

Selective Immobilization of Multivalent Ligands for Surface Plasmon Resonance and Fluorescence Microscopy

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Cell surface multivalent ligands, such as proteoglycans and mucins, are often tethered by a single attachment point. *In vitro*, however, it is difficult to immobilize multivalent ligands at single sites due to their heterogeneity. Moreover, multivalent ligands often lack a single group with reactivity orthogonal to other functionality in the ligand. Biophysical analyses of multivalent ligand–receptor interactions would benefit from the availability of strategies for uniform immobilization of multivalent ligands. To this end, we report the design and synthesis of a multivalent ligand that has a single terminal orthogonal functional group and we demonstrate that this material can be selectively immobilized onto a surface suitable for surface plasmon resonance (SPR) experiments. The polymeric ligand we generated displays multiple copies of 3,6-disulfogalactose, and it can bind to the cell adhesion molecules P- and L-selectin. Using SPR measurements, we found that surfaces displaying our multivalent ligands bind specifically to P- and L-selectin. The affinities of P- and L-selectin for surfaces displaying the multivalent ligand are five- to sixfold better than the affinities for a surface modified with the corresponding monovalent ligand. In addition to binding soluble proteins, surfaces bearing immobilized polymers bound to cells displaying L-selectin. Cell binding was confirmed by visualizing adherent cells by fluorescence microscopy. Together, our results indicate that synthetic surfaces can be created by selective immobilization of multivalent ligands and that these surfaces are capable of binding soluble and cell-surface-associated receptors with high affinity.

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Multivalent ligands, such as highly glycosylated mucins and proteoglycans, are mediators of many important interactions at the cell surface (1–5). Methods such as surface plasmon resonance (SPR)² are valuable for monitoring interfacial interactions (6, 7). Unfortunately, SPR has not been widely applied to the study of multivalent ligands (8) because it is often difficult to attain uniform immobilization of these materials. Uniform immobilization simplifies the interpretation of SPR data by providing surfaces with homogeneous binding sites (9–11). Multivalent ligands, however, are often structurally heterogeneous and have multiple sites for surface attachment (Fig. 1). The development of defined multivalent ligands that could be immobilized homogeneously on surfaces via a single covalent bond would facilitate investigations into multivalent binding.

We envisioned that multivalent ligands derived from the ring-opening metathesis polymerization (ROMP) would fulfill these requirements (12). ROMP provides synthetic polymers that mimic natural multivalent ligands in structure and activity (2, 13–15). Additionally, ruthenium carbene initiators are functional group tolerant (16) and can be used to generate polymers of low polydispersity (17, 18). Materials generated by ROMP can be modified readily to display a variety of binding elements (19). Recent advances in ROMP have provided synthetic protocols for the introduction of unique functionality at the termini of the polymer chains (20–22). We set out to determine if the bifunctional capping strategy (22) could be used to orient ROMP-derived polymers on surfaces, thereby facilitat-

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² Abbreviations used: SPR, surface plasmon resonance; ROMP, ring-opening metathesis polymerization; EDC, *N*-ethyl-*N*-(diethylaminopropyl)carbodiimide; NHS, *N*-hydroxysuccinimide; HBS, Hepes-buffered saline; GGBP, glucose/galactose-binding protein; EGF, epidermal growth factor; CMD, carboxymethyl dextran.

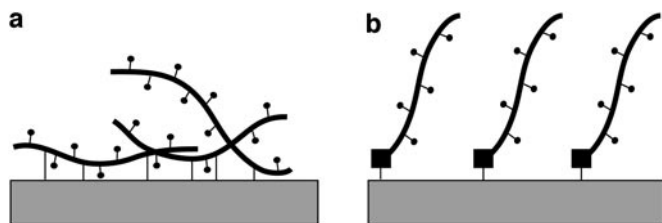


FIG. 1. Strategies for immobilization of multivalent ligands to surfaces. (a) Random covalent coupling yields a surface displaying an ensemble of multivalent ligand orientations. (b) Specific coupling can be achieved by using a unique terminal functional group.

ing investigations of multivalent interactions using SPR.

To determine whether end-functionalized polymers can be used to generate surfaces for binding studies, we investigated the immobilization of polymers bearing multiple copies of the carbohydrate 3,6-disulfogalactose. Polyvalent displays of this sulfated saccharide are ligands for P- and L-selectin, two members of the selectin family of adhesion proteins (23, 24). The selectins are cell surface proteins that mediate the calcium-dependent adhesion of leukocytes to the endothelium during the inflammatory response (25–27). The natural ligands of P- and L-selectin are highly glycosylated mucins; ligands for L-selectin display multiple copies of sulfated saccharides (27, 28), and P-selectin glycoprotein ligand-1 interacts with P-selectin through sulfated tyrosines and glycans in its N-terminal domain (29). Sulfated glycolipids bind to both P- and L-selectin (30).

