1 \text{cm}^3} = 4.4 \times 10^{21} \frac{\mu \text{m}^3}{\text{mole} \cdot s}$$

$$= 4.4 \times 10^{21} \frac{\mu \text{m}^3}{\text{mole} \cdot s} \cdot \frac{1 \text{mole}}{6.023 \times 10^{23} \text{molecules}} = 0.0073 \frac{\mu \text{m}^3}{\text{molecules} \cdot s}$$

Then divide by the reaction volume and multiply by the number of molecules on the substrate in the reaction volume. Calculated for a 20 nm separation distance and cross-bridge length of 100 nm. The reaction volume is the volume of the cylinder under the bead (up to the molecular cross-bridge length away from the substrate) minus the partial volume occupied by the sphere in that cylinder.

$$\text{reactvol} (\mu \text{m}^3) = bl l^2 \cdot \pi \cdot CL - (\frac{3}{2} \cdot \pi \cdot R^3 - \pi (bl2 \cdot R^2 - \frac{1}{2} \cdot bl2^3))$$

$$= 0.85^3 \cdot \pi \cdot 0.1 - (\frac{3}{2} \cdot \pi \cdot 4.57^3 - \pi (4.5 \cdot 4.57^2 - \frac{1}{2} \cdot 4.5^3)) = 0.14 \mu \text{m}^3$$

The number of sites on the substrate in the reaction volume (cross-sectional area of cylinder).

$$\text{molecules} = \text{sitedensity} \cdot bl l^2 \cdot \pi = 170 \text{sites} / \mu \text{m}^2 \cdot 0.85^2 \cdot \pi$$

$$= 388 \text{molecules}$$

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The per second $k_{on}$ becomes:

$$k_{on}(s^{-1}) = 0.0073 \frac{\mu m^3}{molecules \cdot s} \cdot \frac{1}{0.14 \mu m^3} \cdot 388 \text{molecules} = 20 s^{-1}$$

Code for Simulation

The following is the MATLAB 5 code for the “single, $k_{off}$ only” and the “single, $k_{on}$ and $k_{off}$” case of simulated bead rolling. The code for all cases of the simulation is very similar and commented sections are denoted by green text and “%” designations. Only one bond could form at each bonding opportunity and rebonding could not occur. The “single, $k_{off}$ only” case assumed a bond at each time step and bonding opportunity and therefore the probability of formation of a bond was one. The “single, $k_{on}$ and $k_{off}$” did not assume a bond at each opportunity and a fractional probability was calculated as described in the Materials and Methods section.

```matlab
% Monte Carlo Simulation of bead rolling using association and dissociation kinetics for single bonds. We use GCB for motion of sphere between bonds and the geometry between bead surface and substrate surface to get $k_{on}$ per sec. The force on the bond is calculated from the geometry and the $k_{off}$ is calculated from experimental results. Each time a bonding opportunity exists it is tested for formation and then breakage.

sitedensity=170; %Specify site density of substrate + sites $\mu m^{-2}$
disnextbond=1/sqrt(sitedensity); %distance between bonds set by site density

gcb, geometry parameters:......................
```

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Q=.2;  # Flow rate ml/min to get 3CB
R=4.57;  # Velocity for free flow
r=radius of particle, micrometers

h=R+.08;  # Height of particle center from surface, micrometers
CL=0.100;  # Cross bridge length=110nm for Peal, PSGL
da=(h-R)/R;  # COB ratio of separation distance per radius of bead
u=0.01;  # Viscosity of water(DIC g cm^2 sec^-1)
S=6*(Q/60)/((0.026)^2*(1));  # Shear rate sec^-1, determined for flow
S=6*(Q/60)/((0.026)^2*1.5);  # Shear rate for flow chamber with gasket 1
tau=u*S;  # Wall shear stress dyn cm^-2

