Tunable Leuko-polymersomes That Adhere Specifically to Inflammatory Markers

Gregory P. Robbins,† Randi L. Saunders,‡ Jered B. Haun,‡ Jeff Rawson,§ Michael J. Therien,§ and Daniel A. Hammer*†,‡,§

†Department of Chemical and Biomolecular Engineering, School of Engineering and Applied Sciences, University of Pennsylvania, Philadelphia, Pennsylvania 19104, ‡Department of Bioengineering, School of Engineering and Applied Sciences, University of Pennsylvania, Philadelphia, Pennsylvania 19104, and §Department of Chemistry, Duke University, Durham, North Carolina 27708. Current address: Center for Systems Biology, Massachusetts General Hospital, Harvard Medical School, 185 Cambridge Street, Boston, Massachusetts 02114.

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The polymersome, a fully synthetic cell mimetic, is a tunable platform for drug delivery vehicles to detect and treat disease (theranostics). Here, we design a leuko-polymersome, a polymersome with the adhesive properties of leukocytes, which can effectively bind to inflammatory sites under flow. We hypothesize that optimal leukocyte adhesion can be recreated with ligands that mimic receptors of the two major leukocyte molecular adhesion pathways, the selectins and the integrins. Polymersomes functionalized with sialyl Lewis X and an antibody against ICAM-1 adhere avidly and selectively to surfaces coated with inflammatory adhesion molecules P-selectin and ICAM-1 under flow. We find that maximal adhesion occurs at intermediate densities of both sialyl Lewis X and anti-ICAM-1, owing to synergistic binding effects between the two ligands. Leuko-polymersomes bearing these two receptor mimetics adhere under physiological shear rates to inflamed endothelium in an in vitro flow chamber at a rate 7.5 times higher than those to uninfamed endothelium. This work clearly demonstrates that polymersomes bearing only a single ligand bind less avidly and with lower selectivity, thus suggesting proper mimicry of leukocyte adhesion requires contributions from both pathways. This work establishes a basis for the design of polymersomes for targeted drug delivery in inflammation.

Introduction

Inflammation is the process by which the body recruits and activates leukocytes at sites of infection, but an overzealous inflammatory response can create deleterious physiological effects. Therefore significant effort has been made toward developing targeted therapies to treat inflammation.1,2 The two major classes of adhesion molecules upregulated during inflammation, adhesion molecules and selectins, are natural targets for diagnostic and therapeutic particles,3–5 but particles must be designed to bind sites of inflammation selectively.6 Intercellular adhesion molecules-1 (ICAM-1), which is upregulated during inflammation, is expressed at low levels throughout uninfamed endothelium,7 so targeting this molecule alone with a high affinity probe would result in binding to healthy endothelium. P-selectin-mediated adhesion plays a major role in leukocyte recruitment8,9 and, unlike ICAM-1, is only present in inflamed tissues. Selectin-mediated bonds, however, are fast, weak catch-slip interactions that do not typically mediate firm adhesion by themselves.10–13 In this paper, we explore the design of a colloidal mimetic of leukocytes that combines two molecules and thus is designed to preferentially bind to inflamed tissues that express P-selectin and upregulate ICAM-1 with specificity and yield.

Because blood cells, such as neutrophils, lymphocytes, and platelets, have evolved to use two adhesion molecules simultaneously, one can question if there is an inherent advantage for using two adhesion molecules rather than one. Our laboratory previously showed that the simultaneous targeting of both

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


selectins and ICAM-1 results in super adhesion of porous polymeric particles compared with particles targeting one molecule alone. For example, it was shown that firm adhesion to surfaces coated with P-selectin and ICAM-1 could be greatly enhanced with particles that bore the same concentration of anti-ICAM-1 antibody if sialyl Lewis X (sLe X), a carbohydrate that mediates rolling adhesion, was added to the particles. The concept that rolling can mechanistically facilitate firm adhesion has also been predicted by computer simulations of adhesion in our laboratory.  

