A General Synthetic Route to Defined, Biologically Active Multivalent Arrays

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Abstract: A new, general strategy for the synthesis of biologically active multivalent arrays displaying diverse functionality has been developed. This method exploits the ability of ring-opening metathesis polymerizations to produce polymers of defined lengths. By incorporating *N*-hydroxysuccinimide esters into the polymers, recognition epitopes bearing nucleophilic functional groups, in this case an α-mannose derivative, can be attached. The synthesis, characterization, and biological evaluation of mannose-bearing polymers demonstrated the utility of this new methodology. This strategy will facilitate the creation of diverse multivalent libraries and the large-scale production of multidentate ligands.

Introduction

The binding of cell surface receptors to multivalent ligands can trigger a wide variety of biological responses. Such multivalent binding events can have unique consequences that are dramatically different than those elicited by monovalent interactions[1,2] For instance, signaling through growth factors[3] and the shedding of cell surface proteins[4] can be facilitated by multivalent ligands. Further, multidentate saccharide-substituted ligands can exhibit increased avidity and specificity in protein−carbohydrate recognition processes.[1,5] Thus, the ability to synthesize defined, multivalent arrays of biologically relevant binding epitopes provides a means for exploring and manipulating physiologically significant processes.

Because they can span a range of distances, linear multidentate displays of varying length and epitope density are particularly useful for probing structure−function relationships in biological systems. Chemical and chemoenzymatic routes have been developed for the generation of di- and trivalent ligands,[6,7] dendrimers,[8,9] and high molecular weight polymers,[10,11] but well-defined, linear oligomers have proven more difficult to synthesize. Here, we report the development of a general strategy to create diverse arrays of multivalent materials by the ring-opening metathesis polymerization (ROMP).

ROMP has been used to generate defined materials with potent and unique biological activities that range from inhibiting protein−carbohydrate recognition events to promoting the proteolytic release of cell surface proteins.[12−17] The assembly of multidentate displays by ROMP has several advantages over classical methods. Specifically, ROMP can be a living polymerization[18] and, if the rate of initiation is faster than that of propagation, variations in the monomer-to-initiator ratio (M:I) can yield materials of defined lengths. This approach has been successfully applied with the Grubbs catalyst **2**[19] to generate materials with narrow polydispersities.[20] There are, however, inherent disadvantages in the use of standard approaches that rely on ROMP to assemble biologically active materials. A new, functionalized bicyclic substrate for ROMP must be synthesized for each new polymer class to be produced. The physical properties of each monomer, such as its solubility and the electron density and strain of the cyclic olefin, result in different rates of initiation, propagation, and nonproductive termination.[21] In addition, purification of the desired products can be complicated and is dependent on the structure of the monomer unit. Expedient, large-scale syntheses of multiple oligomeric displays are hindered by these technical complications. We therefore set out to develop a general method for synthesizing oligomers that addresses these issues. Our objective was to develop a single set of reaction and purification conditions that could be used for both large-scale oligomer production and the generation of libraries of oligomers.

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Results and Discussion

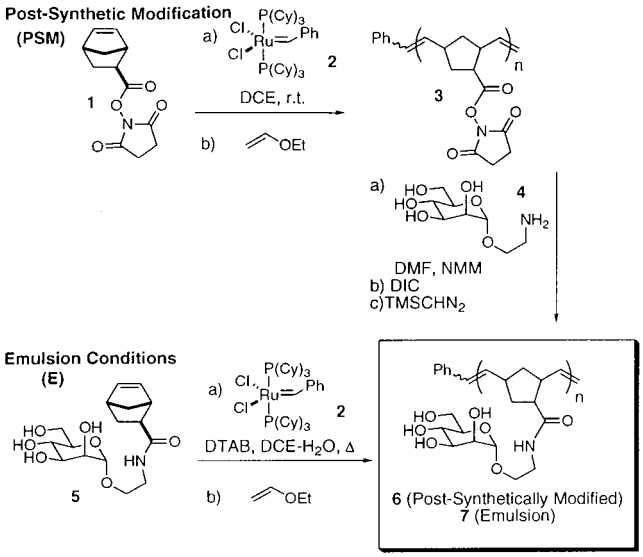
Design of Multivalent Displays with Activated Pendant Functional Groups. To accomplish our synthetic goals, we explored post-polymerization modification of an oligomer backbone generated by ROMP.[22,23] This strategy has the key advantage of requiring the synthesis of a single polymerization template. For this approach to be successful, the process of recognition element attachment must be general, convenient, and efficient. Amide bond formation is a reaction that meets these criteria. To avoid the difficulties of oligomer synthesis and purification associated with highly water-soluble materials,[24] such as those possessing carboxylic acids, we explored nonpolar monomers containing activated esters.

