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Effects of Membrane Rheology on Leuko-polymerosome Adhesion to Inflammatory Ligands

Gregory P. Robbins[†], Dooyoung Lee[‡], Joshua S. Katz[‡], Paul R. Frail[¶], Mike J. Therien^{||}, John C. Crocker[†], and Daniel A. Hammer^{†,‡,§}

[†]School of Engineering and Applied Sciences, Dept of Chemical and Biomolecular Engineering, University of Pennsylvania, Philadelphia, Pennsylvania 19104

[‡]School of Engineering and Applied Sciences, Dept of Bioengineering, University of Pennsylvania, Philadelphia, Pennsylvania 19104

[¶]School of Arts and Sciences, Dept of Chemistry, University of Pennsylvania, Philadelphia, PA 19104

^{||}Department of Chemistry, Duke University, Durham, North Carolina 27708

Summary

A strategy for treating inflammatory disease is to create micro-particles with the adhesive properties of leukocytes. The underlying rheology of deformable adhesive microspheres would be an important factor in the adhesive performance of such particles. In this work the effect of particle deformability on the selectin-mediated rolling of polymer vesicles (polymerosomes) is evaluated. The rheology of the polymerosome membrane was modulated by cross-linking unsaturated side-chains within the hydrophobic core of the membrane. Increased membrane rigidity resulted in decreased rates of particle recruitment rather than decreased average rolling velocities. Reflective interference contrast microscopy of rolling vesicles confirmed that neither flaccid nor rigid vesicles sustained close contacts with the substrate during rolling adhesion. A variable-shear rate parallel-plate flow chamber was employed to evaluate individual vesicles rolling on substrates under different flow conditions. Analysis of the trajectories of single flaccid vesicles revealed several distinct populations of rolling vesicles; however, some of these populations disappear when the vesicle membranes are made rigid. This work shows that membrane mechanics affects the capture, but not the rolling dynamics, of adherent leuko-polymerosomes

Keywords

membrane tether; sialyl Lewis X; deformation; inflammation; P-selectin; RICM

Introduction

The adhesion of leukocytes under flow is critical to the functioning of the immune system. The homing of neutrophils to inflammatory sites, the trafficking of T-cells to immune organs, and the homing of stem cells to bone marrow vasculature all require the adhesion of blood cells under flow via specific adhesion receptors.¹ The first step in the adhesion cascade is leukocyte rolling, which is mediated by selectin adhesion molecules that react quickly yet are mechanically weak.² The second step is firm adhesion, which is mediated by

[§]To whom correspondence should be addressed, hammer@seas.upenn.edu, Phone: 215-573-6761, Fax: 215-573-2093, Room 311A Towne Building, 220 South 33rd Street, University of Pennsylvania, Philadelphia, PA 19104-6393.

activated integrins that are mechanically stronger than selectins.³ The roles of these molecules in leukocyte adhesion have been confirmed by molecular biology, antibody blocking, and reconstitution into cell-free systems in which adhesion molecules can be attached to the surfaces of inert colloidal particles.⁴ Rolling can be recreated by attaching selectin ligands to both polystyrene microspheres and porous polymeric microspheres, suggesting that rolling is driven primarily by the mechanochemical properties of the molecules, and secondarily by cellular structural features. The role of particle elasticity and rheology on adhesion under flow, however, has not been rigorously assessed.

The fluidity and deformability of the cell membrane facilitates several stress-response modes and allows a cell to change shape in shear flow. These factors are thought to be significant in regulating adhesion during inflammation.^{5, 6} Therefore, membrane deformation of vesicles and cells during shear flow has been the subject of intense investigation.^{7, 8} Intravital microscopy,⁹ *in vitro* parallel plate flow chamber experiments,^{5, 10, 11} and simulations^{6, 12} all show that leukocytes deform during adhesion under flow. Membrane deformation changes the hydrodynamic radius of a cell (or vesicle),^{13, 14} the contact area between membrane and substrate, the direction and magnitude of hydrodynamic forces that act on the cell, and the direction and magnitude of forces acting on the adhesion molecules.^{15, 16} Additionally, membrane fluidity allows the cell membrane to 'tank tread' (movement of the membrane independent of the fluid inside the cell) rather than the entire cell rotating in response to torque,¹⁷ and nano-tube tethers can readily form between membranes (cells, liposomes and polymersomes, which are held together by entropic forces) and substrates with which they interact adhesively.^{18–23} Thus, when reconstituting leukocyte adhesion into inert capsules, it is important to assess the role that underlying capsule mechanics has on the dynamics of adhesion.

Up to this point, it has been difficult to experimentally investigate the effects membrane rigidity on adhesion because the experimental toolbox has been limited. Liposomes have been the primary experimental tool used to investigate membrane physics.^{13–15, 17, 24, 25} Liposomes, however, are held together by relatively weak intermolecular forces; they are known to be mechanically weak, and their mechanical elasticity is not easily modified. Interesting work by Needham and co-workers showed that the addition of cholesterol to liposomes facilitates changes in the rigidity of the membranes.²⁶ Blended membranes, however, introduce additional potential variables into the system such as changes in the surface ligand site density, organization of the two components into domains, and vesicle-to-vesicle variation in the ratio of components (thus vesicle-to-vesicle variation in the membrane rigidity). Polymersomes, fully-synthetic analogs of liposomes, are more robust and customizable than their lipid counterparts.²⁷ The intermolecular forces holding polymersome membranes together are much stronger than in liposomes,²⁸ and these vesicles can have an area expansion modulus several orders of magnitude larger than for liposomes.²⁹ This increased strength facilitates the attachment of antibodies and large polysaccharides without destroying the vesicle structure.³⁰

Recently, we created leuko-polymersomes, polymersomes which mimic the adhesive properties of leukocytes.^{31, 32} We showed that we can attach high site densities of adhesive ligands found on activated leukocytes to polymersome surface through an avidin-biotin bridge, and by tuning the ratio of these ligands (sialyl Lewis X and anti-ICAM-1 antibody) on the vesicle surface, we can tune the adhesivity of a vesicle on ICAM-1 and P-selectin substrates. We also showed that we can simultaneously achieve high rates of adhesion under flow on inflamed HUVECs with these leuko-polymersomes while maintaining a low rate adhesion of leuko-polymersomes on normal HUVECs.