Here, we describe the preparation and performance of leuko-polymersomes, in which two adhesion molecules are attached to a polymersome. Polymersomes, fully synthetic and biocompatible analogs of liposomes assembled from block copolymers, are an ideal choice as the underlying colloid for a leukocyte mimic. Polymersomes have been used as an in vivo imaging agent and drug carrier. Polymersomes are significantly stronger and have much thicker membranes than liposomes, allowing them to carry large amounts of hydrophobic cargo within the membrane core, as well as aqueously soluble agents within the vesicle lumen. Ligands, such as antibodies and peptides, can be attached to the exterior of these vesicles without destruction of the vesicular structure. Storage of large proteins and activated release of contents have also been demonstrated in polymersome systems. In this work, we show that the ratio of rolling and firm adhesion ligands on the polymersome surface can be tuned and that we can adjust the adhesivity of a leuko-polymersome to a specific substrate by adjusting this ratio of ligands on the vesicle surface. We demonstrate how our tunable design allows us to increase the adhesivity of a vesicle to endothelium bearing inflammatory molecules while simultaneously decreasing the adhesivity of these particles for uninflamed endothelium. Finally, we show that one of our optimal leuko-polymersome constructs binds selectively to inflamed HUVECs compared to uninflamed cells in vitro under hydrodynamic flow.

Materials and Methods  

Polymersome Assembly. The polymersomes were prepared as described previously. Briefly, the biocytin-terminolated polymer (PEO(1300)-b-PBD(2500)) (PEO = polyethyleneoxide; PBD = polybutadiene); Polymer Source, Inc., Montreal, Quebec) and the fluorophores (PZn2) were dissolved in methylene chloride at a 7.5:1 molar ratio of polymer to fluorophore. The solution was vortexed after heating yielding a solution of vesicles ranging in size from approximately 80 nm to 30 μm diameter. Vesicles used for human umbilical vein endothelial cell (HUVEC) experiments were hydrated with 2 mL of 290 mOsm sucrose +20 μL 10 mg/mL Alexa Fluor-488-labeled 3000 Da dextran (Invitrogen, Carlsbad, CA).

Vesicles used as size standards for flow cytometry were further prepared with serial extrusion using a Liposofast Basic hand-held extruder equipped with 5 μm, 1 μm, and 400 nm polycarbonate membranes (Avestin Inc., Ottawa, Ontario). Relative concentrations of polymersomes were determined based on Beer’s Law and the established extinction coefficient of PZn2 at 485 and 708 nm (εPZn2(485) = 294 875 M⁻¹ cm⁻¹, εPZn2(708) = 59 000 M⁻¹ cm⁻¹).  

Synthesis of Biocytin Modified Polymer. The terminal end of the PEO block was modified to display biocytin through a two-step synthesis previously described. Briefly, 4-fluoro-3-nitrobenzoic acid was attached to the hydroxyl polymer terminus through an esterification in methylene chloride. After high-performance liquid chromatography (HPLC) purification, biocytin was attached to the modified polymer through a nucleophilic aromatic substitution in 50% tetrahydrofuran (THF)/50% deionized (DI) water. This polymer was purified after the reaction using HPLC separation. Characterization of the final polymer using NMR showed that 88% of the polymer was modified with biocytin.  

Association of Ligands and Separation of Free Ligand. One milliliter volumes of polymersomes post assembly (approximately 0.1 mM polymer) were diluted in 9 mL phosphate-buffered saline (PBS) +1% bovine serum albumin (BSA). Fifty microliters of NeutrAvidin at 10 mg/mL in DI water was added to solution, and vesicles were allowed to bind 1 h in a tube rotator at room temperature (RT). Vesicles were then separated twice. The first separation was performed by adding a cushion (20% Optiprep density gradient medium, Sigma-Aldrich, 80% 270 mOsm sucrose) to the bottom of a centrifuge tube containing diluted polymersome tubes. Tubes were spun 30 min at 7500 rpm and 1–2 mL volume of concentrated vesicles was removed. Nine milliliter density gradients ranging from 100% Optiprep/sucrose to 100% PBS were made. Concentrated vesicles were separated again at 7500 rpm for 30 min. Vesicles diluted to 10 mL in PBS +1% BSA, and α-ICAM-1 and/or sLe X were added to solution. Binding and separation steps were identical to the steps described for association/separation of...
NeutrAvidin. Vesicles were dialyzed in 2 L of Dulbecco’s (DPBS) + Mg^{2+} + Ca^{2+} with two changes of buffer over a period of 36 h before adhesion experiments. Concentrations were verified using absorbance of Pzn in a membrane bilayer prior to experiments. Vesicles used for HUVEC experiments were diluted to approximately 4.8 × 10^6 particles/mL for all experiments; vesicles were diluted at a ratio of 2:1 media to diazylized vesicles using L-15 media (Lonza, Walkersville, MD).

