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Synthesis and Applications of End-Labeled Neoglycopolymers

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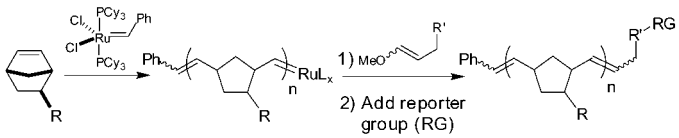
ABSTRACT



Neoglycopolymers that vary in length and contain a single fluorescent reporter group were synthesized using ring-opening metathesis polymerization (ROMP). The utility of these materials is demonstrated by the development of a cellular binding assay for L-selectin, a cell surface protein that plays a role in inflammation. The data reveal that these multivalent ligands interact with multiple copies of L-selectin.

Multivalent binding events are ubiquitous in biological systems. For example, multivalent protein−carbohydrate complexation events are important in cell adhesion, host−pathogen interactions, and the immune response.[1] Despite the importance of multivalent binding, mechanistic information is often lacking. One critical issue that arises when a multivalent ligand interacts with a cell-surface protein is whether it binds multiple copies of the target receptor. Though this binding mode is often invoked with multivalent ligands, it is difficult to determine the stoichiometry of these receptor−ligand complexes.[2] We sought to address this issue by examining multivalent ligand binding to cell-surface L-selectin.

L-Selectin is displayed on the cell-surface of leukocytes where it participates in the recruitment of these cells to the inflamed endothelium.[3,4] The natural ligands for L-selectin are multivalent; they display multiple copies of sulfated carbohydrates.[4] L-Selectin is localized to regions on the cell-surface,[5] and dimerization of L-selectin enhances its ability to bind to endothelial cells.[6] These data implicate multivalency in the interaction of L-selectin and its ligands at the cell surface, but direct evidence has been difficult to obtain.

Synthetic multivalent displays have emerged as powerful tools for investigating multivalent binding.[7] Polymeric ligands that incorporate both binding elements and a reporter group are especially useful for visualizing and quantitating binding interactions.[8] A reporter moiety, such as a fluorescent or biotin group, is typically introduced through the polymerization of an appropriately substituted monomer or through

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random conjugation to the polymer backbone.[9] Although useful for many purposes, these strategies provide limited control over the number and location of the introduced functionality. Polymers possessing a single end-label, however, can be selectively immobilized on surfaces to provide novel materials for physical[10] or biological studies.[11] We envisioned that multivalent ligands varying in valency but containing a single fluorophore per ligand could provide unique mechanistic information. If multivalent binding occurs, saturating concentrations of ligands differing only in their valency would give rise to different cell surface fluorescence intensities (Figure 1). In contrast, monovalent

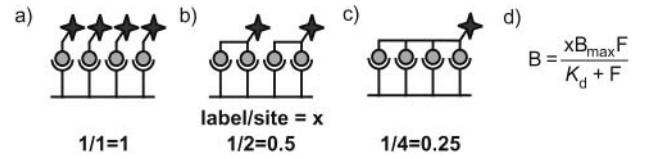


Figure 1. End-labeled materials can be used to reveal multivalent interactions. The number of labels bound depends on the number of binding sites occupied per multivalent ligand (parts a−c). The binding of end-labeled multivalent ligands at saturation (B_{max}) will vary according to valency (equation shown in part 1d, B is moles of bound ligand, F is the free multivalent ligand concentration).

binding interactions would yield identical fluorescence intensities at saturation.

ROMP[12] provides a means to generate end-labeled materials varying in valency. When ROMP is living,[13] an active carbene (e.g., **3**) remains at the polymer terminus after consumption of the monomer (Figure 2). The metal alky-

