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Assembly of P-Selectin Ligands on a Polymeric Template

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Summary

High-affinity receptor-ligand interactions frequently involve molecular interactions at two distinct sites. A derivatized polyacrylic-based polymer was synthesized to allow substitution with multiple ligands (e.g., L^1 and L^2) on the backbone. Two-site P-selectin-ligand interactions were first studied with $SiaLe^x$ (L^1) and tyrosine sulfate (L^2) covalently incorporated onto the flexible polymer. In competition assays, a marked synergistic inhibitory effect was observed when the polymer presented both L^1 and L^2 as opposed to either ligand alone. In a second approach, the $SiaLe^X$ ligand was reduced in complexity so that L^1 was fixed as Le^x or Le^a, and alternative L^2 groups (to mimic sialic acid) were investigated. Certain combinations of L^1 and L^2 were better antagonists of P-selectin than $SiaLe^x$ itself. These approaches offer the potential of facilitating the discovery of novel inhibitors of receptors or enzymes.

Introduction

Many receptor-ligand interactions are complex, especially when the ligand is a macromolecule such as a protein or a complex glycan. The complexities are exemplified with receptors such as chemokine receptors and P-selectin, for which molecular interactions with ligands occur at two or more distinct sites on the receptor. The complexity of these interactions has hampered the discovery of effective receptor antagonists in these cases. For example, standard "black box" screening procedures for antagonists frequently target a single site on a receptor and ignore the potential for targeting both sites. Furthermore, the identity of a ligand interacting at a second site may be unknown. Rational drug design is resource-intensive and requires a knowledge of the spatial organization of the active center of a receptor or enzyme.

Recently, substituted polyacrylamide-based polymers have been developed for presenting carbohydrate ligands to selectins [1]. These neoglycoconjugates proved valuable in evaluating selectin-mediated interactions in vitro and in establishing screening assays for selectin inhibitors [2]. The relatively facile chemical derivatization of these polymers opens up the possibility of further applications for their use in studying receptor-ligand interactions, for example in searching for alternative or new ligands interacting at a specific site and for dissecting complex macromolecule ligands into simpler, active constituents. The work described in the current paper evaluates these proposals by using P-selectin as a model receptor.

Selectins and their ligands mediate leukocyte recruitment in inflammatory processes [3]. The oligosaccharide $SiaLe^x$ borne on the glycoprotein PSGL-1 is a minimal ligand for P-selectin (see Figure 1 for the structure of $SiaLe^x$ and other selectin ligands). High-affinity interactions between PSGL-1 and P-selectin require an additional anionic domain, which has been identified as a motif containing three sTyr (tyrosine-*O*-sulfate) residues on PSGL-1 ([4]; see Figure 2). The syntheses of a number of analogs and mimetics of selectin carbohydrate-based ligands have been reported (for examples, see [5]). As a result, it has been shown that the key "pharmacophores" for recognition of $SiaLe^x$ by P-selectin are the hydroxyl groups of fucose and the carboxylic acid group of sialic acid [6]. A number of rationally designed glycomimetics have incorporated a fucose residue on a template with acidic peptides. Such compounds have shown moderate to good activity as selectin inhibitors [7, 8]. Other strategies have involved the synthesis of compounds in which an α-D-mannosyl moiety (isostere to L-fucose) is linked to a carboxy group on a biphenyl template (i.e., they are situated in tight proximity). These compounds have similar (or better) selectin-blocking activity to that of the parent $SiaLe^x$ [9].

Exploration of P-selectin ligand recognition is a convenient model for evaluating the proposals outlined above for two main reasons: first, the two-site interaction model is well documented [4]; second, chimeric molecules L^1-Y-L^2, where L^1 is $SiaLe^x$, L^2 is tyrosine sulfate, and Y is a rigid template corresponding to the distance between the binding sites on selectin, have already been synthesized and shown to bind to P-selectin [10].