Polymersomes are an ideal system for studying the coupling between adhesion and membrane rheology, because the material properties of the membrane are widely tunable.^{28, 33, 34} The membrane rheology of the polymersome can be adjusted either by changing the polymer building block chemistry, size or by creating a chemically cross-linked network in the hydrophobic membrane core to increase the rigidity of the membrane.^{33, 34} Although receptor-coated hard spheres can recreate the basic dynamics of leukocyte rolling,³⁵ numerous calculations show that macroscopic deformation can have a significant effect on the shear-rate dependence of leukocyte rolling. Specifically, deformable cells seem to be more adherent at higher shear rates because they can form a large contact area.⁶ Until now, it has been difficult to systematically test the role of rheology on adhesion, but with polymersomes this is now possible.

In this work, we compare rolling adhesion of two populations of adhesive vesicles. These vesicles are made from identical polymer and coated with sialyl Lewis X (sLe^x) ligand using identical coating procedures for each population. One population was cross-linked using a membrane-encapsulated UV-activated free radical initiator, and the other population was left flaccid. This system facilitates direct evaluation of the effects of membrane fluidity on adhesion under shear flow. We found that increased membrane rigidity hinders vesicle recruitment to the substrate and decreases the area of close vesicle-substrate contact of firmly adhered vesicles. Increased membrane rigidity, however, does not substantially affect rolling velocities or firm adhesion.

Results and Discussion

Vesicle membrane cross-linking

Flaccid vesicles decorated with high site densities of the adhesion ligand sialyl Lewis X (approx. 3,000 sites μm^{-2}) were previously assembled and reported.^{31, 32} We showed that the membrane toughness could be adjusted by chemically cross-linking unsaturated polymers in the hydrophobic core.³³ In these studies, a soluble initiator-redox combination was used to generate free-radicals needed for membrane polymerization.³⁶ Concerns about the effect these ions may have on the activity of adhesive ligands attached to the vesicle surface prompted the use of a UV-activated free radical initiator that could be fully encapsulated in the hydrophobic core of the vesicle membrane. This cross-linking method is used extensively in the formation of hydrogels³⁷ but has been sparingly used to modify polymer vesicles.³⁸ By optimizing this method, we ensured minimal damage to proteins or functional groups attached to the polymer from free-radicals in solution.

The molar ratio of free radical initiator to polymer was systematically changed, and after exposure to UV light, the rigidity of cross-linked vesicles was evaluated using micropipette aspiration. Although concentrations of initiator as low as 1:1 (molar ratio initiator:polymer) resulted in membrane stabilization, we found an initiator to polymer ratio of 10:1 to be the minimum concentration of initiator required to impart full rigidity to vesicle membranes as assessed by micropipette aspiration. Vesicles were considered fully rigid when we were unable to pull membrane extensions using micropipette aspiration with pipettes on the order of 5 μm (I.D.) at suction pressures exceeding 100 cm-H₂O, as illustrated in Figure 1. Cross-linking was optimized using biotinylated polymer vesicles with no additional surface functionality, but fully functional sLe^x-functionalized vesicles were also found to be rigid when cross-linked using similar concentrations of photo-initiator. Cross-linking affects only the hydrophobic phase of the polymersome membrane. This chemical modification results in an enthalpically stabilized vesicle which resists deformations in the plane of the membrane (i.e. force-induced deviations from the spherical shape) as well as nano-tube pulling, but since the PEO-brush layer is unaffected, the membrane retains some spring-like quality (Figure 2A, B). The area expansion modulus (K_a) of vesicles used for this study range from

$60 \pm 5 \text{ mN/m}^{39}$ to an estimated value $\gg 10^2$.³³ These values are within the range of values measured for leukocytes (0.035 mN/m) and erythrocytes (500 mN/m).⁴⁰

Vesicle-substrate contact area measurements through RICM

Experiments using leukocytes and numerical simulations have shown that shear flow can induce significant increases in membrane-substrate contact area.^{5, 6, 10–12} Consequently, we expected the flaccid vesicles to flatten during rolling adhesion in response to forces generated by fluid flow pushing the vesicle membranes into the substrate. Static tests using reflective interference contrast microscopy (RICM) were performed to determine the extent of membrane spreading induced by sLe^x-P-selectin bonds. In these experiments, vesicles were flowed into the chamber; the flow was terminated and the vesicles were allowed to settle onto the P-selectin substrate. We measured the two-dimensional area of closest contact visualized in RICM (darkest region, Figure 3) and compared this area to the two-dimensional area of a vesicle slice at the maximum vesicle diameter, visualized using DIC optics. For flaccid vesicles, this ratio was 0.231 ± 0.058 (11 measurements, Figure 3A). The ratio of contact area to vesicle area for rigid vesicles approached zero because the rigid vesicles touched at a single point, and the interference pattern was dominated by Newtonian rings (Figure 3B). This pattern is expected for a spherical object.⁴¹ In addition to the differences in contact area, we also note that while the shape of the rigid vesicle contact area was always circular, the shape of the flaccid vesicle contact area was often asymmetric, an additional indication of membrane deformation. In several cases, deformation did occur in the direction of flow, but other vesicles deformed randomly. This result suggests that once a strong contact was made, by the membrane touching the substrate during a random oscillation, the bonds between ligand and receptor were strong enough to kinetically trap the membrane on the substrate. These results confirm vesicle deformation of flaccid vesicles, but the extent of vesicle deformation was much less than we expected.