**Flow Cytometry.** Ligand-coated vesicles were assembled according to method described in this work. Vesicles (0.5 mL) were diluted to 5 mL in PBS + 1% BSA and either 20 μL of 0.5 mg/mL mouse antihuman CD15s (BD Pharmingen) or 20 μL of 0.5 mg/mL fluorescein isothiocyanate (FITC)-rat antimouse Ig, κ light chain monoclonal antibody (BD Pharmingen), was added to the solution and allowed to bind 1 h in the tube rotator. For SLe^a vesicles labeled with antihuman CD15s, vesicles were separated once in a centrifuge using an OptiPrep/sucrose cushion. Vesicles were immediately diluted to 5 mL in ice-cold PBS + 1% BSA, and 20 μL of 0.5 mg/mL FITC-rat antimouse Ig, κ light chain monoclonal antibody (BD Pharmingen) was added to the solution and allowed to bind 1 h in tube rotator at 4 °C. Vesicles were separated again using an OptiPrep/sucrose cushion.

Flow cytometry was performed on a Guava EasyCyte flow cytometer (Guava Technologies, Hayward CA.; CyteSoft version 3.6 software) immediately after separation. Samples diluted to approximately 500 vesicles/μL in PBS, and 20,000 points were collected for each sample. Extruded samples of known size distributions were analyzed to calibrate forward scatter with approxi-mately 4.8 × 10^6 particles/mL for all experiments; vesicles were diluted at a ratio of 2:1 media to diazylized vesicles using L-15 media (Lonza, Walkersville, MD).

**Confocal Light Scanning Microscopy (CLSM).** Experiments were carried out on an Olympus Fluoview FV1000 confocal microscope (Center Valley, PA), equipped with a UPLFLN 40× oil objective lens. A 488 nm laser was used to image FITC and Alexa-488 labeled proteins, and 633 nm laser was used to image Pzn-loaded polymersome membrane. Typical scan speeds were between 10 μs/pixel and 20 μs/pixel, and 3–4 scans using a Kahlman filter were used to acquire final images.

**Substrate Site Density Determination.** The surface ELISA method was used to characterize the site densities of P-selectin/Fc and ICAM-1/Fc. Flexiperm gaskets were placed in the wells of 12-well untreated polystyrene plates. Substrates were prepared as previously described using 165 μL of receptor solution rather than 375 μL. After substrate prep, each well was then washed two times with 1 mL of ice-cold PBS, and gaskets were removed. Each well was then washed one time with 2 mL of ice-cold PBS. Two milliliters of ice-cold starting buffer protein blocking buffer (Pierce Biotechnology) was added to each well and aspirated. The wells were washed again two times with 2 mL of ice-cold PBS. A 0.5 μL portion of 5 μg/mL antibody in PBS (mouse antihuman P-selectin monoclonal ab or mouse antihuman ICAM-1 monoclonal ab, R&D Systems) was added to each well. Antibodies were allowed to bind 1 h on a laboratory rocker at 4 °C. The antibody solution was aspirated, and each well was washed with ice-cold PBS three times (1 mL 1X, 2 mL 2X). A 0.5 μL portion of horseradish peroxidase (HRP) rat-antimouse IgG monoclonal antibody (50:1 dilution in PBS, BD Pharmingen) was added to each well. Antibodies were allowed to bind 1 h on a laboratory rocker at 4 °C. The buffer aspirated, and the surfaces were washed three times (1 mL 1X, 2 mL 2X). 100 μL of PBS + 300 μL of tetramethylbenzidine (TMB) substrate (TMB Substrate Kit, Pierce Biotechnology) was added to each well. Reactions were allowed to proceed 10 min and 20 s on a laboratory rocker at RT. The reaction was quenched with 1 mL 1 N H2SO4. The absorbance was read on a plate reader (Tecan Infinite M200 Männedorf, Switzerland) at 450 nm. Twelve-well tissue culture-treated polystyrene plates were used to create the calibration curve. Wells blocked with 2 mL of starting block were then washed with 2 mL of PBS per well four times. A 100 μL portion of HRP biotin (Pierce Biotechnology) was added to wells at concentrations between 0 and 0.1 μg/mL. TMB substrate (300 μL) was added to each well. Reactions were allowed to proceed 10 min and 20 s on a laboratory rocker at RT. Reactions were then quenched with 1 mL of 1 N H2SO4. The absorbance was read on a plate reader at 450 nm.