One Principle of Approaching the Design of Neo-Ligands

A number of copies of a small-molecule partial ligand (or a component of a complex ligand) (L^1) for a receptor can be attached to a flexible polymer. Multiple copies of a second ligand (L^2) can be attached to the same polymer (P). The ligands can be selected either randomly

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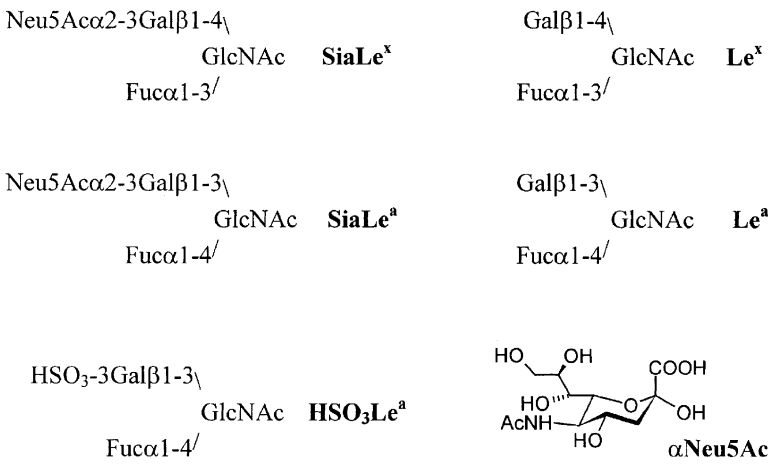

Figure 1. Formulae and Designations of the Oligosaccharides Used in this Work

Although SiaLe^x is the natural selectin ligand, we have also used SiaLe^a, which has a higher affinity for the selectins, and the sulpho-analog, HSO_3Le^a. Neu5Ac is N-acetylneuraminic acid, GlcNAc is N-acetyl-D-glucosamine, Fuc is L-fucose, and Gal is D-galactose.

or rationally. If the receptor recognizes a second ligand (L^2), the binding of L^1-P-L^2 with the receptor becomes much stronger than with L^1-P alone, i.e., a favorable combination of L^1 with L^2 produces a synergistic effect on binding to the receptor. Polymer flexibility is crucial because only a flexible template allows both ligands to adopt a conformation to accommodate the receptor binding sites (see Figure 3). Although the interaction of two flexibly bound ligands with a receptor is entropically unfavorable, this unavoidable price is not a barrier in the initial search for a successful L^1/L^2 combination. Once an initial ligand pair L^1/L^2 is found, one can progress to the second stage of ligand (inhibitor) design, namely the synthesis of a limited library L^1-Y-L^2 where Y is a small rigid template.

Results and Discussion

P-Selectin Binding Properties of Mono- or Bi-Ligand-Substituted Polymers

The ability of mono- and bi-ligand-substituted polymers to inhibit P-selectin was first evaluated with a flexible soluble polyacrylamide (PAA) carrier decorated with either SiaLe^a (L^1) or sTyr (L^2) or both ligands. Previous work has shown that SiaLe^a is a better ligand for P-selectin than SiaLe^x [2]. The mono- and bi-substituted polymers were evaluated as P-selectin ligands in a cell-free competition assay [2]. The mono-substituted ligands, SiaLe^a-PAA or sTyr-PAA, inhibited P-selectin with an IC_{50} value of 30 μM (Figure 4). A simple additive effect was observed when both mono-substituted polymers were included together in the assays. However, a marked synergistic inhibitory effect was found with the bi-substituted ligand, SiaLe^a-PAA-sTyr (IC_{50}, 4 μM; Figure 4). These results illustrate that occupancy of both binding sites on P-selectin with tethered ligands is required for optimal antagonism.

Reconstitution of SiaLe^x-Like Ligands on a Polymeric Backbone

Using knowledge of the key requirements for selectin binding (see Introduction), we have constructed a model for the design of a SiaLe^x mimetic (Figure 5). It is possible in principle to reassemble components of a complex ligand in close proximity on a polymer backbone (see Figure 3). The gain in entropy due to the beneficial mutual orientation of the $L^1 + L^2$ pair complexed with a receptor compensates for the entropy loss. A third interaction is crucial for the recognition of SiaLe^x by selectins. This is mediated by a Ca^{2+} ion, which makes contacts with both the protein and the carbohydrate. Therefore, calcium ions, which are present in the medium during the assay, could play a positive role in modulating the geometry of $L^1 + L^2$ on the polymer.