We also visualized the contact area of rolling vesicles (both flaccid and rigid) using RICM, but surprisingly, few instances of close vesicle-substrate contact areas during rolling were observed. Flaccid vesicles remained approximately 100–129 nm above the substrate surface for the entire rolling portion of their trajectories (supplemental information; Video SV1); in fact, regions of close contact (approx. 26.5 nm) appeared only after the vesicles had firmly adhered to the substrate. Similar to the trajectories of the flaccid vesicles, rigid vesicles also rolled at separation distances in the range of 100–129 nm, but unlike flaccid vesicles, rigid vesicles periodically formed close contact throughout the rolling portions of their trajectories (supplemental information, Figure S1, Video SV2, SV3). The 29–100 nm separation distance is reasonable because the length of a P-selectin molecule is approximately 38 nm,⁴² and the sLe^x-PAA polymer (estimated 10–15 nm), NeutrAvidin (approx. 7.2 nm⁴³), and extended PEO-brush (10.5 nm PEG, estimated based on a 0.35 nm persistence length per monomer chain⁴⁴) should facilitate additional separation distance between vesicles and substrate during rolling motions (Figure 2C). We were unable to visualize substrate-induced vesicle deformation of flaccid vesicles during rolling because of these large separation distances. Occasionally, we observed the vesicle-substrate contact area of firmly adherent flaccid vesicles grow after capture under shear flow (Figure 4), effectively stabilizing the firm adhesion⁴⁵. However, we did not observe the growth of close contact after firm adhesion in rigid vesicles.

A possible explanation for the distant separation of flaccid vesicles from the substrate during rolling is that flaccid vesicles form nano-tube extensions. Nano-tubes are known to form between colloidal structures and substrates when an adhesive interaction is formed at a point and the structure is pulled away.¹⁸ Hochmuth and co-workers calculated that the tension required to pull a 55 angstrom diameter nano-tube from red blood cell is as low as 0.19 dyne cm^{-1} ,¹⁹ and the phenomenon of tether formation has been observed *in vitro*.⁴⁶ In this

system, sLe^x is attached to the vesicle through NeutrAvidin proteins which are bound to individual polymer chains in the membrane. In contrast, cross-linked membranes cannot form nano-tube extensions because the polymer chains are covalently linked within the membrane, and the result is that rolling adhesion is less continuous. Like the flaccid vesicles, the rigid vesicles form bonds with the substrate, but rigid vesicles cannot form nano-tubes attached to mature bonds (presumably at the back of the vesicles) because the individual polymer chains in the membrane are covalently cross-linked. Instead of remaining at a constant separation distance, vesicles are periodically forced into the substrate, due to torque induced by bonds at the back end of the contact zone of rigid vesicles. These experiments show particle deformation is not required for the maintenance of rolling adhesion; instead, deformation-induced changes in vesicle-substrate contact area are most important in stabilizing firm adhesion.

Changes in adhesive flux

Although vesicle rigidity did not affect the rolling velocity, we tested whether vesicle rigidity controlled the capture of vesicles on the substrate. Capture is quantified by the adhesive flux, which was previously measured using a constant shear rate parallel plate flow chamber and flaccid vesicles.^{31, 32} These experiments were repeated using rigid vesicles so that a direct comparison between the adhesive flux of the two systems could be performed. The flow chamber used for these experiments is shown schematically in Figure 5A. In these tests, *firm adhesion* was defined as when the vesicle centroid remained stationary for at least 5 consecutive seconds. Other categories of motion were *stable rolling*, defined as when a vesicle moves at a velocity 45% or less than the hydrodynamic velocity of fluid (the fluid velocity at a distance from the wall equal to the vesicle radius) for at least 10% of the total time the vesicle was tracked, and *unstable rolling*, when the vesicle moves at a velocity 45% or less than the hydrodynamic velocity of fluid but the vesicle does not roll for 10% of the total time tracked. Unstable rolling motions were typically saltatory (characterized by brief, jumpy interactions with the substrate). *Rolling + binding* vesicles ultimately were firmly bound but rolled at some point during their trajectories. The results shown in Figure 6 demonstrate that rigid vesicles adhere less well than flaccid vesicles, but that the difference becomes less significant with decreasing substrate receptor site density. Additionally, as the ligand density decreases, there was a change in the types of adhesion. This data, illustrated in terms of percentage of total adhesive flux, is shown in Table 1. Analysis reveals that increasing the rigidity of vesicle membranes causes the strength of adhesion to decrease, and with both the 100% and 50% P-selectin substrate, there is an increase in the percentage of vesicles in unstable rolling. The rate of firm adhesion events in rigid vesicle systems occurs more frequently than expected. Selectin bonds are understood not to mediate firm adhesion,⁴⁷ but we previously noted that high sLe^x site density on the vesicle is the likely cause of firm adhesion in this system³² because the vesicles become kinetically trapped.⁴⁵

The change in adhesion rate and adhesive interaction type after formation of a rigid membrane could be the result of reduced membrane flexibility in the nanomechanics of adhesion and the role of membrane elasticity on vesicle capture. Although we expected that flaccid, deformable vesicles would result in slower rolling velocities, histograms of rolling velocities of both flaccid and rigid vesicles at two shear rates, $\gamma = 130 \text{ s}^{-1}$ and $\gamma = 210 \text{ s}^{-1}$, shown in Figure 7, indicate that rolling velocity of rigid vesicle does not substantially change compared to flaccid vesicles.