The activated ester groups examined were those that we hypothesized would be stable to the polymerization conditions. A norbornene derivative equipped with an *exo* pentafluorophenyl ester group (bicyclo[2.2.1]hept-5-ene-*exo*-2-carboxylic acid pentafluorophenyl ester) could be oligomerized by ROMP in methylene chloride. Unfortunately, the resulting products were films that were difficult to manipulate and afforded low yields in the subsequent amide bond-forming step. In contrast, a monomer possessing an *N*-hydroxysuccinimide (NHS) ester (**1**) underwent facile oligomerization upon treatment with metallocarbene **2** generating materials that could be readily functionalized (Scheme 1). These results further extend the range of functional groups compatible with the highly tolerant ruthenium catalyst **2** and demonstrate the feasibility of this approach for producing a new class of polymers.

Synthesis of Carbohydrate-bearing Arrays. To investigate the preparation of bioactive multivalent ligands, we examined the attachment of carbohydrate recognition elements to this oligomer backbone. The NHS-substituted materials **3** were treated with a mannose derivative **4**, which has an aglycon linker terminating in a primary amine (Scheme 1). A carbodiimide coupling agent (DIC) was included to increase the efficiency of linking the desired recognition elements thereby maximizing the yield of multivalent **6**. To minimize the possibility that nonspecific Coulombic interactions would interfere with subsequent biological assays, the resulting postsynthetically modified (PSM) polymers were then treated with (trimethylsilyl)-diazomethane to convert any unreacted free acid groups into methyl esters. The final reaction sequence is general and has been applied to additional recognition epitopes, including neutral and charged carbohydrate epitopes including galactose and sulfated carbohydrate derivatives.

Evaluation of Synthetic Routes. Analytical and biological assays were employed to evaluate the utility of the postsynthetic modification strategy: The PSM polymers were compared to

Scheme 1. Two Synthetic Routes Used To Generate Analogous Multivalent Mannose Displays[a]



[a] One method involves polymerization of the activated ester template **1** to afford the pre-formed polymer backbone **3** followed by postsynthetic modification with carbohydrate **4** to yield **6** (PSM). The alternate route involves polymerization of the polar carbohydrate substituted monomer **5** under emulsion conditions (**7**, E). (DCE = 1,2-dichloroethane; NMM = 4-methylmorpholine; DIC = diisopropylcarbodiimide; DTAB = dodecyltrimethylammonium bromide).

those synthesized from the corresponding carbohydrate-substituted monomer **5** under emulsion conditions (Scheme 1, E). Because a key feature of ROMP is that it can provide oligomers of varying average lengths and because such oligomers can illuminate biological recognition processes, we generated a series of NHS-substituted materials differing in average length (degree of polymerization, DP) using three monomer-to-initiator ratios (10:1, 25:1, and 50:1). All polymerization reactions proceeded efficiently, consuming all of the monomer. The mannose epitopes were appended by treatment of the activated oligomer backbones with amine **4** to afford oligomer series **6** (Scheme 1). The related materials were generated by the conventional method under emulsion polymerization conditions employing the same monomer-to-initiator ratios used for the NHS ester-substituted norbornene **1** (Scheme 1, **7**). No variations in the macroscopic physical properties of the oligomers prepared by the two methods were detected, and no differences were observable by ^{1}H NMR spectroscopy.

The DPs of the materials generated by each method were assessed by gel permeation chromatography (GPC). The carbohydrate polymers **6** and **7** were acetylated to convert them into organic soluble derivatives, which are more readily evaluated by GPC. Analyses of the materials suggested that the polymers generated under emulsion conditions are slightly shorter than those produced by postsynthetic modification (Figure 1). Each method, however, provides a linear correlation between polymer length and monomer-to-initiator ratios (M:I), an indication that the polymerization reactions are living.[18] Thus, the PSM protocol can be used to prepare multivalent assemblies varying in length. The GPC data also suggest that the shortest polymers made by each method are within ~3 unit lengths of one another, while the longest polymers are within ~12 units. The minor discrepancies in the lengths of the emulsion and PSM polymers highlight how subtle differences in the monomers can give rise to variations in the polymerization reaction. The new PSM procedure is important because materials with identical backbones can be generated from reaction of a template with a