**ICAM Immunostaining and Upregulation Quantification.** HUVECs were cultured in EGM endothelial growth media (Lonza, Walkersville, MD) supplemented with 0.4% bovine brain extract (BEE) with heparin, 0.1% human epidermal growth factor (h-EGF), 0.1% hydrocortisone, 0.1% gentamicin sulfate (GA-1000), and 2% fetal bovine serum (FBS). Cells were maintained in plastic culture flasks at 37 °C in a humidified atmosphere containing 5% CO2 in air and were subcultured when the flasks were 70–90% confluent. HUVECs were used between passages 5–7. Cells were activated by incubating with recombinant tumor necrosis factor-α (TNF-α; R&D Systems) at 10 ng/mL for 6 h. This incubation causes HUVEC cells to upregulate expression of inflammatory markers. ICAM-1 was measured using a surface ELISA as described previously. Vesicles were dialyzed 2 L of Dulbecco’s (DPBS) + Mg2+ + Ca2+ with two changes of buffer over a period of 36 h before adhesion experiments. Concentrations were verified using absorbance of Pzn in a membrane bilayer prior to experiments. Vesicles used for HUVEC experiments were diluted to approximately 4.8 × 10^6 particles/mL for all experiments; vesicles were diluted at a ratio of 2:1 media to diazylized vesicles using L-15 media (Lonza, Walkersville, MD).

**Cell Culture and Activation.** HUVECs were cultured in EGM endothelial growth media (Lonza, Walkersville, MD) supplemented with 0.4% bovine brain extract (BEE) with heparin, 0.1% human epidermal growth factor (h-EGF), 0.1% hydrocortisone, 0.1% gentamicin sulfate (GA-1000), and 2% fetal bovine serum (FBS). Cells were maintained in plastic culture flasks at 37 °C in a humidified atmosphere containing 5% CO2 in air and were subcultured when the flasks were 70–90% confluent. HUVECs were used between passages 5–7. Cells were activated by incubating with recombinant tumor necrosis factor-α (TNF-α; R&D Systems) at 10 ng/mL for 6 h. This incubation causes HUVEC cells to upregulate expression of inflammatory markers. ICAM-1 was measured using a surface ELISA as described previously.
The fluorescence intensity was then read on a LICOR Odyssey and analyzed with the LICOR Odyssey analysis software version 2.1: background fluorescence was subtracted from all signals. HUVECs treated with recombinant TNF-α had an adjusted fluorescence intensity of 0.946, and the untreated HUVECs had an adjusted fluorescence intensity of 0.748. A Student t test with a p-value of 0.022 shows that incubation with TNF-α statistically increases the level of ICAM expressed by HUVECs above the basal level.

**Monolayer Assembly.** A culture surface was created by attaching a 30 × 5 mm flexiPERM slide to a clean 75 × 25 mm microscope slide. The chamber was then filled with 0.5 mL of 0.1 mg/mL fibronectin (Sigma-Aldrich, St. Louis, MO) in PBS and incubated at room temperature for 1.5 h. The fibronectin solution was then aspirated, and 90,000 cells/chamber were plated and incubated for 2 days to create a confluent monolayer.