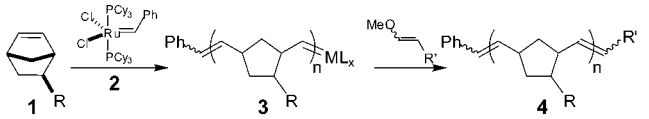


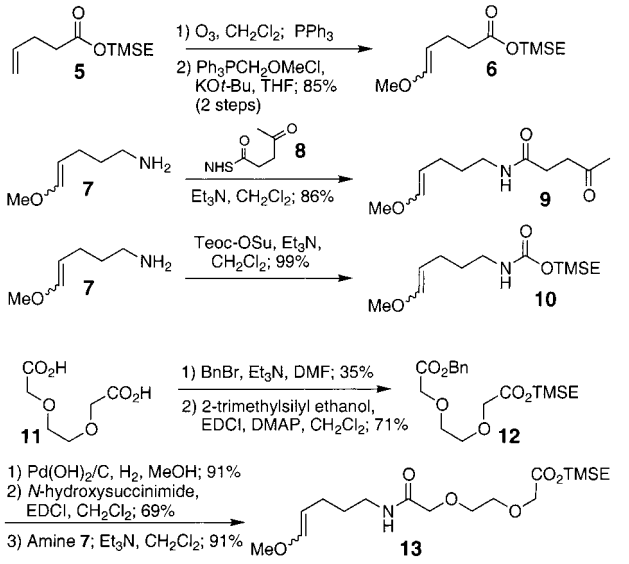
Figure 2. Termination of ruthenium carbene-initiated ROMP reactions with an enol-ether provides an end-labeled material (**4**).

lidene **3** can undergo further transformations. For example, Schrock and co-workers treated a polymer possessing a terminal molybdenum carbene with aldehydes to provide materials with unique redox and luminescence properties.[10] The highly functional group tolerant ruthenium initiator **2** developed by the Grubbs group also has been used to synthesize end-labeled polymers. Ruthenium alkylidene-initiated reactions conducted in the presence of chain-transfer agents provide symmetrically terminated materials.[14] Alternatively, a terminal aldehyde can be installed by treatment of **3** with molecular oxygen.[15] We have shown that a substituted enol ether can be used to produce specifically, end-labeled polymers, such as **4**.[16]

Here, we demonstrate the effectiveness of this strategy for the synthesis of labeled polymers varying in length and terminal functionality.

Termination Efficiency. Because our objective was to generate multivalent ligands of different lengths, the capping process must be efficient. To identify effective termination agents, enol ether derivatives possessing unique functional groups, including masked acid (**6**, **13**), ketone (**9**), and masked amine (**10**) groups, were synthesized (Scheme 1).

Scheme 1. Synthetic Routes to Acid-, Ketone-, and Amine-Substituted Capping Agents



The functional groups chosen provide the means to install reporter groups under mild conditions using commercially available reagents. The capping agents were assembled from simple building blocks (Scheme 1). Enol ether **6** was obtained in excellent yield from the known 2-trimethylsilyl ethyl (TMSE) ester (**5**) of 4-pentenoic acid.[17] Ozonolysis of **5** followed by Wittig reaction yielded the desired vinyl ether **6** as a 1:3 *cis:trans* mixture. Although *cis*-enol ethers have been shown to react more quickly with ruthenium carbene

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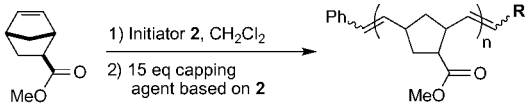
2,[18] the isomers were not separated because an excess of the mixture (15 equiv) was used in the termination step (vide infra). Capping agents **9**, **10**, and **13**, were all generated from known amine **7**.[19] The ketone-containing capping agent **9** could be synthesized in a single step from **7** and the *N*-hydroxysuccinimide (NHS) ester of levulinic acid (**8**).[20] Similarly, compound **10** could be accessed from the reaction of **7** with 2-trimethylsilylethyl *N*-succinimidyl carbonate (Teoc-OSu).[21] Preparation of the ethylene glycol-based capping agent (**13**) required desymmetrization of the diacid **11** to give the diester **12**. The benzyl ester was converted to the NHS ester via the acid. The enol ether was installed under standard amide bond forming conditions to afford capping agent **13**. The efficiencies of **6**, **9**, **10**, and **13** in terminating ROMP reactions were then assessed.