Single vesicle analysis

Analysis of adhesive flux indicated that increased vesicle rigidity decreases the recruitment rate rather than altering the dynamics of rolling after capture. RICM experiments showed that the contact area between a flaccid vesicle and substrate does not increase substantially

during rolling adhesion. These results may differ from our predictions because the membranes of flaccid vesicles used in these experiments have little excess membrane, making them more reminiscent of erythrocytes than leukocytes. Dong and co-workers note that, while shear flow driven membrane-substrate contact area spreading is significant in leukocytes, which have excess membrane in folds and ruffles, erythrocytes, which have little excess membrane, experience very little deformation during flow.¹⁰ Our data suggests that very little membrane spreading occurs in flaccid, adherent vesicles, and rather than affecting the dynamics of rolling adhesion and firm adhesion rate, increased membrane rigidity results in decreased vesicle recruitment. Presumably, the increased compliance of the flaccid membrane increases the contact area and/or contact time of a vesicle and substrate during recruitment and decreases the loading rate on a particular bond.

Population heterogeneity may also play a role in the effects that were observed. Chang and Hammer performed adhesive dynamics simulations on the capture and rolling of populations of molecularly coated polystyrene beads.⁴⁸ They showed that the rolling velocity of a population with a single site density increases with increasing shear rate in accordance with what would be predicted from force balances. When experiments were performed in a parallel plate flow chamber on a population of beads with a distribution of site densities, the rolling velocities increased with increasing shear rate at a lower rate than predicted by force balances. This phenomenon was a result of changes in bead recruitment rate. As the shear rate increased, beads with low ligand site densities, which would have demonstrated fast rolling velocities, could no longer bind. Thus, these fast rollers are excluded from the population of adhesive beads in adverse adhesion conditions. We observe similar results in our rolling velocity distributions (Figure 7); specifically, we found that the while distributions of rolling velocities in flaccid and rigid populations were similar, the adhesive flux of rigid vesicles was substantially lower than the adhesive flux of flaccid vesicles (Figure 6).

We also observed a shift from firm adhesion to stable rolling and from stable to unstable rolling in our adhesive flux in response to forming rigid membranes (Table 1). A distribution of ligand site densities exists in polymersome populations³² as well as a distribution in vesicle size.³² We hypothesize that by increasing the membrane rigidity of a vesicle population, particle adhesion becomes more difficult; therefore, similar to the bead experiments, we suspect we were measuring the adhesion of only a subset of the vesicle population in the flow chamber experiments described previously and quantified in Figure 6 (and Table S1). Additionally, the difference in the adhesive flux between these two populations disappears as the substrate receptor concentration is decreased, and the shift in adhesive mode changes as a function of substrate. This data suggests that substrate identity, in addition to vesicle rigidity, effects the sub-population of vesicles that bind, but this data also shows that rigidity plays a role in vesicle recruitment at multiple substrate site densities. It is impossible to select for identical distributions of vesicles with which to perform population-level comparisons between flaccid and rigid vesicles, so to evaluate this hypothesis, measurements were made on individual vesicles under variable shear rate flow conditions.

For these experiments, a tapered flow channel was employed (Figure 5B–C). This chamber has been used to evaluate adhesion at multiple shear rates in a single experiment.^{35, 49} We measured the rolling velocity of individual flaccid and rigid vesicles as they traveled through the flow chamber from high ($\gamma = 390 \text{ s}^{-1}$) to low ($\gamma = 130 \text{ s}^{-1}$) shear rates. Force balances predict that rolling velocity should decrease with decreasing shear rate, so the slope of rolling velocity vs. shear rate was used to compare adhesion of individual vesicles.

Figure 8 illustrates the results of these experiments. Interestingly, flaccid vesicles were characterized by two distinct slopes relating rolling velocity to shear rate, whereas rigid vesicles that responded to decreasing shear rate with decreasing rolling velocities were characterized by only one slope. The ‘trajectories’ (rolling velocity vs. shear rate) for three representative vesicles, one from each group in Figure 8A, are shown in Figure 8B. These results show that tightly bound vesicles (shallow slopes) are in both flaccid and rigid populations, but weakly associated vesicles (with steep slopes) are only seen in the flaccid populations. Presumably, the tightly binding vesicles make a sufficient number of contacts with the substrate during initial recruitment, and minor changes in the number of total bonds or the total force exerted on the vesicle resulting from membrane deformation does not translate to changes in the characteristic vesicle rolling velocity. Figure 8 shows that similar velocity-shear rate relationships are measured in the flaccid vesicle populations to the relationship measured in the rigid vesicle population. We suspect that the interfacial deformation of flaccid vesicles facilitates the rolling characteristics unique to flaccid vesicles.

Two additional shear rate-rolling velocity relationships were measured. The first relationship was slow, constant rolling velocity, independent of shear rate. These vesicles, observed in both rigid and flaccid populations, rolled for long periods of time at a single velocity and typically ended their trajectories with firm arrest. Several of these trajectories are shown in Figure 9A, and these vesicles presumably bind the substrates more tightly than the vesicle populations in which rolling velocity and shear rate were correlated. The other shear rate-rolling velocity relationship was characterized by random, jumpy motion. These vesicles, presumably the weakest binding ‘rolling’ vesicles observed, were only observed in the flaccid vesicle populations, presumably because rigid vesicles were unable to be repeatedly captured to maintain a fast, jumpy velocity of rolling.