The selectin ligands are associated with cell surfaces (27). We sought to reproduce this arrangement by synthesizing multivalent, end-functionalized 3,6-disulfogalactose derivatives by ROMP and incorporating these materials onto surfaces. Using SPR, we found that the resulting surfaces interact specifically with soluble P- and L-selectin and also bind to cells displaying L-selectin. We confirmed the cell binding results by visualizing cell adhesion to the derivatized surface with fluorescence microscopy. Additionally, the immobilization of multivalent ligands yields a synthetic surface with a five- to sixfold better affinity for P- or L-selectin than a surface displaying an equivalent amount of monovalent ligand. This finding suggests that the valency of the natural selectin ligands may be important in determining the strength of the selectin–ligand interaction.

MATERIALS AND METHODS

Sensor chips (CM5) were purchased from BIAcore (Uppsala, Sweden). SPR experiments were performed on a BIAcore 2000 instrument. Reagents for immobilization (*N*-ethyl-*N*′-(diethylaminopropyl)carbodiimide (EDC), *N*-hydroxysuccinimide (NHS), and ethanol-

amine) were purchased from Aldrich (Milwaukee, WI). Running buffer for SPR was HEPES-buffered saline (HBS, 10 mM HEPES, 150 mM sodium chloride, pH 7.4). Buffers were filtered (0.25 μm) prior to use. The control protein, glucose/galactose-binding protein (GGBP), was produced by osmotic shock, as described previously (31). The carbohydrate recognition domain and first EGF-like domain of P- and L-selectin were produced in baculovirus (32). The molecular weight of GGBP is 35 kDa and those of P- and L-selectin are approximately 65 kDa. Protein concentrations were determined by UV-visible spectroscopy at 280 nm. Jurkat cells were maintained as described previously (22).

The surface was prepared for conjugation to polymer as follows. The carboxymethyl dextran (CMD) matrix of a CM5 chip was activated by injection of a solution of EDC and NHS (70 μL , 200 mM EDC, 50 mM NHS) at a flow rate of 5 $\mu\text{L}/\text{min}$ (33). The resulting surface was treated with a solution of ethylene diamine (50 μL , 1 M, pH 8.5) to generate a surface bearing free amine groups.

The synthesis of a multivalent ligand with a terminal protected acid was performed as described previously (22). From the acid-bearing polymer 6, an activated ester was generated by treatment with NHS/EDC (50 mM NHS, 200 mM EDC) for 15 min at 23°C in HBS. The resulting polymer bearing a single NHS-ester was purified by G-50 size-exclusion chromatography and immediately injected (0.04 μM in HBS) over the amine-bearing CMD surface described above. The SPR response obtained from polymer conjugation to the surface was used to monitor the reaction. The control lane was prepared by analogous treatment without addition of polymer. The monomer-derivatized lane was generated by injection of 30 μL of 5 (2 mM in HBS, pH 7.4) over an NHS-activated CMD surface. The coupling of the monomer was less efficient at pH 4.5 or 8.5.

To measure protein binding by SPR, solutions of P- or L-selectin or GGBP in HBS were injected over the derivatized and control lanes of the modified CM5 surface at a flow rate of 5 $\mu\text{L}/\text{min}$. P-selectin was injected at 4, 3, 2, 1, 0.5, and 0.1 μM . L-selectin was injected at 2 and 1 μM . GGBP was injected at 4 μM . The regeneration buffer employed was 10 mM Tris, 1 mM EDTA, 150 mM NaCl, pH 8.5. The theoretical R_{max} for selectin binding to a surface bearing 82 RU of polymer 7 is approximately 140. Global fits to determine the dissociation constants were performed in BIAevaluation software using a 1:1 binding model (34).

For cell-binding experiments, Jurkat cells were washed 3 \times with ice-cold HBS and then injected at a concentration of 10,000 cells/mL. Three injections of 10 μL were performed. A representative sensorgram is shown (Fig. 4). The response was recorded 100 s after injection.