**Parallel Plate Flow Assay.** Substrates were blocked with SuperBlock protein blocking buffer (Pierce Biotechnology), 1 mL per well three times. A parallel-plate flow chamber similar to those described previously,24 mounted on the stage of a Nikon Diaphot inverted microscope (Nikon, Tokyo, Japan), was used for laminar flow assays. Flow was initiated using a syringe pump (Harvard Medical Systems, Montvale, NJ). Experiments were recorded using a Cool-Snap HQ cooled CCD camera and Sony SVO-9500MD S-VHS recorder (Sony 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 wall shear stress obtained. A shear rate of 130 s⁻¹ was used for all experiments because this is representative of flow within post-capillary venules.32 Vesicle interactions with the surface were observed along the center axis of the flow chamber and recorded for later analysis. Experiments with HUVEC monolayers were performed using the same flow chambers and microscope (cells maintained at 37 °C in L-15 media buffered for use in non-CO₂ equilibrated environments). Four or five fields of view were chosen for each experiment, and confluent regions of cells (at similar locations within the chamber for each experiment) were chosen. Fluorescent imaging was used so that vesicles could be easily identified. Images were collected every 6 s.

**Image Analysis and Particle Tracking.** For HUVEC experiments, firmly adhered vesicles were counted. The particle tracking algorithm (used for all adhesion experiments except those performed on cell monolayers) 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. Particles were 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 was then compared to each particle in frame n+1 to construct trajectories and classify the type of movement (firm adhesion, rolling, transient adhesion) based on the particle size and free stream velocity at the vesicle centroid. After particle tracking was complete, broken trajectories were reconstructed, and noise was filtered by eliminating any particle that interacted for less than 30 frames (1 s) or did not roll or firmly adhere during the trajectory. Firm binding is classified as the centroid of a particle moving less than 1.5 pixel.

**Results and Discussion**

Ligand-coated emissive polymersomes22 were built by first assembling vesicles from a biotin-terminated block copolymer and PZn₃ fluorophore,30 then saturating the surfaces with NeuAvidin (referred to as “avidin”) and biotinylated ligands in subsequent steps, as illustrated in Figure 1. A previously published reaction—an esterification followed by an aromatic substitution (Supporting Information)—was used to attach biotin to the hydrophilic (polyethylene-oxide) end of the copolymer.24,29 The final reaction efficiency was determined to be 88% by NMR. Aliquots of this product (biotin-polyethyleneoxide-b-polyybutadiene) were used, without further modification or blending, for all experiments in order to ensure consistency between samples, and synthesis of a biotin-terminated copolymer allows for the assembly of an effectively fully biotinylated polymersome surface. CLSM was used to confirm the presence of both avidin and the target ligand on vesicle surfaces, and there was no evidence of ligand clustering when both ligands were attached to the vesicle surface (Supporting Information).

Quantitative surface site-density measurement of the targeting ligands sLeX (PSGL-1 analog) or anti-ICAM-1 antibody (LFA-1 analog) on avidin-coated vesicles was determined using flow cytometry. First, the total number of accessible biotin-binding pockets on avidin-coated vesicles was determined by binding FITC-tagged 3000 Da biotinylated dextran to a population of vesicles and comparing to calibrated fluorescent standards.35 Second, a FITC-labeled κ-light chain specific monoclonal antibody was bound to 100% α-ICAM-1-coated vesicles to determine


the site density of the α-ICAM-1 antibody on the vesicle surfaces. These measurements yield site densities of 3560 ± 760 sites/μm² and 2890 ± 150 sites/μm² for the 100% dextran and 100% α-ICAM-1 antibody-coated polymersomes, respectively. This density is approximately 50% of the maximum possible biotin-binding sites, based on closest packing of avidin molecules on a flat surface. The maximum sLe^x site density is approximated to be 3560 ± 760 sites/μm², the measured maximum site density of dextran. sLe^x site densities for α-ICAM-1/sLe^x vesicles were calculated using the site density balances (Figure 2c); while we saw positive anti-sLe^x antibody signals (Figure 2a), we were unable to achieve strong enough binding of antibodies to sLe^x to specifically quantify the number of sLe^x sugars on the vesicle surface. These maximal achievable site densities are far greater than those measured for activated leukocytes, which have site densities of approximately 50–350 sites/μm² LFA-1 and 60–200 sites/μm² PSGL-1. Having an excess in ligand density on our leuko-polymersome is warranted since leukocytes can cluster adhesion ligands, which leads to more effective adhesion than uniformly coated cells would achieve. Histograms of FITC signals from populations of α-ICAM-1/sLe^x vesicles in which the α-ICAM-1 antibodies were labeled are shown in Figure 2b; this figure clearly shows an increased fluorescent signal corresponding to increased concentrations of α-ICAM-1 on polymersome surfaces (B). Site densities for these different populations were determined based on the mean fluorescence intensities of the various samples and a calibration curve based on FITC-coated beads (C), and these site densities show that the ratio of ligands on the polymersome surfaces closely correlates to the ratio of ligand in solution during association of biotinylated ligand with avidin-coated polymersomes (D). Data represent the mean ± standard error of the mean (SEM) for eight experiments.