The results of these experiments confirm that rigid vesicles adhere less avidly than flaccid vesicles, but rolling velocities of rigid vesicles are similar to rolling velocities of flaccid vesicles. Again, these results are consistent with observations in cells. The rolling velocities of leukocytes, with soft membranes and significant excess membrane in folds and ruffles,⁴⁰ are significantly affected by shear rate, membrane deformation, and membrane spreading on substrates while erythrocytes, with taut membranes, are not significantly affected by shear flow induced deformation.¹⁰

Experimental

Materials

Polyethylene oxide-polybutadiene (PEO₃₀-PBD₄₆, denoted OB29) di-block copolymer was purchased from Polymer Source (Montreal, Quebec, Canada). Density gradient medium (Optiprep®, iodixanol solution), Dulbecco’s phosphate buffered saline (DPBS, +Mg²⁺, +Ca²⁺), 4-fluoro-3-nitrobenzoic acid, tetrahydrofuran (THF) (HPLC grade), triethylamine, biocytin, fibronectin, phosphate buffered saline (PBS) tablets, dimethylaminopyridine, dicyclohexylcarbodiimide, and 2,2-dimethoxy-2-phenylacetophenone (DMPA) were purchased from Sigma-Aldrich (St. Louis, MO). Methylene chloride (HPLC grade) and sucrose were purchased from Fisher Chemicals (Pittsburgh, PA). 20,000 Da MWCO Slide-A-Lyzer dialysis cassettes, Superblock, and NeutrAvidin were purchased from Pierce Biotechnology (Rockford, IL). Biotinylated-sLe^x multivalent polymer was purchased from GlycoTech (Rockville, MD). P-selectin/Fc and ICAM-1/Fc were purchased from R & D Systems, (Minneapolis, MN). Calcein was purchased from Invitrogen (Carlsbad, CA). The meso-to-meso ethyne-bridged (porphinato)zinc(II)dimer (PZn₂) was synthesized in the laboratory of Dr. Michael J. Therien (Duke University), where its photophysical properties were previously characterized.⁵⁰ All materials were used as received.

Synthesis of Biocytin Modified Polymer

The terminal end of the PEO block was modified to display biocytin through a two step synthesis, first with 4-fluoro-3-nitrobenzoic acid, followed by biocytin. In a round-bottomed flask, 100 mg polymer was added to 1.1 equivalents of 4-fluoro-3-nitrobenzoic acid, with catalytic dimethylaminopyridine, followed by the addition of 35 mL dry methylene chloride. To a dried septum-seal vial we added 1.1 equivalents of dicyclohexylcarbodiimide and 5 mL methylene chloride, which was then injected into the round-bottomed flask with the polymer. The reaction was allowed to proceed for 48 hours, and the resulting solution was purified by filtration and HPLC (Varian, Palo Alto, CA with a PLgel size exclusion column and THF as the carrier solvent). Polymer-4-fluoro-3-nitrobenzoic acid was dissolved in 50% THF (HPLC grade)-50% DI water (by volume) at 5 mM in glass screwtop vial. Polymer-4-fluoro-3-nitrobenzoic acid, biocytin and triethylamine were combined at 1:1.5:10 (molar ratio, polymer-biocytin-triethylamine). The reaction was allowed to proceed at 37–40°C, while stirring, for 24 hours. The mixture was dried by rotary evaporation to remove water and THF, resuspended in 100% THF at 0.1 mg/mL and then filtered and purified by HPLC.^{31, 32, 51} Characterization of the final polymer using NMR showed that 88% of the polymer was modified with biocytin.

Polymersome Assembly

The biocytin terminated copolymer (PEO(1300)-*b*-PBD(2500)) and either PZn₂ (flaccid vesicles, ~7:1 ratio) or the 2,2-dimethoxy-2-phenylacetophenone radical initiator (DMPA, for rigid vesicles only) were dissolved in methylene chloride at a 1:10 molar ratio of polymer to initiator. The solution was then deposited onto a roughened Teflon square and dried overnight under vacuum. 2 mL of 290 mOsm sucrose + 1 mM Calcein fluorophore (Calcein used only in rigid vesicles) was then added to a glass vial containing the film; the vial was then sealed and allowed to hydrate at 65°C for 24 hours. Solutions were vortexed after heating yielding a solution of vesicles ranging in size from approximately 800 nm to 30 μ m diameter. Vesicles were cross-linked by exposing solutions to high intensity UV-light (Omnicure spotcure system, 365 nm, 55 mw cm², using a collimating lens) for 30 min while gently stirring after all assembly and purification steps (described later). Vesicles were suspending in slightly hypo-osmotic solutions during cross-linking to ensure that most vesicles were locked into a spherical shape. Cross-linked vesicles populations were evaluated on an inverted light microscope using DIC optics for changes resulting from UV-illumination and vesicle cross-linking. No differences from flaccid vesicle populations were observed in the size distribution, vesicle morphology, or aggregation.

Labeling with NeutrAvidin

1 mL of the polymer vesicle solution was diluted in 9 mL 290 mOsm PBS (Mg²⁺, Ca²⁺) with 1% BSA (lyophilized, suitable for ELISA). The solution was mixed in tube rotator 10 min. NeutrAvidin was dissolved in DI water at 10 mg/mL and partitioned into 50 μ L aliquots. One aliquot was sonicated for 15 min and spun for 10 min at 10,000 rpm. A volume of 48 μ L was removed from top of tube, added to diluted polymer vesicles, immediately mixed, then placed in a tube rotator for 1 hour.