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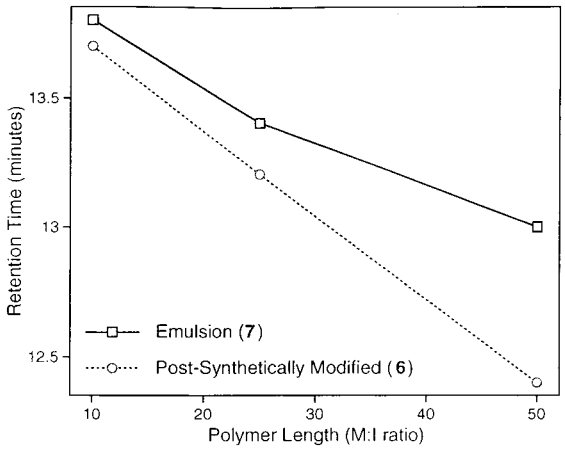

Figure 1. GPC data reveal a linear correlation between the degree of polymerization and the monomer-to-initiator ratio. Emulsion conditions (**7**, E) yield polymers of shorter relative length than the postsynthetic modification (**6**, PSM). Average lengths (DPs) as determined by NMR integration of the alkene protons to the phenyl ring protons are as listed: For emulsion conditions M/I = 10:1, DP = 21; M/I = 25:1, DP = 38; and M/I = 50:1, DP not determined. For postsynthetic modification conditions M/I = 10:1, DP = 18; M/I = 25:1, DP = 45; and M/I = 50:1, DP not determined.

wide range of different recognition elements. Such substances will facilitate the elucidation of structure−function relationships.

Biological Activity of Multivalent Displays. We sought to further investigate this new method by comparing the biological activity of oligomers derived from the new process to those synthesized by the standard approach. The mannose-substituted polymers were designed to interact with the well-characterized lectin Concanavalin A (Con A).[25] Con A is a homotetramer at pH 7 that can facilitate the agglutination of red blood cells via simultaneous interactions with mannose residues on the surfaces of adjacent cells. The ability of soluble carbohydrate ligands to inhibit cell agglutination can be measured, and we have previously shown that the efficacies of ROMP-derived oligomers in a Con A inhibition assay depend on their lengths.[21,26] Hemagglutination assays,[27] therefore, provide a convenient format to assess the activities of materials generated from the two distinct preparation methods.

The Con A inhibitory potencies of the materials generated by the emulsion and PSM protocols were compared on a saccharide residue basis using monovalent α-methyl mannopyranoside as a standard. Within a single series, either polymer **6** or **7**, the most effective oligomers were those produced with a 50:1 monomer-to-initiator ratio (Figure 2). This result is consistent with previous studies, which revealed that the most potent inhibitors are those that can span two saccharide binding sites on Con A.[21,26] At each M:I ratio, the PSM oligomers are slightly more active than those prepared under emulsion conditions. For example, a 400-fold increase over α-methyl mannopyranoside was seen for the polymer derived from the 50:1 monomer-to-initiator ratio in the emulsion polymerization, but an enhancement of 550-fold was found for the related material made by postsynthetic modification. Because longer oligomers are more active inhibitors, this finding is consistent with the GPC data that indicate the average length of the PSM oligomer exceeds that of the material generated under the emulsion polymerization conditions. Overall, our results indicate that the PSM protocol can be used to synthesize biologically active materials with potencies that match or surpass those of substances generated by standard ROMP approaches.

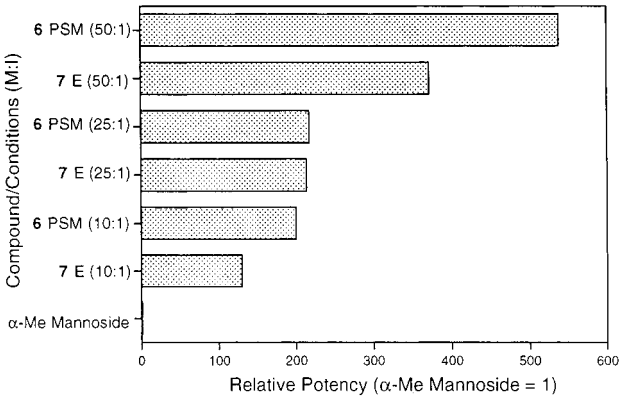

Figure 2. Dependence of hemagglutination inhibition on polymer length. Relative IC_{50} values are reported on a saccharide residue basis with α-methyl mannopyranoside as a standard. The reported results are the average of a minimum of five experiments, and the error associated with the dose determination is a factor of 2, as dictated by the 2-fold dilutions in the assay.