# Association kinetics parameters:
theta=asin((R+(h-R))/(R+CL));  # Calculating the angle for the reaction to divide the 3D
beadlength1=R*cos(theta);  # kon to get a per second kon
beadlength2=R*sin(theta);  # theta is angle between bond and substrate, beadlength1 is distance from centerline of bead to bond, beadlength2 is distance from center of bead to beadlength1
reactheight=h-beadlength2;  # Reactheight is the height of the cylinder of reaction potential
kon=(((sitedensity/6.023e23)*4.4e6*(1/reactheight)^1000*10000^3);  # kon is 1 of the bond based upon site density and separation distance and a 3D kon of 4.4e6 Ms Menta et al, JBC, 1998 for P-selectin

# Dissociation kinetics parameters:
Fb=180*tau;  # Specify the force pN on the bond
koff=1.5378*exp(-.090688*Fb);  # Specify the kon s^-1 of the bond based on what we measure experimentally

# Es1_koff=3.4299*exp(.0943361*Fb);  # koff kon for PMN,180 for bead
# Peal_koff=2.4007*exp(.095605*Fb);  # neutrophil

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\$P_{sel\_koff}=1.53^{-6}*\exp(-0.90688*F_b) \quad \text{**dead**}
\$P_{nad\_koff}=4.26^{-6}*\exp(-0.2714*F_b)
\$L_{sel\_koff}=2.19^{-8}*\exp(-0.124*F_b)

\text{**GCB Near a Wall**}

\[ F_{tn}=-(1-9/16/(R/h))^{1/3} \quad \text{**GCB tabulated values for Near a Wall**}
\]
\[ T_{tn}=3/32*(R/h)^4 \quad \text{**plus these approximations into full velocity eqns. below**}
\]

\[ F_{rn}=1/8*(R/h)^4 \quad \text{**Near**}
\]
\[ T_{rn}=-(1+5/16*(R/h)^3) \quad \text{**Near**}
\]

\[ F_{sn}=1+9/16*(R/h) \quad \text{**Near**}
\]
\[ T_{sn}=1-3/16*(R/h)^3 \quad \text{**Near**}
\]

\[ v_n=(0.5*(R/h)*F_{rn}*T_{sn}-F_{sn}*T_{rn})/(F_{tn}*T_{rn}-F_{rn}*T_{tn}))*h*S \]
\[ \text{**GCB Near Wall velocity**}
\]
\[ \text{**Microns/s**}
\]

\[ \omega_{n}=(2*(h/R)*F_{sn}*T_{tn}-F_{tn}*T_{sn})/(F_{tn}*T_{rn}-F_{rn}*T_{tn}))*0.5*S \]
\[ \text{**GCB Near Wall angular velocity rad/s**}
\]

\text{**GCB Almost Touching a Wall**}

\[ F_{tt}=(8/15)*\log(da)-.9588 \]
\[ T_{tt}=-1*\log(da)-.1895 \]

\[ F_{rt}=-(2/15)*\log(da)-.2526 \]
\[ T_{rt}=(2/5)*\log(da)-.3817 \]

\[ F_{st}=1.7005 \]
\[ T_{st}=.944 \]

\[ v_t=(0.5*(R/h)*F_{rt}*T_{st}-F_{st}*T_{rt})/(F_{tt}*T_{rt}-F_{rt}*T_{tt}))*h*S \]
\[ \text{**GCB Near Wall angular velocity rad/s**}
\]

\[ \omega_{gt}=(2*(h/R)*F_{st}*T_{tt}-F_{tt}*T_{st})/(F_{tt}*T_{rt}-F_{rt}*T_{tt}))*0.5*S \]