Separation of vesicles from free NeutrAvidin

A density separation solution with 20% Optiprep density gradient medium, 80% 270 mOsm sucrose solution (v/v) was prepared. The vesicle-NeutrAvidin mixture was transferred to a thin wall polyallomer ultracentrifuge tube (Beckman-Coulter, Fullerton, CA). Using a long syringe, 1.5 mL density separation solution was slowly injected into bottom of tube. The tube was spun in an ultracentrifuge for 30 min at 7500 rpm. Vesicles appeared as a band at the buffer interface and were removed by puncture of the tube and aspiration with a needle

and syringe. A 9 mL density gradient was created in a fresh thin wall polyallomer ultracentrifuge tube, ranging from a 100% density separation solution to 100% PBS solution, using multi-pump head attached to an FPLC (ÄKTA Basic, P-900, GE Healthcare, Schenectady, NY). The solution from the first separation was added to top of tube and was spun in an ultracentrifuge for 30 min at 7500 rpm to create a vesicle band. 1–2 mL final volume of vesicles was removed.

Addition of biotinylated ligand

NeutrAvidin-coated vesicles were diluted in 290 mOsm PBS + 1% BSA (lyophilized, suitable for ELISA) resulting in a final volume of 10 mL. Biotinylated sLe^x was added to diluted vesicles (20 µL, 1 mg/mL in PBS). The vesicle and ligands were immediately mixed, then placed in a tube rotator for 1 hour. Free ligand was removed using the centrifugation as described above, and buffer was exchanged by dialysis with a 20,000 Da MWCO Slide-A-Lyzer dialysis cassette (Pierce Biotechnology) in 1 L DPBS + Mg²⁺ + Ca²⁺. Dialysis buffer was changed twice over a 36 hour period, and the samples were kept at 4°C on stirring plate. Concentrations were verified using absorbance measurements of PZn₂ in the membrane bilayer prior to experiments.³⁹ Ligand concentration and size distribution of vesicles is reported in separate work.³² Cross-linked vesicles could not be independently verified for surface ligand site density because the prolonged UV-illuminated destroyed the activity of any fluorophores.

Micropipette Aspiration

Micropipettes made of borosilicate glass tubing (Friedrich and Dimmock, Milville, NJ) were prepared using a needle/pipette puller (Model 720, David Kopf Instruments, Tujunga, CA) and microforged using a glass bead to give the tip a smooth and flat edge. The inner diameters of the pipettes ranged from 4.7 to 10.4 µm and were measured using optical microscopy and computer imaging software. Pipettes were filled with 290–295 mOsm PBS, and the surfaces were blocked with DPBS + 1% BSA for 2 min. The pipettes were then connected to an aspiration station mounted on the side of a Zeiss inverted microscope, equipped with a manometer, Validyne pressure transducer (models DP 15–32, and DP 103–14, Validyne Engineering Corp., Northridge, CA), digital pressure readouts, micromanipulators (model WR-6, Narishige, Tokyo, Japan), and MellesGriot millimanipulators (coarse *x*, *y*, *z* control). Suction pressure was applied via a syringe attached to the manometer. Vesicle-containing solutions in sucrose/PBS buffer (290–295 mOsm) were diluted with an equal volume of 290–295 mOsm PBS. Pipettes were used to select single vesicles. Pressure was increased stepwise in 5 cm-H₂O increments, and the membrane was allowed one min after each pressure change to equilibrate. Experiments were imaged using DIC optics with a 40x objective and a Cohu black-and-white CCD camera (Cohu, Inc., San Diego, CA). ImageJ software was used to measure membrane extensions and vesicle diameters.⁵² Calculations of tension and area extension were performed using force balances discussed extensively by Evans and coworkers.⁵³

Reflective Interference Contrast Microscopy

A microfluidic chamber fabricated in poly(dimethylsiloxane) was assembled on a P-selectin-coated glass coverslip (#1.5), and the channel was initially filled with 0.22 µm filtered DPBS + Mg²⁺ + Ca²⁺ + 1% BSA buffer. The vesicle solution (diluted in the same DPBS buffer) was then placed at the inlet of the channel and withdrawn by a syringe pump. The contact between vesicles and the glass substrate was visualized by reflective interference contrast microscopy (RICM) using an inverted microscope (Axiovert 200, Karl Zeiss, Gottingen, Germany) with an antilex 63x oil immersion objective and appropriate polarizers. Images were recorded using a CCD camera (Retiga Exi Fast Cooled Mono 12-bit camera. 32–0082B-128 QImaging, Burnaby, Canada) and IPLab software (BD Biosciences,

Franklin Lakes, NJ). The distance between the vesicle membrane and substrate is estimated from the grayscale interference pattern arising from the reflective interference pattern which is a result of the substrate-buffer interface and the membrane-buffer interface. The intensity of the reflective interference, I , depends on the height, h . The mathematics used to calibrate I with h are described elsewhere.^{54, 55} Briefly, the distance between vesicles, membrane and substrate is calculated from the grayscale interference pattern arising from the reflective interference from the substrate-buffer interface and the membrane-buffer interface. The formalized reflectance (or intensity), R_{norm} depends on the height, h as

$$R_{norm} = y_0 - A \cos \left(4\pi \frac{n_{out}}{\lambda} (n - h_0) \right), \quad (1)$$

$$A = 2 \frac{r_{12}}{r_{01}} (r_{01}^2 - 1) \sqrt{1 + \gamma^2 + 2\gamma \cos \delta_2}, \quad (2)$$

$$h_0 = \frac{-\lambda}{4\pi n_{out}} \tan \frac{\gamma \sin \delta_2}{1 + \gamma \cos \delta_2}. \quad (3)$$