To test the effectiveness of our capping strategy, monomer **14** was polymerized using ruthenium initiator **2**, and the reaction was terminated by the addition of a capping agent. Methyl ester **14** was used as a monomer because the resulting polymer (**15**) could be readily separated from excess capping agent by silica gel chromatography. Each of the capping agents was used in a 15-fold excess as a mixture of *cis*:*trans* isomers. The termination reaction was monitored by ^{1}H NMR spectroscopy. Complete consumption of the propagating carbene by each terminating agent occurred within 3 h. After purification of the resulting polymers, the capping efficiencies were determined by comparing the integration of the signal due to the polymer phenyl protons relative to a diagnostic signal from the capping agent (the trimethylsilyl group for compounds **6**, **10**, and **13** or the protons α to the ketone for **9**). The termination efficiencies were excellent for **6** and **10**, good for **9**, and moderate for compound **13** (Table 1). The lower efficiencies for **9** and **13**

Table 1. Capping Efficiencies for the Polymerization of Alkene **14**



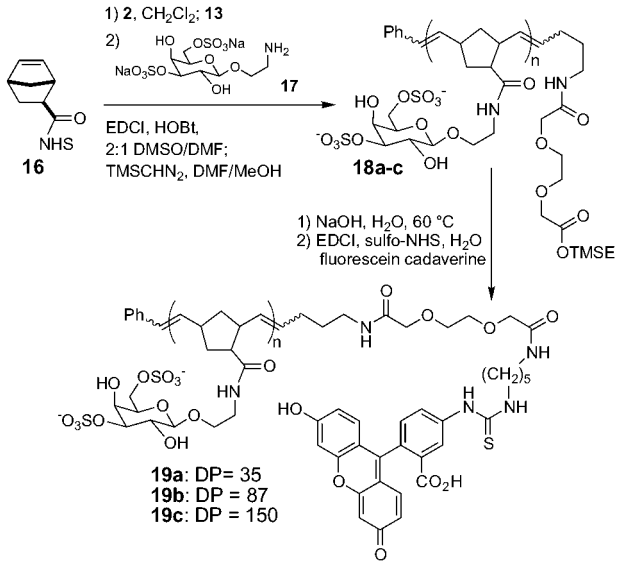
entry	capping agent	capping efficiency[a]
1	**6**	>95%/93%
2	**9**	80%/86%
3	**10**	>95%/92%
4	**13**	64%/68%

[a] Efficiencies are an average of a minimum of two separate experiments and were determined for monomer-to-catalyst ratios of 15:1/50:1.

may arise from their amide groups, which may chelate to the ruthenium alkylidene species.[22] The termination reaction of the carbamate-containing capping agent **10**, which is less Lewis basic than an amide,[23] is more efficient.

End-Labeled Polymers as Cell-Surface Probes. Given that end-labeled polymers could be generated efficiently, these materials were used to investigate ligand binding to L-selectin-displaying cells. We found previously that multivalent ligands presenting 3,6-disulfogalactose residues bind to L-selectin.[16,24] We envisioned that fluorescent derivatives could be used to assay binding[16,25] and to assess the importance of multivalent interactions. A series of polymers in which the degree of polymerization was varied was generated by controlling the ratio of monomer **16**[26] to ruthenium alkylidene initiator **2**. The polymerization reactions were terminated by the addition of **13** to afford electrophilic polymers that could be sequentially functionalized. Treatment with the amine-bearing 3,6-disulfogalactose derivative (**17**)[27] yielded the desired polymers (**18a**−**c**) (Scheme 2). Hydrolysis of the terminal ester and conjugation of fluorescein cadaverine to the resulting amine group provided the target labeled ligands (**19a**−**c**).[16] The interaction of these materials with Jurkat cells displaying L-selectin was then assessed.

Scheme 2. Synthesis of Labeled 3,6-Disulfogalactose-substituted Polymers **20a**−**c** for L-Selectin Binding Assay. The Degree of Polymerization (DP[30]) Was Determined from the ^{1}H NMR Spectra



Jurkat cells were exposed to polymers **19a**−**c** or a fluorescently labeled anti-L-selectin antibody (Figure 3A)