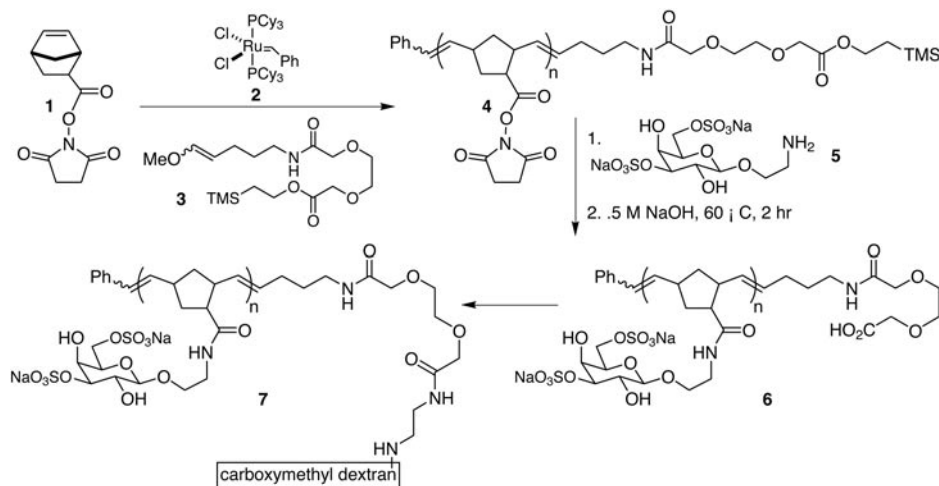


FIG. 2. The synthesis of an end-capped multivalent ligand was performed as described previously (22). The reaction of polymer 6 with an amine-modified surface is described under Materials and Methods. Valency (n) was approximately 85, as determined by integration of the ^1H NMR peaks corresponding to the backbone alkenes and the terminal phenyl group.

For the fluorescence microscopy experiments, Jurkat cells were washed twice with ice-cold HBS- Ca^{2+} (10 mM Hepes, 100 mM NaCl, 1 mM CaCl_2 , pH 7.5). Cells were resuspended to a density of approximately 5×10^6 cells/mL in HBS- Ca^{2+} containing 250 $\mu\text{g}/\text{mL}$ Hoescht stain. Cells were stained for 30 min at 37°C and washed with ice-cold HBS- Ca^{2+} to remove unbound dye. The polymer-modified gold-glass surface was removed (35) from the plastic housing of the derivatized CM5 biosensor chip. The surface was placed on a clean glass slide and moistened with HBS- Ca^{2+} . Cells at approximately 1×10^7 cells/mL in HBS- Ca^{2+} were added to the surface of the slide. The addition of labeled cells directly to the modified chip allowed for rapid screening to determine optimum washing conditions; however, we anticipate that cell binding could be achieved by using injection on the BIAcore. Cells bound nonspecifically were removed by washing with HBS- Ca^{2+} ; ice-cold buffer was added to one side of the chip and solution was removed from the other side by capillary action with a KimWipe. This process was repeated with approximately 5 vol of buffer. A cover slip was then placed on the chip. The chip was viewed on a Zeiss Axioscope outfitted with the appropriate filter set. Biosensor lanes were clearly visible at $200\times$ magnification. Images were collected in IPLab Spectrum and artificially colored in Adobe Photoshop 5.0.

RESULTS AND DISCUSSION

Our synthetic strategy for oriented immobilization of multivalent ligands involves three steps: generation of an amine-bearing surface from the CMD layer of a commercially available CM5 chip (BIAcore), synthesis of a multivalent ligand with a terminal carboxylic acid

(22), and immobilization of that ligand by reacting the acid with the amines of the modified surface (33).

To implement the strategy, we prepared the amine-modified CMD surface. The carboxyl groups of the CMD matrix were activated with NHS/EDC and the resulting NHS esters were treated with ethylenediamine to afford a surface displaying primary amines. The end-functionalized polymer was synthesized using a bifunctional enol ether bearing a protected acid 3 as a capping agent (Fig. 2) (22). This compound was used to terminate ruthenium carbene 2-initiated ROMP of monomer 1. The resulting polymer 4 was treated with an amine-functionalized 3,6-disulfogalactose derivative 5, and the terminal ester group was hydrolyzed to yield polymer 6. This material was activated by treatment with NHS/EDC and conjugated to the amine-modified CMD surface.