$\lambda = 546$ nm (white light emitted by a HBO lamp is filtered using a green filter), $\delta_2 = 4\pi n_2 d / \lambda = 0.1368$ (where $n_2 = 1.486$ and $d = 4$),

$$r_{01} = \frac{(n_0 - n_1)}{(n_0 + n_1)}, \quad (4)$$

$$r_{12} = \frac{(n_1 - n_2)}{(n_1 + n_2)}, \quad (5)$$

$$r_{23} = \frac{(n_2 - n_3)}{(n_2 + n_3)}. \quad (6)$$

Refractive indices:

$$n_0 = n_{\text{glass}} = 1.532$$

$$n_1 = n_{\text{out}} = n_{\text{PBS}} = 1.332$$

$$n_2 = n_{\text{lipid}} = 1.486$$

$$n_3 = n_{\text{in}} = n_{\text{sucrose}} = 1.347$$

$$y_0 = \gamma = \frac{r_{23}}{r_{12}} (1 - r_{12}^2). \quad (7)$$

h_0 is the shortest distance between the vesicle membrane and substrate, and this corresponds to the minimum intensity. For this experiment, the refractive index of polymer was assumed to be approximately 1.486, the reflective index of lipid membranes. The darkest regions correspond to approximately 26.5 nm separation distance, and the lightest regions correspond to 100 nm or larger separation distance.^{54, 55} This minimum distance is non-zero because the refractive indices of the inner and outer buffer are similar. Subsequent rings

around close areas of contact (Newtonian rings pattern) correspond to larger separation distances.

Adhesive Substrate Preparation

Double well, rectangular, flexiperm gaskets (Sigma-Aldrich, Vivascience) were placed on untreated polystyrene slides. #1.5 glass coverslips were treated with 3-aminopropyl trimethoxysilane (silane) according to established protocols⁵⁶ to increase absorption of receptor to substrate surface. Substrates were washed with 0.1 M NaHCO₃ buffer adjusted to pH 9.2 (binding buffer). 375 µL of ICAM-1/Fc at 0, 5, or 10 µg/mL in binding buffer was added to lower well, and 375 µL binding buffer was added to upper well (ICAM-1/Fc was used to titrate the concentration of P-selectin). Receptor was allowed to bind on a laboratory rocker for 2 hours at room temperature. Slides were placed in refrigerator (4°C) overnight. Substrates were then allowed to warm on rocker at room temperature 1 hr. Buffer was then aspirated, and 1 mL binding buffer was used to wash each well. 375 µL P-selectin/Fc at 10 µg/mL in binding buffer was added to lower wells, and 375 µL binding buffer was added to upper wells (except 100% ICAM-1 surface). Receptor was allowed to bind 2 hr on laboratory rocker at room temperature.

Parallel Plate Flow Assay

Substrates were blocked with SuperBlock protein blocking buffer, 1 mL per well 3x. A parallel-plate flow chamber similar to those described previously,⁴ mounted on the stage of a Nikon Diaphot-300 inverted microscope (Nikon, Tokyo, Japan) was used for laminar flow assays. Both straight- and tapered-channel parallel-plate flow chambers were used for laminar flow assays (Figure 5 A–B). The channel templates were cut from 0.01-inch-thick Duralastic sheeting (Allied Biomedical, Goose Creek, SC). For each flow experiment, the template was placed over a P-selectin/ICAM-1 coated polystyrene slide. The template and slide were placed in the bottom well of the flow chamber, and the top was secured with nuts and bolts. The precise location along the flow chamber was recorded digitally at 1 second intervals throughout each experiment during variable shear rate experiments so that the shear rate could be calculated during data analysis. Details regarding the dimensions of the tapered channel are described elsewhere.^{35, 49, 57} Briefly, the tapered channel allows the shear stress to vary along the length of the channel (Figure 5C), and the shear stress is defined according to

$$\tau_w = \frac{6\mu Q}{h^2 w_1} \left(1 - \frac{z}{L}\right), \quad (8)$$

where μ is the fluid viscosity, Q is the volumetric flow rate, h is the channel height, w_1 is the channel width at the entrance in cm, z is the axial position from the inlet, and L is the channel length. For this chamber, $h = 0.023$ cm, $w_1 = 0.1$ cm, and $L = 6.6$ cm. For each experiment using the straight-channel flow chamber, wall shear stress (τ_w) was calculated according to

$$\tau_w = \frac{6\mu Q}{h^2 w}, \quad (9)$$

where μ is the fluid viscosity, Q is the volumetric flow rate, h is the channel height, w is the channel width at the entrance in cm, z is the axial position from the inlet, and L is the channel length. For this chamber, $h = 0.023$ cm, $w = 0.1$ cm. Flow was initiated using a syringe pump (Harvard Apparatus, South Natick, MA), and positions in the flow chamber were monitored using Nikon stage calipers Nikon Inc., Melville, NY). Experiments were

recorded using a Cool-Snap HQ cooled CCD camera and Sony SVO-9500MD S-VHS recorder (Sony Medical Systems, Montvale, NJ). For each experiment, chamber height (gap width) and flow rate were measured to calculate the range of wall shear stresses obtained. A shear rate of 130 or 190 s^{-1} was used for all experiments with the straight-channel flow chamber because this is representative of flow within postcapillary venules.⁵⁸ Vesicle interactions with the substrate were observed along the center axis of the flow chamber and recorded for later analysis.