To investigate the importance of the component blocks, we have synthesized an Le^x-containing polymer bearing additional carboxy groups (L^2 = -$(CH_2)_5$COOH or -Neu5Acα, Table 1, compounds 5 and 6) or sulfate

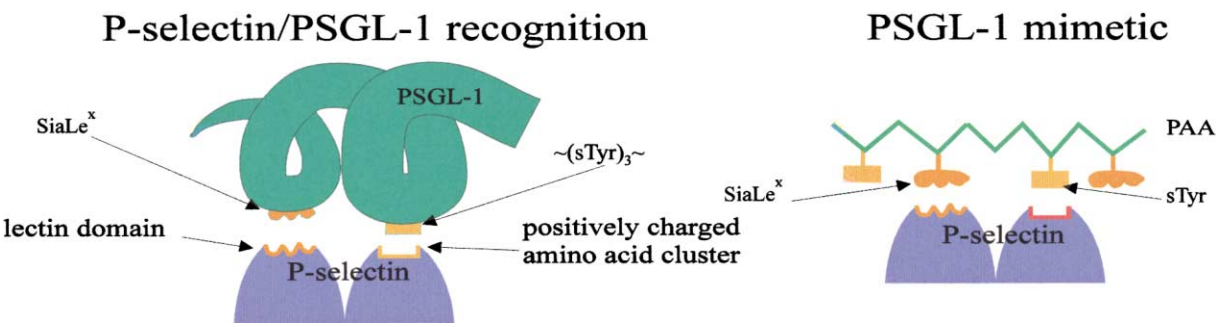

Figure 2. Scheme of the Two-Site Interaction of P-Selectin with PSGL-1 and the Design of PSGL-1 Mimetic by Attachment of Multiple Copies of Oligosaccharide and Tyrosine Sulfate to a Flexible Polymer PAA

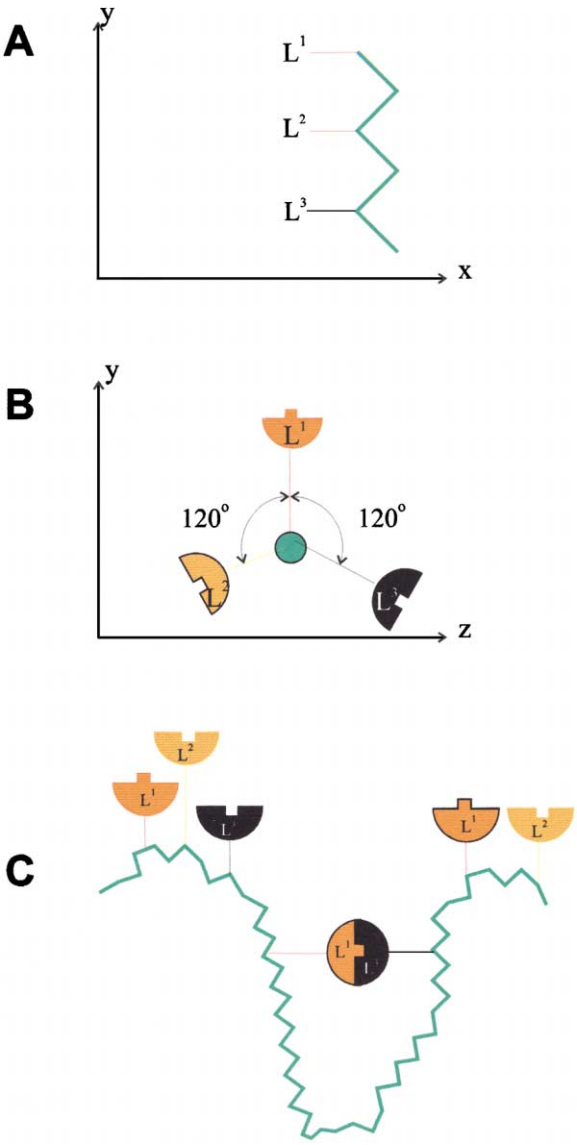

Figure 3. Presentation of Ligands in a Polymeric Chain

(A and B) Adjacent ligands, for example as L^1/L^2 or L^1/L^3 pairs, cannot be assembled closely because they are arrayed at an angle of about 120°.