\text{if (h-R)<0.0146}
\text{\quad Decide to use the Touch Eqns.}
\text{\quad v=v_t;}
\text{\quad dt=(disnextbond/v_t);}
\text{\quad \# Specify the time resolution or time step}
\text{\quad \# which also sets the site density}
\text{\quad totime=5/dt;}
\text{\quad T=dt*ttotime;}
\text{\quad Time step through T by dt}
\text{\quad \# Decide to use the Touch Eqns.}
\text{\quad else}
\text{\quad v=v_n;}
\text{\quad dt=(disnextbond/v_n);}
\text{\quad totime=5/dt;}
\text{\quad T=dt*ttotime;}
\text{\quad end}

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Monte Carlo

randnumbform=rand(200,T/dt);  % Generate array of random numbers
  % that will
  % be used to compare to probabilities
  % of formation
probbond=1-exp(-k_bond*dt);  % Probability that a bond forms
probnobond=1-exp(-k_off*dt);  % Probability that a bond breaks
for cell=1:200,
    newbond=0;
    z=1;
    while z<=T/dt
        if randnumbform(cell,z)<probbond & z<=T/dt
            % Probability < probbond then a bond
            while newbond==0 & z<=T/dt
                if rand<probnobond & z<=T/dt
                    % Probability < probnobond
                    % then
                    dis(cell,z)=v*dt;
                    % a bond does
                    % NOT exist and
                    % motion
                    z=z+1;
                    % no bond
                    % therefore
                    % Motion and
                    % travel time and
                    % distance
                    % before
                    % encountering
                    % the next
                    % opportunity
                    % for bonding
                elseif z<=T/dt
                    dis(cell,z)=0;
                    % bond No
                    % motion and
                    % cell remains
                    % stationary
                    z=z+1;
                    newbond=0;
                end
            end
            newbond=1;
        else
            dis(cell,z)=v*dt;
            % bond did not
            % form so
            % cell moves
            % forward
            z=z+1;
        end
    end
end

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end
rollvel(cell)=sum(dis(cell,:))/T;
end

The following is the MATLAB 5 code for the “multi-no re” case of simulated bead rolling. Up to two bonds could form at each bonding opportunity but rebonding could not occur.

* Monte Carlo Simulation of bead rolling using association and dissociation kinetics for up to two bonds at a time. We use JCB for motion of sphere between bonds and the geometry between bead surface and substrate surface to get concentration per sec. The force on the bond is calculated from the geometry and the force is calculated from experimental results. Up to two bonds can form and the probability of either one of those bonds breaking is the same and independent. But once a bond breaks, it cannot reform, so no rebonding exists.

sitedensity=170;  %specify site density of substrate sites microm^-2
disnextbond=2/sqrt(sitedensity);  %distance between bonds set by site density

*RJB geometry parameters

Q=.6;  %flow rate ml min to get JCB velocity for free flow
R=4.57;  %radius of particle micrometers
for x= 0.019 0.020 0.021 0.022 0.023 0.024 0.025 0.026 0.027 0.028 0.033 0.034 0.035 0.036
  h=R+.05;  %height of particle center from surface micrometers
  CL=0.100;  %cross bridge length=100nm for PSEL-PSGL-L1
  da=(h-R)/R;  %JCB ratio of separation distance per radius of bead
  u=0.01;  %viscosity of water=10C g cm/sec
  S=6*(Q/60)/((0.026)^2*(1));  %shear rate sec^-1, determined by flow chamber with gasket D
  kS=6*Q/60 1.0E-0.02 0.5;  %shear rate for flow chamber with gasket D
  tau=u*S;

*Association kinetics parameters

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theta = asin((R + (h - R)) / (R + CL));  # Calculating the volume for the
# reaction to diffuse the i-1
beadlength1 = R * cos(theta);  # km to get a per second km.
# theta is angle between bond
beadlength2 = R * sin(theta);  # km substrate. beadlength1 is
# distance from center line of bead
reactheight = h - beadlength2;  # to bond. beadlength1 is distance
# from center of bead to beadlength1
# reactheight is the height of the
cylinder of reaction potential

kon = ((sitedensity / 6.023e23) * 4.4e6 * (1 / reactheight) * 1000 * 10000^3);
# kon s-1 of the bond based upon
# site density and
# separation distance and a 3-D kon
# of 4.4e6 l Ms
# seminal. JBC:1395. for
# P-selectin