Conclusions

Our results indicate that the PSM method can provide access to a wide range of materials with interesting biological functions. Significantly, this approach could be used to generate libraries of oligomeric substances that differ in appended functionality as well as in length. Moreover, the application of the PSM protocol to a living polymerization has significant advantages. For example, the ability to control the number of recognition elements in a multidentate binding display can provide valuable insight into structure−function relationships in biological systems. In addition, unique block copolymers can be generated in which some blocks are held invariant while others are diversified through PSM. We have demonstrated the utility of the PSM approach for the preparation of biologically active materials. We anticipate that this method can be used to rapidly and efficiently synthesize a wide range of polymeric displays with diverse properties.

Experimental Section

Reactions were carried out in oven-dried glassware under nitrogen atmosphere, except as otherwise noted. ACS grade 1,2-dichloroethane (DCE) was used as received. Solvents used in polymerization reactions were deoxygenated with a minimum of 3 freeze−pump−thaw cycles prior to use. Distilled, deionized (dd or MQ) water and 500 MWCO dialysis tubing was used for the polymer purification. Chromatography solvents were ACS grade; dichloromethane, acetone and hexanes were distilled. Dodecyltrimethylammonium bromide (DTAB) was recrystallized from acetone. Gel permeation chromatography was run with THF as the eluent (2 mL/min) over three columns: 10^3, 10^4, and 10^5 Å pore sizes. Peaks were detected by UV (254 nm). Analytical thin-layer chromatography (TLC) was performed on 0.25 mm precoated silica gel plates (60F-254), and flash chromatography on silica gel (230−400 mesh). Visualization of TLC was done with ultraviolet light and *p*-anisaldehyde stain. ^{1}H and ^{13}C NMR spectra were recorded on 300 or 500 MHz spectrometers; chemical shifts are reported downfield from tetramethylsilane in parts per million (δ). ^{1}H NMR data are assumed to be first order with apparent doublets and triplets reported as d and t, respectively. Resonances that appear broad are designated as br.

Bicyclo[2.2.1]hept-5-ene-*exo*-2-carboxylic acid *N*-hydroxysuccinimide ester, **1**: The norbornene acid (151.8 mg, 1.1 mmol), *N*-

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hydroxysuccinimide (172.5 mg, 1.49 mmol), and EDCI (278.1 mg, 1.45 mmol) were stirred in CH_2Cl_2 (3.6 mL) overnight under nitrogen. The solvent was removed under reduced pressure and the residue was subjected to flash silica gel chromatography with CH_2Cl_2 as the solvent. A white solid was isolated (186.7 mg, 0.88 mmol). Yield 80%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 6.19 (dd, J = 5.7, 2.9 Hz, 1H), 6.17 (dd, J = 5.7, 3.1 Hz, 1H), 3.25 (br s, 1H), 2.98 (br s, 1H), 2.82 (d, J = 1.65 Hz, 2H), 2.49 (ddd, J = 10.48, 4.78, 1.65 Hz, 1H), 2.03 (ddd, J = 11.95, 4.23, 4.2 Hz, 1H), 1.55−1.41 (m, 3H); EI m/z 235.0847 [235.2395, calcd for $C_{12}H_{13}NO_4$].

Polymerization of bicyclo[2.2.1]hept-5-ene-*exo*-2-carboxylic acid *N*-hydroxysuccinimide ester, **3**, n = 10: The *N*-hydroxy ester (98.3 mg, 0.425 mmol) **1** was dissolved in 1,2-dichloroethane (2.1 mL). To this was added a solution of $[(Cy)_3P]_2Cl_2Ru{=}CHPh$ in DCE (35 mg in 2.1 mL). The reaction was stirred under nitrogen at room temperature for 45 min. The reaction appeared complete by TLC, and an excess of ethyl vinyl ether was added. The reaction mixture was filtered through a small plug of silica gel with CH_2Cl_2 as eluent. The solvent was removed under reduced pressure to afford a brown solid (96.8 mg) that was used without further purification. Yield 98%; ^{1}H NMR (300 MHz, $CDCl_3$) δ 7.3 (m), 5.7−5.2 (m), 3.5−0.90 (br m).