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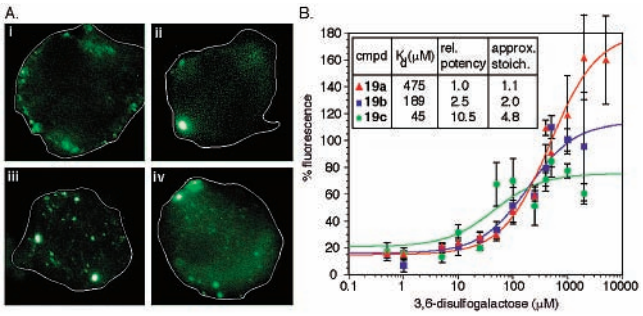

Figure 3. (A) Treatment of Jurkat cells with (i) fluorophore-labeled anti-L-selectin antibody, (ii) polymer **19a**, (iii) **19b**, or (iv) **19c**. (B) Results from the L-selectin binding assay. The binding of **19a**−**c** is reversed upon addition of unlabeled 3,6-disulfogalactose or sialyl Lewis x-bearing polymers (data not shown). Concentrations refer to 3,6-disulfogalactose residue concentration. The approximate stoichiometry is defined as the number of copies of L-selectin that interact simultaneously with a polymer of a given DP.[28]

toscreen for ligand binding. As anticipated,[16] fluorescence microscopy experiments indicate that **19a**−**c** interact with Jurkat cells displaying L-selectin. To quantify the amount of bound ligand, Jurkat cells were treated with **19a**−**c** and washed, and the intensity of the fluorescence emission was assessed (Figure 3B). The resulting data were fit to the equation in Figure 1d to determine the dissociation constant (K_d) for each compound.[28] The K_d values for the polymers[29] were dependent on the polymer length. Polymer **19c** (degree of polymerization,[30] DP of 150) had a potency that was ca. 10-fold greater than the shortest polymer (**19a**, DP of 35) on a saccharide residue basis. Although useful for comparison, this calculation underestimates the relative increase in potency. The individual saccharide (3,6-disulfogalactose) does not bind to L-selectin—a multivalent display is required.

Because the polymers possess a single fluorophore label, their ability to bind multiple copies of L-selectin at the cell surface could be determined. The concentration of polymer required to saturate the fluorescence intensity (B_{max}) was measured. Dividing the average amount of L-selectin on a Jurkat cell-surface by the calculated concentrations of bound ligand provides an estimate of the relative stoichiometry of the L-selectin−ligand complexes. By this analysis, polymer **19c** (DP of 150) bound approximately five copies of L-selectin while polymer **19a** (DP of 35) bound only one or two copies of L-selectin. These data indicate that compounds **19a**−**c** engage in multivalent interactions with L-selectin and that the stoichiometry of the resulting complex depends on the valency of the ligand. This result is consistent with studies demonstrating that neoglycopolymers can cluster proteins in solution and in the cell.[2,31] Compounds with more complex binding epitopes[32] may more effectively interact with multiple copies of cell-surface L-selectin.

Our previous studies indicate that multivalent but not monovalent ligands induce L-selectin shedding from white blood cells.[24] One explanation for this difference is that polymeric ligands cluster multiple copies of L-selectin but monovalent ligands do not. Because the ligands used in these studies induce L-selectin downregulation,[24] our results suggest that the ability of these ligands to modulate cell-surface levels of L-selectin depends on clustering.[33] This finding offers new opportunities for the design of L-selectin antagonists.[34] Our results also suggest mechanisms by which natural multivalent ligands may bind L-selectin.

In conclusion, end-labeled polymers are valuable probes of multivalent ligand−receptor interactions. Our data reveal that multivalent ligands varying in length with single end-labels can be generated using ROMP. The investigations described here highlight the utility of such materials for probing the mechanisms underlying multivalent binding at the cell surface. The ligands we have generated provide the means to investigate mechanistic aspects of cell-surface receptor−ligand interactions that have been inaccessible. We anticipate that the strategies outlined can be used to synthesize polymers that will be useful in a wide range of materials science and in vitro and in vivo biological applications.

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Supporting Information Available: Experimental procedures and characterization data for compounds **6**, **9**, **10**, **12**, **13**, **15**, **18**, and **19**. This material is available free of charge via the Internet at http://pubs.acs.org.

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(28) See the Supporting Information.
(29) K_d values were calculated on a saccharide residue basis.
(30) Degree of polymerization (DP) was determined from the integration of ^{1}H NMR signals in the phenyl region against those in the alkene region.

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