Figure 2. FITC intensity histogram for a fully sLe^x-coated polymersome labeled with a primary monoclonal mouse antibody against sLe^x and a secondary FITC-labeled κ-light chain specific antibody confirms sLe^x is present on vesicle surfaces (A). FITC intensity histogram for various α-ICAM-1 + sLe^x-coated vesicles tagged with an FITC-labeled κ-light chain specific antibody. Results show an increased fluorescent signal corresponding to increased concentrations of α-ICAM-1 on polymersome surfaces (B). Site densities for these different populations were determined based on the mean fluorescence intensities of the various samples and a calibration curve based on FITC-coated beads (C), and these site densities show that the ratio of ligands on the polymersome surfaces closely correlates to the ratio of ligand in solution during association of biotinylated ligand with avidin-coated polymersomes (D). Data represent the mean ± standard error of the mean (SEM) for eight experiments.

physiological shear rate of 130 s$^{-1}$, with 24 combinations of vesicle/substrate functionalities. Substrate site densities were determined using surface ELISA. The specific site densities for the four substrates used in this study are indicated in Table 1.

These four surfaces provide a range of inflammatory marker concentrations similar to those measured for endothelial cells during cytokine induced inflammation. In addition to quantifying the total amount of adhesion, we quantified the amounts of rolling, firm adhesion, and transient adhesion (or saltation) to gain insight into the mechanisms of adhesion. The use of these substrates facilitated repeatable experiments with uniform ligand site density and uniform shear force, and the simplicity and cleanliness of the substrates allowed for the collection of accurate rates of transient and rolling adhesion. The total rates of binding for all vesicle/substrates combinations tested are shown in Figure 3 (note: ordinate scales are different for each panel so that details are not obscured). Supporting Information Figures S5 and S6 show aggregates of the data in Figure 3 and representative trajectories, respectively.

The inclusion of sLe$^\alpha$ on vesicles is required for significant adhesion on any substrate that bears P-selectin. It is known that under physiological conditions, sLe$^\alpha$–P-selectin interactions mediate tethering and rolling owing to the fast on-rate for sLe$^\alpha$ with P-selectin, which facilitates these interactions.

As expected, interactions between sLe$^\alpha$ on vesicles and P-selectin is important for significant adhesion. Indeed, the absence of sLe$^\alpha$ (on 100% α-ICAM-1 vesicles) shows that the characteristic interactions between antibody and ICAM-1 are a significant impediment to binding under flow. The 100% α-ICAM-1 vesicles only bound in significant numbers on high ICAM-1 density substrates, but even small decreases in the site density of α-ICAM-1 significantly impair the ability of vesicles to bind to this substrate, as demonstrated by the 20% and 35% sLe$^\alpha$ vesicles (80% and 65% α-ICAM-1). In fact, these two vesicles interacted sparsely on most substrates because neither of the site densities of sLe$^\alpha$ were high enough to promote rolling on P-selectin substrates nor were the α-ICAM-1 site densities high enough to promote firm adhesion on high density ICAM-1 substrates.