Reaction between the activated ester of the polymer with surface amines provided up to 175 RU (approximately $10.0 \text{ fmol}/\text{mm}^2$) of immobilized polymer 7. Coupling occurred rapidly (10- to 20-min contact times), despite the potential for repulsion between the negative charge of the polymer and the unmodified carboxylic acid groups of the CMD matrix. To minimize re-binding events, a surface bearing a lower density of polymer 7 was used to investigate binding to the selectins. This surface had 82 RU (approximately $4.7 \text{ fmol}/\text{mm}^2$) of immobilized polymer.

To test the activity of immobilized polymer 7, soluble P- and L-selectin were introduced. The derivatized surface bound the selectins (Figs. 3b and 3d). As would be expected for specific binding, this surface did not bind the control protein, GGBP. GGBP does not bind to charged saccharides and, accordingly, little protein ac-

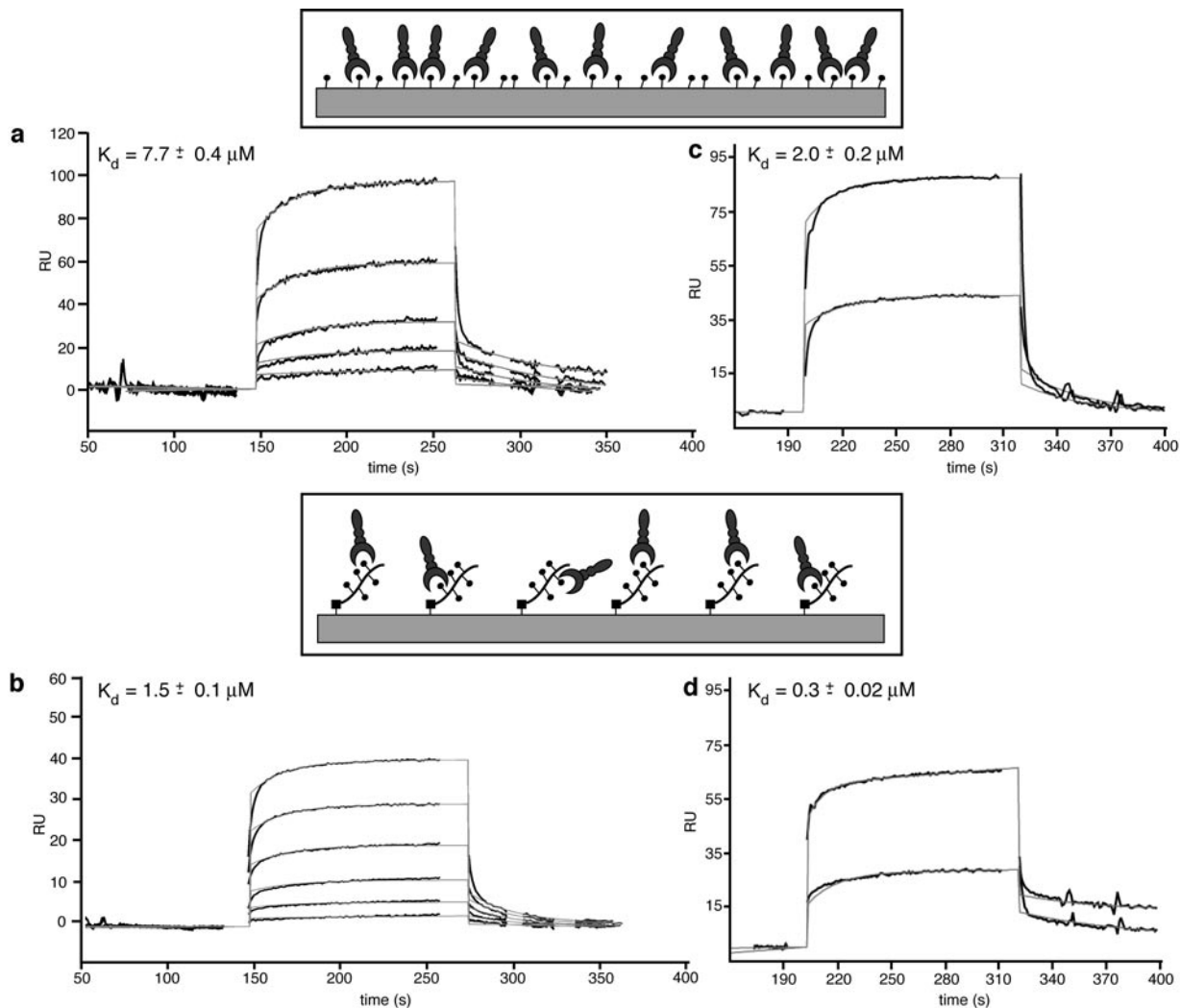


FIG. 3. Adhesion of proteins to immobilized ligands. (a) SPR response obtained with a surface bearing monovalent ligand 5 in the presence of P-selectin. (b) SPR response obtained with a surface bearing multivalent ligand 7 in the presence of P-selectin. (c) SPR response obtained with a surface bearing monovalent ligand 5 in the presence of L-selectin. (d) SPR response obtained with a surface bearing multivalent ligand 7 upon L-selectin treatment. The response units (ΔRU) are determined by subtraction of the signal due to a modified surface from that obtained with an underivatized control. Interestingly, the monomer surface bound more copies of P- and L-selectin than the polymer surface. The monomer-bearing surface accumulated approximately 90 RU of P-selectin and only about 60 RU bound the polymer-bearing surface. These results are consistent with monomer-coupled surfaces displaying more total molecules, as depicted in the figure.

cumulated on the derivatized surface (3.5 RU). The selectin's ability to bind carbohydrate ligands depends on the presence of calcium. As anticipated, the selectin-surface interaction was sensitive to the presence of calcium (1 mM EDTA prevented binding). Additionally, 60 μM multivalent 3,6-disulfogalactose derivative 6 added to a solution of P-selectin acted as a competitive ligand (binding was reduced by 40%). These results demonstrate that uniformly immobilized polymers are active as ligands and that they are specific for P- and L-selectin.

Our goal was to develop a strategy to selectively immobilize multivalent displays to surfaces for SPR. However, *monovalent* ligands can also be immobilized

selectively. Immobilization of monovalent ligands on a polymeric scaffold, such as CMD, effectively generates a multivalent binding partner. Despite this apparent similarity, we hypothesized that the surface displaying the multivalent ligands might have a greater avidity for protein than the surface substituted with the corresponding monovalent derivative. Whereas attachment of monovalent ligands to CMD would generate a multivalent surface with randomly spaced sites, synthetic multivalent ligands such as 7 have a close (approximately 3–5 Å) (36) and controlled spacing between epitopes. To test if binding site spacing affects selectin-ligand interactions, we compared the surface displaying 82 RU of immobilized polymer 7 to a surface