# Dissociation kinetics parameters: 1111
Fb = 180 * tau;  # Specify the force pN on the bond
# 125 for PMN, 150 for bead
koff = 1.5378 * exp(-0.090688 * Fb);  # Specify the koff s-1 of the bond
# based on what
# we measure experimentally

# JCB Near a Wall: 1111
Esel_koff = 1.4323 * exp(-0.03155 * Fb);  # JCB Tabulated values for Near
# a Wall
Psel_koff = 1.4323 * exp(-0.03155 * Fb);  # Psel_koff = 1.4323 * exp(-0.03155 * Fb)
Pnha_koff = 4.0174 * exp(-0.03155 * Fb);  # Pnha_koff = 4.0174 * exp(-0.03155 * Fb)
Lsel_koff = 1.4323 * exp(-0.03155 * Fb)

Ftn = -(1 - 9/16*(R/h))^-1;  # JCB Near Wall velocity
Ttn = 3/32*(R/h)  # plug these approximations into
# full velocity
eqns. below

Fn = 1/8*(R/h)^4;  # Near
Tr = -(1 + 5/16*(R/h)^3);  # Near
Fsn = 1 + 9/16*(R/h);  # Near
Tsn = 1 - 3/16*(R/h)^3;  # Near

vn = ((0.5*(R/h) * Fn * Tsn - Fsn * Tn) / (Ftn * Trn - Fn * Ttn)) * h * S;
# JCB Near Wall velocity
# microns s
omegan = ((2*(h/R) * Fsn * Ttn - Ftn * Tsn) / (Ftn * Trn - Fn * Ttn)) * 0.5 * S;

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*GCB Near Wall angular velocity radians

\[
\begin{align*}
F_{tt} &= (8/15) \log(da) - .9588; \\
T_{tt} &= -1.1 \log(da) - .1895; \\
F_{tt} &= -(2/15) \log(da) + .2526; \\
T_{tt} &= -(2/5) \log(da) + .3817; \\
F_{st} &= 1.7005; \\
T_{st} &= .944; \\
vt &= (0.5 * (R/h) * F_{tt} * T_{st} - F_{st} * T_{tt}) / (F_{tt} * T_{tt} - F_{st} * T_{st}) * h * S; \\
\text{omeg} &= ((2 * (h/R) * F_{tt} * T_{st} - F_{st} * T_{tt}) / (F_{tt} * T_{tt} - F_{st} * T_{st})) + 0.5 * S;
\end{align*}
\]

if \((h-R) \leq 0.0146\)  \hspace{1cm} \text{\textit{\textcolor{red}{Decide to use the Touch Eqns.}}}
\[v = vt;\]
\[dt = (\text{disnextb}ond / \text{vt});\]
\[\text{tottime} = 5 / dt;\]
\[T = dt \times \text{tottime};\]

else \hspace{1cm} \text{\textit{\textcolor{red}{Decide to use the Near Eqns.}}}
\[v = vn;\]
\[dt = (\text{disnextb}ond / \text{vn});\]
\[\text{tottime} = 5 / dt;\]
\[T = dt \times \text{tottime};\]
end