Image Analysis and Particle Tracking

The particle tracking algorithm is based in the MATLAB software suite, using the image processing toolbox. Two minute avi files were created for each location in the flow chamber observed during an adhesion experiment: three locations on the functionalized surface, two locations on the control surface. Automated particle tracking is described elsewhere.^{31, 32} Briefly, particles are identified in each frame by first thresholding to create a binary image and then using intrinsic MATLAB functions to count and determine properties for each particle (i.e. diameter, eccentricity, solidity). Each particle in frame n is then compared to each particle in frame $n+1$ to construct trajectories and classify the type of movement (firm adhesion, rolling, saltation) based on the particle size and free stream velocity at its centroid. After particle tracking is complete, broken trajectories are reconstructed and noise is filtered by eliminating any particle that interact for less than 30 frames (1 second) or do not roll or firmly adhere during the trajectory. Firm binding is classified as the centroid of a particle moving less than 1.44 μm between frames for 150 consecutive frames or more (5 seconds). Stable rolling is classified as a particle centroid moving more than 1.5 pixel but less than 45% the free stream velocity at the particle centroid (calculated based on Poiseuille flow) for greater than 10% of the entire trajectory of the particle. Transient rolling is classified as a particle that interacts for at least 30 frames but roll for less than 10% of the trajectory of the particle. Rolling+binding vesicles are classified as particle that meets the criteria for firm binding and makes rolling movements during the trajectory.

Conclusion

We show that vesicle rigidity affects the capture and rolling of sLe^x-coated vesicles on P-selectin-coated substrates. Populations of flaccid vesicles and single flaccid vesicle experiments illustrate an increased rate of recruitment and a preponderance of rolling vesicles, compared to rigid vesicles. Sialyl Lewis X-coated vesicles that do roll, roll with similar velocities in both flaccid and rigid vesicle populations, showing that rolling velocity is not strong a function of interfacial rigidity. Site densities of sLe^x are high enough that firm adhesion occurs in both flaccid and rigid vesicle populations.

Additionally, RICM experiments show that vesicle rolling of flaccid vesicles on P-selectin substrates does not require close approach between the vesicle and the surface. Instead, vesicles remain approximately 100 nm above the substrate and only form close contacts after they come to full arrest. Finally, adhered flaccid vesicles do not spread substantially on substrate surfaces, suggesting the adhesive energy cannot overcome the membrane elasticity, even in flaccid vesicles.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Micropipette aspiration was used to test the rigidity of polymer vesicles. Flaccid vesicles extended into the pipette in response to suction pressure, 7 μm ID, 30 cm H_2O (A). Rigid vesicles did not extend into the pipette in response to pressure, 5.4 μm ID, >90 cm H_2O (B).

Figure 2.

Flaccid membranes bend in response to force (A). Rigid membranes do not bend in response to force, but the PEO brush is unaffected by cross-linking and is able to move and extend (B). Sialyl Lewis X coated vesicles rolling over P-selectin-coated substrates can remain separated from the substrate by approximately 100 nm because the PEO brush, NeutrAvidin, sLe^x polymer and P-selectin can all be fully extended (C).

Figure 3.

Flaccid sLe^x-coated vesicles bound to P-selectin substrates with large asymmetric shaped regions indicated by the dark region in the center (upper panel). The size and shape of these regions indicate that these vesicles deformed as they bound the substrate. Rigid vesicles adhered with small circular regions (lower panel). Note that the center of the bound rigid vesicle remains spherical as indicated by the well-defined Newton's ring pattern. Flow moved from right to left in these images.

Figure 4.

Still images from RICM flow experiments on flaccid vesicles at $\gamma = 510 \text{ s}^{-1}$ on 100% P-selectin substrate (A) and $\gamma = 200 \text{ s}^{-1}$ on 30% P-selectin substrate (B). Spreading occurred over approximately 30 seconds in both cases. Images were collected over a period of approximately 30 sec, and images proceed in time from the left to right panel. Flow moved from right to left in these images.

Figure 5. Flow chambers used in this work. Straight channel flow chamber allows for single shear rate adhesion data (A). The tapered channel design (Usami flow chamber) (B) allows for variable shear rate experiments in which the shear rate is dependent on the z -distance from the start of the chamber (C).

Figure 6. Adhesive flux of flaccid and rigid sLe^x vesicles on various P-selectin/ICAM-1 substrates (ICAM-1 served to titrate the concentration of P-selectin). A minimum of 6 measurements were performed for each bar.

Figure 7. Rolling velocity distributions of stable rolling flaccid and rigid vesicles on 100% P-selectin substrates at two shear rates (130 s^{-1} and 190 s^{-1}). Distributions represent between 284 and 1540 individual vesicles each.

Figure 8. Slopes of rolling velocity as a function of shear rate for individual flaccid and rigid vesicles. Slopes for these two populations are plotted to show the distributions (A). Representative trajectories for each population of vesicles (flaccid tight-binding, flaccid weak binding, rigid tight-binding) that responded to decreasing shear rate with decreasing rolling velocity (B).

Figure 9.

Representative trajectories for vesicles which were unresponsive to changing shear rate and rolled for long periods of time at a single velocity and typically ended their trajectories with firm arrest.

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Table 1

Relative percent of total adhesive events for sLe^x-coated vesicles adhering to P-selectin substrates in constant shear flow, $\gamma = 130\text{ s}^{-1}$. A minimum of 6 measurements were performed for each column in this table.

	100% P-sel		50% P-sel		30% P-sel	
	Flaccid	rigid	flaccid	rigid	flaccid	rigid
unstable rolling	19 ± 5	28 ± 5	16 ± 3	16 ± 1	5 ± 2	19 ± 5
stable rolling	63 ± 13	61 ± 7	64 ± 6	73 ± 2	28 ± 7	55 ± 12
rolling+binding	10 ± 3	13 ± 3	4 ± 1	2 ± 1	0 ± 0	0 ± 0
firm adhesion	27 ± 6	24 ± 5	24 ± 3	12 ± 2	67 ± 54	24 ± 5