Table 1. Substrate Site Densities Determined by Surface ELISA

<table>
<thead>
<tr>
<th>substrate name</th>
<th>sites/ $\mu$m$^2$</th>
</tr>
</thead>
<tbody>
<tr>
<td>100% ICAM-1</td>
<td>861 ± 21$^a$</td>
</tr>
<tr>
<td>P-selectin/Fc/P-sel/Fc</td>
<td>0</td>
</tr>
<tr>
<td>50% P-selectin</td>
<td>547 ± 188*</td>
</tr>
<tr>
<td>ICAM-1/Fc/Psel/Fc</td>
<td>542 ± 135*</td>
</tr>
<tr>
<td>30% P-selectin</td>
<td>970 ± 163*</td>
</tr>
<tr>
<td>ICAM-1/Fc/P-sel/Fc</td>
<td>424 ± 30*</td>
</tr>
<tr>
<td>100% P-selectin</td>
<td>476 ± 37$^a$</td>
</tr>
<tr>
<td>ICAM-1/Fc/P-sel/Fc</td>
<td>0</td>
</tr>
</tbody>
</table>

$^a$ Data points represent the mean ± SEM for four experiments ($^o$) or two experiments ($^*o$).

it is not surprising that the 100% sLe$^\alpha$ vesicles bind at a higher rate on P-selectin than any other vesicle and that sLe$^\alpha$ is required for significant vesicle accumulation. Indeed, the absence of sLe$^\alpha$ (on 100% α-ICAM-1 vesicles) shows that the characteristic interactions between antibody and ICAM-1 are a significant impediment to binding under flow. The 100% α-ICAM-1 vesicles only bound in significant numbers on high ICAM-1 density substrates, but even small decreases in the site density of α-ICAM-1 significantly impair the ability of vesicles to bind to this substrate, as demonstrated by the 20% and 35% sLe$^\alpha$ vesicles (80% and 65% α-ICAM-1). In fact, these two vesicles interacted sparsely on most substrates because neither of the site densities of sLe$^\alpha$ were high enough to promote rolling on P-selectin substrates nor were the α-ICAM-1 site densities high enough to promote firm adhesion on high density ICAM-1 substrates.

As expected, interactions between α-ICAM-1 antibody and ICAM-1 promote predominantly firm adhesion, and sLe$^\alpha$–P-selectin interactions result in primarily rolling interactions. Perhaps owing to the extremely high density of sLe$^\alpha$ achievable on leuko-polymersomes, 100% sLe$^\alpha$ vesicles firmly bind to 100% P-selectin and 50% P-selectin substrates. Firm adhesion is not

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often observed in selectin-mediated adhesion. Although the \( sLe^x \) polymer used is a tetramer, it is unlikely that more than one sugar on each polymer could bind a P-selectin molecule simultaneously because of steric hindrances. The close proximity of multiple sugars to any \( sLe^x \)–P-selectin bond, however, increases the association rate of a new \( sLe^x \)–P-selectin bond as mature bonds break, thus facilitating the observed rates of firm adhesion.

The goal of tuning the adhesion of a population of vesicles for a target substrate using a specific combination of two ligands is demonstrated in the adhesion of mixed-ligand vesicles. Vesicles made with either 50% \( sLe^x \) or 65% \( sLe^x \) exhibit the highest rates of binding for substrates that imitate inflamed tissue. The 50% \( sLe^x \) vesicle showed a high rate of firm adhesion to the 30% P-selectin substrate, presumably because of a high number of \( \alpha \)-ICAM–ICAM-1 interactions, while the 65% \( sLe^x \) vesicles bound most frequently to the 50% P-selectin substrate, presumably because the increased \( sLe^x \) could more effectively engage P-selectin. The main difference between these two vesicles is the increased rate of rolling adhesion observed between the 65% \( sLe^x \) vesicles and high P-selectin density substrates. It is clear from these experiments that there is a threshold number of \( \alpha \)-ICAM–ICAM-1 bonds required to bring a slow rolling vesicle to firm arrest, but once enough \( \alpha \)-ICAM-1 is present on this vesicle, \( sLe^x \)–P-selectin bonds are more important in increasing the total binding flux. On the 30% P-selectin substrate, in which the 50% \( sLe^x \) vesicles bind most frequently, a threshold number of \( sLe^x \)–P-selectin bonds are required to slow the vesicle, but the concentration of P-selectin on the substrate is not sufficiently high to stop vesicles at a high rate. In this case, \( \alpha \)-ICAM-1–ICAM-1 interactions are needed to promote significant firm adhesion. These results indicate that the composition of the biomimetic surface has to be carefully tuned to maximize adhesion to a target substrate.