\text{\textit{\textcolor{red}{Monte Carlo}}}\hspace{1cm} \text{\textit{\textcolor{red}{Generate array of random numbers}}}
\[\text{ran} \text{\textit{d} numbform1 = ran} \text{\textit{d}(100, T/dt);}\]
\[\text{ran} \text{\textit{d} numbform2 = ran} \text{\textit{d}(100, T/dt);}\]
\[\text{probbond} = 1 \text{\textit{-exp}(-kon*dt);}\]
\[\text{probnobond} = 1 \text{\textit{-exp}(-koff*dt);}\]
\[\text{Fb2} = \text{Fb} / 2;\]
\[\text{koff} = 1.5378 \text{\textit{exp}(0.0090688*Fb2);}\]
\[\text{probnobond} = 1 \text{\textit{-exp}(-koff2*dt);}\]

pack

for \text{cell} = 1:100,
\text{cell}

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```c
bonds=0;
z=1;
while z<=T/dt
  if bonds==0 & randnumbform1(cell,z)<probbond |
    randnumbform2(cell,z)<probbond
    ; if 1 bonds has formed
    if randnumbform1(cell,z)<probbond &
      randnumbform2(cell,z)<probbond
      ; 2 bonds have formed
      bonds=2;
      ; list is shared between 2 bonds
      knif is less
      ; probability is greater than 1
      bond breaks
  else
    bonds=1;
  end
  while bonds==2
    if rand<probnobond2
      ; Probability =
      ; probnobond2 then
      dis(cell,z)=0;
      ; a bond has broken and
      ; test for new bond
      z=z+1;
      ; formation and breakage
      ; at next time step
      bonds=1;
      ; bond therefore no
      ; motion and
      ; bead remains
      ; stationary
      ; 1=bond motion and
      ; bead moves at critical
      ; velocity
      ; to next bond
      if rand<probnobond
        dis(cell,z)=v*dt;
        z=z+1;
        bonds=0;
      end
    else
      dis(cell,z)=0;
      z=z+1;
      bonds=2;
    end
  end
  if bonds==1 & rand<probnobond
    dis(cell,z)=v*dt;
    z=z+1;
    bonds=0;
  else
    dis(cell,z)=0;
    z=z+1;
    bonds=1;
  end
```

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 elseif bonds==1
    if rand<probnobond
        dis(cell,z)=v*dt;
        z=z+1;
        bonds=0;
    else
        dis(cell,z)=0;
        z=z+1;
        bonds=1;
    end
    else
        dis(cell,z)=v*dt;  % bond did not form so cell moves
        z=z+1;             % forward
    end
end
rollvel(cell)=sum(dis(cell,:))/T;
end

The following is the MATLAB 5 code for the “multi-re” case of simulated bead rolling. Up to two bonds could form at each bonding opportunity and rebonding could occur.

% Monte Carlo Simulation of bead rolling using association and
% dissociation kinetics for up to two bonds at a time. We use JCB for
% motion of sphere between bonds and the geometry between bead surface
% and substrate surface to get rate per sec. The force on the bond is
% calculated from the geometry and the Koff is calculated from
% experimental results. Up to two bonds can form and the probability of
% either one if those bonds breaking is the same and independent. The
% second bond can form any time that there is one bond already existing,
% so rebonding can occur.

sitedensity=170;  % Specify site density of substrate

% sites/μm^2

% JCB: geometry parameters: --------------------------

Q=.6;  % Flow rate μl/min to get JCB

% velocity for

% free flow

R=4.57;  % radius of particle μmeters

% for x = 0.001 0.005 0.005 0.005 0.01 0.01 0.01 0.01 0.015
% 0.01 0.01 0.01 0.01 0.02 0.026