Aminoethyl-α-D-mannopyranoside, **4:** The azidoethyl mannoside has previously been prepared.[28] We used standard conditions for glycosylation with allyl alcohol.[29] The azidoethyl mannoside was reduced with Pearlmann's catalyst in a 1:1 mixture of methanol−water (a modification of the procedure cited above) to give **4**.

Aminoethyl-α-D-mannopyranosyl bicyclo[2.2.1]hept-5-ene-*exo*-2-carboxamide, **5:** The mannose monomer was prepared via the pentafluorophenyl ester and compound **4** under the same conditions previously described.[30] ^{1}H NMR (300 MHz, D_2O) δ 6.19 (dd, J = 5.7, 2.9 Hz, 1H), 4.694 (d, J = 1.65 Hz, 1H), 3.76 (dd, J = 2.94, 1.83, 1H), 3.70 (dt, J = 12.32, 1.9 Hz, 1H), 3.64−3.41 (m, 6H), 3.29 (br m, 1H), 2.76 (br m, 1H), 2.03 (m, 1H), 1.57 (m, 1H), 1.35−1.17 (m, 3H); EI m/z 343.1627 [343.377, calcd for $C_{16}H_{25}NO_7$].

Coupling to product of the polymerization of bicyclo[2.2.1]hept-5-ene-*exo*-2-carboxylic acid *N*-hydroxysuccinimide ester, **6**, n = 10: Aminoethyl mannoside **4** (16.0 mg, 0.0788 mmol), *N*-methylmorpholine (7.7 mL, 0.0702 mmol), and polymer **3** (n = 10, 15.2 mg, 0.0647 mmol) were stirred for 24 h. Diisopropylcarbodiimide (11 mL, 0.0638 mmol) was added and stirring continued overnight. The DMF was removed under reduced pressure, and the resulting solid was washed three times with 1−2 mL of CH_2Cl_2 and ethanol. The solid was dried, and (trimethylsilyl)diazomethane (35 μL, 0.0702 mmol) and methanol (350 mL) were added and the reaction stirred overnight. The reaction was quenched upon addition of water, and the solvent was removed under reduced pressure. The solid was dissolved in MQ water and placed in dialysis tubing. The sample was dialyzed (48 h, four water changes, 1 L each time) to remove impurities from the coupling reaction. The solution was filtered through a 0.25 μm filter and the solvent was removed under reduced pressure to give a tan solid (15.4 mg, 71%).^{1}H NMR (300 MHz, D_2O) δ 7.3 (br m, 0.278 H), 5.5−4.9 (br, 2 H), 4.0−3.0 (br m, 14 H), 2.5−2.15 (br m, 2 H), 1.9−1.4 (br, 2 H), 1.1−0.9 (br, 2H).

Polymerization of aminoethyl α-D-mannopyranosyl bicyclo[2.2.1]-hept-5-ene-*exo*-2-carboxamide, **7**, n = 10: The mannose monomer **5** (19.6 mg, 0.0571 mmol) and DTAB (29 mg, 0.0933 mmol) were dissolved in water (182 mL) and degassed. DCE (181 mL) was added to the ruthenium catalyst **2** (6.1 mg) and this solution (91 mL corresponding to 4.7 mg, 0.00571 mmol of **2**) was added to the solution of **5**. The reaction was stirred at room temperature for 30 min and then was heated at 60 °C for 4 h. Once the reaction was complete by TLC, an excess of ethyl vinyl ether was added to quench the active alkylidene. The reaction mixture was evaporated under reduced pressure, and the solid was washed with dichloromethane and ethanol. The polymer was dissolved in MQ water and dialyzed against 1 L of water for 2 days, changing the water every 12 h. The solution was removed from the dialysis tubing and filtered through a 0.25 μm filter which, after removal of the solvent under reduced pressure, gave a tan solid (18.2 mg). Yield 90%; ^{1}H NMR (300 MHz, D_2O) δ 7.3 (br m, 0.238 H), 5.5−4.9 (br, 2 H), 4.0−3.0 (br m, 14 H), 2.5−2.15 (br m, 2 H), 1.9−1.4 (br, 2 H), 1.1−0.9 (br, 1 H).

Hemagglutination assay: This assay was performed as previously described.[21] The concentrations of the polymer samples used in the assay were determined by ^{1}H NMR integration of the peak at 5.25 ppm with an external sample of NaOAc of known concentration.

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Supporting Information Available: Spectral data for compounds **1**, **3**,and **5**−**7** (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

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