In order to evaluate our hypothesis that two-ligand vesicles adhere at a higher rate and with more selectively than vesicles bearing single ligands, we tested the rate of binding for several populations of vesicles on monolayers of HUVEC cells. Two populations of cells were used, a control population of uninflamed cells, and an inflamed population of cells that had been stimulated with TNF-\( \alpha \) to activate the inflammatory pathway according to established methods. Three vesicular populations, 50% \( sLe^x \)/50% \( \alpha \)-ICAM-1, 100% \( \alpha \)-ICAM-1, and 100% \( sLe^x \), were evaluated for total binding events at \( \gamma = 130 \text{ s}^{-1} \) in a parallel plate flow chamber. These results are shown in Figure 4. An additional fluorophore was encapsulated in the aqueous lumen of these vesicles to allow accurate identification of vesicles on the cell substrates. Fluorescent image capture, however, was too slow to capture fast events such as transient and rolling adhesion, so firm adhesion was used as a measure of binding flux. The best binder was the 50% \( sLe^x \) vesicle with a 7.5-fold increase in binding rate to inflamed versus uninflamed cells. Vesicles coated with 100% \( \alpha \)-ICAM-1 bound at a high rate to the inflamed cells (approximately 66% the rate of the 50% \( sLe^x \) vesicle), but as expected, binding was not selective because these high density \( \alpha \)-ICAM-1 vesicles bound to uninflamed and inflamed cells at equal rates. Additionally, there was a wide distribution in the rate of polymersome binding in different locations on the monolayer, suggesting that local shear rate (dependent on cell confluence) plays a significant role in the binding of these vesicles. It is hypothesized that selectivity could be achieved with \( \alpha \)-ICAM-1 vesicles by decreasing the site density of antibody on the vesicle surface, but this treatment would also cause a decrease in the rate of adhesion to inflamed tissues. The 100% \( sLe^x \) vesicles bound selectively, with a 5.6-fold selectivity in binding between uninflamed and inflamed cells, but these vesicles bound at the lowest rate. Additionally, the shear rate used for these experiments is at the low end of venous shear rates, so we expect that binding rates for 100% \( sLe^x \) vesicles will decrease with increased shear rate, in the mid range of venous shear rates seen in circulation, as \( sLe^x \)–P-selectin bonds are weak and tend not to promote firm binding.

**Conclusions**

We have described the construction and characterization of a leuko-polymersome, a polymersome with the adhesive properties of leukocytes. Through biotin–avidin chemistry, multiple ligands can be titrated onto the surface of these vesicles to create a multifunctional leukocyte mimic.

Additionally, this work demonstrates the simultaneous use of two adhesive ligands, which mimic the adhesive ligands on activated leukocytes, as a strategy to selectively mediate binding of vesicles to inflammatory substrates. The synergy, employed in nature, of fast, weak selectin-mediated bonds in combination with strong adhesion molecule-mediated bonds, allows firm binding to occur on relevant substrates at physiological shear rates. By tuning the ratio of ligands, the rate and type of adhesive interaction can be tuned to the adhesive characteristics of a specific substrate. Binding can be enhanced on certain substrates and simultaneously diminished on other substrates by tuning the ligand ratio on the leuko-polymersome. Finally, we show, using HUVECs, that leuko-polymersomes characterized on synthetic surfaces display selective binding to inflamed endothelium under flow.

The adhesiveness of this polymersome can be combined with other unique features of polymersome technology, such as their ability to encapsulate drugs and image contrast agents to create a theranostic particle for inflammation that can image and deliver drugs to inflammatory sites.

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Supporting Information Available: Two-step reaction used to functionalize the hydroxyl-terminated polymer with biotin; confocal microscopy images of multiple-ligand surfaces revealing no clustering; histogram showing polydispersity of adhesive vesicles; flow chamber used for parallel plate flow chamber experiments; cumulative adhesive flux for each of the 24 vesicle/substrate combinations evaluated; and trajectories representing the four categories of adhesive events. This material is available free of charge via the Internet at http://pubs.acs.org.