% 0.03 0.05 0.065 0.08 0.08

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\[ h = R + 0.099; \]
\[ \text{height of particle center from surface} \]
\[ CL = 0.100; \]
\[ \text{cross bridge length} = \text{nm for R-set} \]
\[ \text{PSL} \]
\[ da = (h-R)/R; \]
\[ \text{C/D ratio of separation distance per radius of bead} \]
\[ u = 0.01; \]
\[ \text{viscosity of water} = 1 \text{cP} \]
\[ S = 6*(Q/60)/((0.026)^2*(1)); \]
\[ \text{shear rate} \text{sec}^{-1} \text{ determined by flow chamber with gasket} \]
\[ \tau = u*S; \]
\[ \text{wall shear stress} \text{ dyn cm}^{-2} \]

\[ \text{Association kinetics parameters} \]
\[ \text{Calculating the volume for the reaction to divide the 3-D} \]
\[ \text{kon to get a per second kon.} \]
\[ \text{theta is angle between bond and substrate. beadlength1 is distance from centerline of bead} \]
\[ \text{reactheight} = h - \text{beadlength2}; \]
\[ \text{distance from center of bead to beadlength1} \]
\[ \text{reactheight is the height of the cylinder of reaction potential} \]
\[ \text{konsl} = (\text{site density}/6.023e23)*4.4e-6*(1/\text{reactheight})*1000*10000^3; \]
\[ \text{upon separation distance and a 1-D kon of 4.4e-6} \text{ Ms Kenta et al., JBC, 1996 for P selectin} \]

\[ \text{Dissociation kinetics parameters} \]
\[ \text{Specify the force \text{pN} on the bond} \]
\[ \text{103 for PMN, 165 for bead} \]
\[ koff = 1.5378*\exp(.0090688*Fb); \]
\[ \text{Specify the koff set of the bond, based on what we measure experimentally} \]
\[ \text{Fb = 180*tau; \text{ Specify the force pN on the bond}} \]
\[ \text{103 for PMN, 165 for bead} \]
\[ \text{Psel_koff = 2.402*exp(1.033*Fp); \text{ neutrophil}} \]

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\[ \text{Psel} \_ \text{xoff}=1.5378 \times \exp \left(-0.3045 - 8 \times Fb\right); \]
\[ \text{Phead} \_ \text{xoff}=1.2936 \exp \left(-0.647 \times Fb\right); \]
\[ \text{Lsel} \_ \text{xoff}=3.4378 \exp \left(-0.1976 \times Fb\right); \]

\[ \text{Ftn}\_\text{Near} = -\left(1 - 9/16 (R/h)^4\right)^{-1}; \]
\[ \text{Ttn}\_\text{Near} = 3/32 (R/h)^4; \]

\[ \text{Frn}\_\text{Near} = 1/8 (R/h)^4; \]
\[ \text{Trn}\_\text{Near} = (1 + 5/16 (R/h)^3); \]

\[ \text{Fsn}\_\text{Near} = 1 + 9/16 (R/h); \]
\[ \text{Tsn}\_\text{Near} = 1 - 3/16 (R/h)^3; \]

\[ \text{vn}\_\text{Near} = \left(0.5 (R/h) \times \text{Frn}\_\text{Tsn} - \text{Fsn}\_\text{Tsn} \right) / \left( \text{Ftn}\_\text{Ttn} - \text{Ftn}\_\text{Tsn} \right) \times h \times S; \]

\[ \text{omegan}\_\text{Near} = \left(2 \times (h/R) \times \text{Fsn}\_\text{Ttn} - \text{Ftn}\_\text{Ttn} \right) / \left( \text{Ftn}\_\text{Ttn} \right) \times 0.5 \times S; \]

\[ \text{Ftt}\_\text{Near} = 8/15 \times \log (da) - 0.9588; \]
\[ \text{Ttt}\_\text{Near} = -1\times \log (da) - 0.1895; \]

\[ \text{Frtn}\_\text{Near} = -2/15 \times \log (da) - 0.2526; \]
\[ \text{Trtn}\_\text{Near} = 2/5 \times \log (da) - 0.3817; \]

\[ \text{Fst}\_\text{Near} = 1.7005; \]
\[ \text{Tst}\_\text{Near} = 0.944; \]

\[ \text{vt}\_\text{Near} = \left(0.5 (R/h) \times \text{Frtn}\_\text{Tst} - \text{Fst}\_\text{Tst} \right) / \left( \text{Ftt}\_\text{Trtn} - \text{Ftt}\_\text{Tst} \right); \]