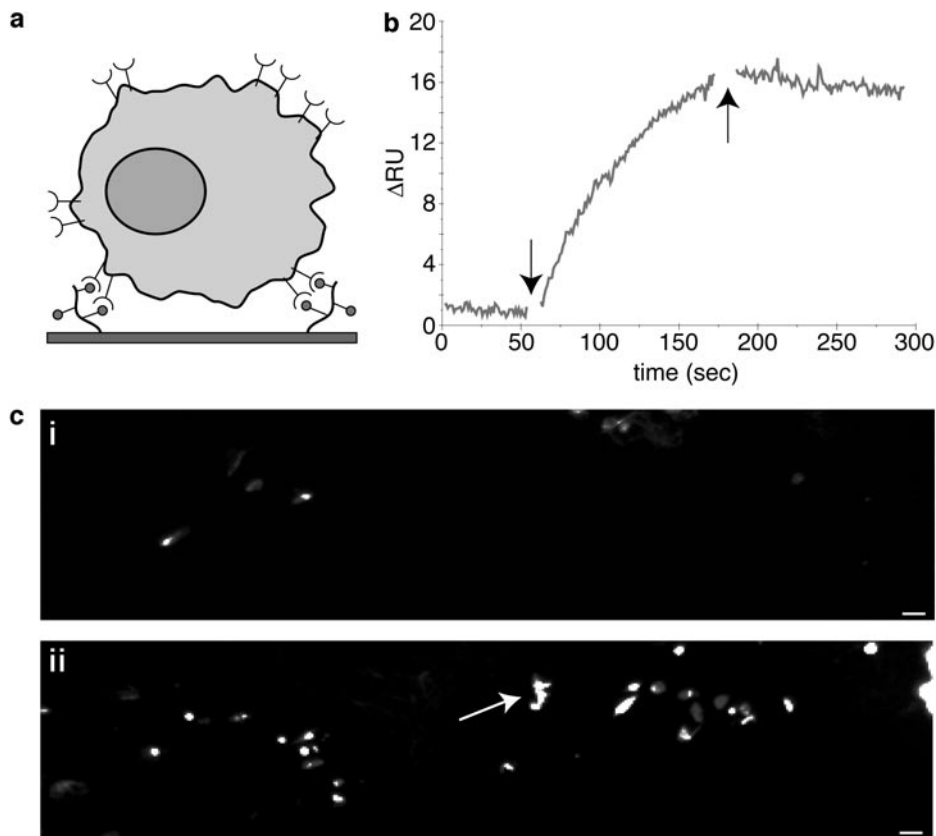


FIG. 4. Binding of Jurkat cells to a multivalent ligand-derivatized CM5 surface. (a) Schematic of cell binding to a derivatized surface. (b) Sensorgram of cell binding to the derivatized surface. The sensorgram was generated by subtraction of the control lane. Arrows signify the start and end of the injection. (c) Fluorescence microscopy of the lanes of the amine-modified CM5 chip. The top panel (i) is an underderivatized lane. The bottom panel (ii) shows Jurkat cells bound to a lane bearing immobilized ligand 7. An arrow points to a cell adhering to the surface. Bar, 10 μ m.

derivatized with the 3,6-disulfogalactose monomer 5. The amine-functionalized monomer 5 was immobilized by coupling to NHS-esters generated from the carboxylic acid groups of CMD. Importantly, we controlled the injection contact times to ensure that the monomer- and polymer-immobilized surfaces displayed the same number of carbohydrate residues. Thus, conditions for immobilization of 70 RU of monomer 5 were found. We injected solutions of P- or L-selectin over the surfaces that displayed polymer 7 or monomer 5. We performed kinetic global fits of the data collected to determine first-order dissociation constants (K_d) for the interactions. By this analysis, the polymer-immobilized surface bound P-selectin fivefold tighter than the monomeric surface. Similarly, L-selectin bound to the polymer-immobilized surface approximately sevenfold better than the monomer-immobilized surface. This result is interesting because the number of molecules immobilized on the monomer-derivatized surface is higher than the number of molecules on the polymer-substituted surfaces. Thus, despite the similar selectivity of immobilization of monomer versus polymer,

surfaces derivatized with polymers have better apparent binding affinity than do surfaces with bound monomers.

SPR can also be used to investigate ligand–cell interactions (37–39). Because the selectin–ligand interaction occurs at cellular interfaces, we sought to determine if our multivalent ligand-bearing surfaces could interact specifically with cells displaying selectin. Accordingly, Jurkat cells, which display L-selectin, were introduced to the polymer-derivatized surface. Cell adhesion was monitored by SPR, and analysis of these data revealed that Jurkat cells interact with the derivatized surface (Fig. 4b). The response from cell binding, after correction for the underderivatized lane, was 13.5 ± 3.2 RU. A low SPR response was expected, because cells are unable to penetrate the CMD matrix (37). Nonetheless, we sought to confirm the surface interaction with cells by another method. Few procedures exist for confirming cell adhesion to commercially available chips. Based on experiments investigating cell binding to self-assembled monolayers (40, 41), we developed a simple method for visualizing the

cells by fluorescence microscopy. To apply this method, the modified SPR chip was removed from its housing and mounted it on a clean glass slide (35). The surface was moistened and fluorescently-labeled Jurkat cells were introduced. Gentle washing removed unbound material and the remaining cells could be visualized (Fig. 4c). The cells that adhered spread on the derivatized lane, a morphology that is indicative of surface attachment. Few cells adhered to the control lane and changes in cell morphology were less apparent. The results confirmed the data indicating cell binding from SPR. Moreover, they highlight the utility of our end-functionalization strategy.

The selectins are targets for the development of anti-inflammatory strategies (26, 42), and additional approaches to screen for inhibitors would facilitate such efforts. The methods we have described are directly applicable to these goals. The natural ligands for P- and L-selectin are multivalent mucins on endothelial cell surfaces (27). Therefore, polymer-derivatized surfaces may provide a structurally relevant platform from which to identify inhibitors. As we have shown, surfaces displaying multivalent ligands have a greater avidity for selectins than surfaces displaying the monovalent ligands. We suggest that the latter do not as effectively mimic natural multivalent displays, such as those of mucins and proteoglycans. We anticipate that this methodology, therefore, will facilitate the biophysical characterization of interactions involving other multivalent ligands and aid in the search for inhibitors of these interactions.

In summary, we have applied bifunctional capping agents to the generation of polyvalent ligands suitable for immobilization on surfaces. SPR and fluorescence microscopy were utilized to demonstrate that the surface displaying immobilized polymers was recognized by both soluble and cell-surface-associated selectin. These results support the use of end-functionalized polymers as a class of molecules for the investigation of multivalent interactions at surfaces.

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