\[ \text{omegat}\_\text{Near} = \left(2 \times (h/R) \times \text{Fst}\_\text{Ttt} - \text{Ftt}\_\text{Ttt} \right) / \left( \text{Ftt}\_\text{Ttt} - \text{Ftt}\_\text{Tst} \right) \times 0.5 \times S; \]

if \( (h-R) < 0.0146 \)

\[ \text{v} = \text{vt}; \]
\[ \text{dt} = (\text{disnextbond} / \text{vt}); \]
\[ \text{totttime} = 5 / \text{dt}; \]
\[ \text{T} = \text{dt} \times \text{totttime}; \]

else

\[ \text{v} = \text{vn}; \]
\[ \text{dt} = (\text{disnextbond} / \text{vn}); \]

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Appendix

tottime=5/dt;
T=dt*tottime;
end

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randnumbform1=rand(100,T/dt);
randnumbform2=rand(100,T/dt);
probbond=1-exp(-kon*dt);
probnobond=1-exp(-koff*dt);
Fb2=Pb/2;
koff2=1.5378*exp(.0090688*Fb2);
probnobond2=1-exp(-koff2*dt);

pack

for cell=1:100,
cell
  bonds=0;
z=1;
  while z<=T/dt
    if bonds==0 & randnumbform1(cell,z)<probbond |
      randnumbform2(cell,z)<probbond
        %1 or 2 bonds have formed
        if randnumbform1(cell,z)<probbond &
          randnumbform2(cell,z)<probbond
            %2 bonds have formed
            bonds=2;
            %load is shared
            %between 2 bonds
            %koff is less
            %probability is
            %greater than 1
            %bond breaks
          elseif
            bonds=1;
          end
        else
          bonds=1;
        end
        %if rand<probnobond2
        %Probability <
        %probnobond2 then
        %a bond has
        %broken and test
        %for new bond
        %formation and
        %breakage at next
        %time step
        %1 bond therefore
        %no motion and
  end
  if z=T/dt
    bonds=0;
  end
  end
  dis(cell,z)=0;
z=z+1;
bonds=1;

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Appendix

*read remains
*stationary
*+bond motion
+and bead moves
*at critical
+velocity
+to next bond

if rand<probnobond
    dis(cell,z)=v*dt;
    z=z+1;
    bonds=0;
end
else
    dis(cell,z)=0;
    z=z+1;
    bonds=2;
end

if bonds==1 & rand<probnobond
    dis(cell,z)=v*dt;
    z=z+1;
    bonds=0;
else
    dis(cell,z)=0;
    z=z+1;
    bonds=1;
    if rand<probnobond
        bonds=2;
    end
endif

elseif bonds==1
    if rand<probnobond
        dis(cell,z)=v*dt;
        z=z+1;
        bonds=0;
    else
        dis(cell,z)=0;
        z=z+1;
        bonds=1;
        if rand<probnobond
            bonds=2;
        end
    end
endif

elseif bonds==2
    while bonds==2
        if rand<probnobond2
            probability <
            probnobond2 then
            a bond has
            broken and

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\[ z = z + 1; \]
\[ \text{bonds} = 1; \]

*test for new bond formation and breakage at next time step
*bond therefore no motion and bead remains stationary
*bond motion and bead moves at critical velocity to next bond

\[
\text{if rand < probnobond}
\]
\[ \text{dis(cell, } z) = v \times dt; \]
\[ z = z + 1; \]
\[ \text{bonds} = 0; \]
\[ \text{end} \]
\[
\text{else}
\]
\[ \text{dis(cell, } z) = 0; \]
\[ z = z + 1; \]
\[ \text{bonds} = 2; \]
\[ \text{end} \]
\[
\text{else}
\]
\[ \text{dis(cell, } z) = v \times dt; \]
\[ z = z + 1; \]
\[ \text{end} \]
\[ \text{rollvel(cell) = sum(discell, :))/T;} \]
\[ \text{end} \]
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