(C) In a flexible polymer, optimal spatial orientation of ligands can be achieved because the two ligand pairs can be located on different coils of the polymer chain.

(L^2 = $-CH_2CH_2OSO_3H$, compound 7) in molarity equivalent to that of Le^x. These negatively charged glycoconjugates did not display consistent inhibitory activity (Table 1). However, if the trisaccharide Le^x or Le^a is attached to polyacrylic acid (compound 1), the inhibitory activity of the macromolecule (IC_{50}, 30 μg/ml) is similar to that of SiaLe^x-PAA (compound 9, IC_{50} value of 40 μg/ml). Note that the polyacrylamide-based Le^a-PAA (compound 2) or Neu5Acα-PAA (compound 4) did not inhibit P-selectin. Polyacrylic acid itself showed some inhibitory effect (compound 3), and coupling of polyacrylic acid with SiaLe^a produced a potent inhibitor (compound 8) having an IC_{50} of 2 μg/ml.

Polymer-Based Neo-Ligands for P-Selectin

To evaluate the potential of assembling neo-ligands for P-selectin on a polymer template, we synthesized additional mono- and bi-substituted polymers (Table 2). Incorporation of a monosaccharide D-mannose residue (instead of Le^x) on the polyacrylic acid was found to give potent inhibition (Table 2, compounds 10 and 12). Interestingly, these compounds are even more active than the polymeric Le^a trisaccharide (compound 1).

The results shown in Table 2 also confirm the previous observation that the carboxy groups in the polymer matrix influence the inhibitory activity of the glycoconjugates. For example, the αMan-polyacrylic acid polymer (compound 13) with a carboxy group content of 20 mol% is several times *less* active than the completely uncharged αMan-polyacrylamides (compounds 15 and 16). Although a charged group on the ligand or on the polymer matrix is not obligatory for inhibitory activity, it is evident that an acidic polymeric backbone markedly increases the potency of inhibition. It is possible that the flexibility of the polymer chain is influenced positively by the degree of charge.

Interestingly, we found a synergistic inhibitory effect on P-selectin by including mannose plus a charged group on the polymer backbone (Table 2). Both Man-PAA (compounds 15 and 16, uncharged) and polyacrylic acid without ligand (compound 3) are at least 100 times less active than the Man-polyacrylic acid conjugates (compounds 10 and 12). It is not possible to predict at this stage whether the charged group of the polymer mimics the carboxy group of SiaLe^x itself or the anionic interaction normally mediated by the cluster of tyrosine sulphates on PSGL-1. Compounds of the αMan-$OCH_2CH_2CH_2$-PAA-COOH-type exhibited low activity, whereas the SiaLe^a-polyacrylic acid has high activity in comparison to SiaLe^a-PAA (compound 19). Thus, polyacrylic-acid mannose derivatives (compound 10) are similar in this respect to the bi-substituted conjugate SiaLe^a-PAA-sTyr (compound 20, the most potent reference compound). Most probably, both types of interaction contribute to the high activity of Man-polyacrylic acid. In other words, there is a contribution from the interaction of the -COOH cluster with the sTyr binding region of P-selectin as well a contribution from Man- and -COOH structures mimicking the SiaLe^x group.

The high activity of the mannose-containing conjugates is particularly noteworthy. Although free mannose did not affect P-selectin even at a concentration of 0.3 M, its polymeric derivatives showed considerable inhibitory potency (Table 2). However, the conjugate αMan-$OCH_2CH_2CH_2$-PAA (compound 16), which does not bear either a negative charge or aromatic groups, had activity comparable to that of SiaLe^x-PAA. It should also be noted that Man-PAA was even more effective as an inhibitor than the corresponding fucose derivative, whereas Le^a-PAA (compound 2, Table 1) showed relatively poor activity. The Man-polyacrylic acid glycoconjugate (compound 10) displayed even greater activity, having an IC_{50} value of less than 1 μM (PAA and polyacrylic acid have identical molecular masses and therefore can be compared on a mass or molarity basis). The aromatic aglycon polyacrylic acid derivative (compound 10) was 10-fold more potent than the corresponding aliphatic derivative (compound 12; Table 2).

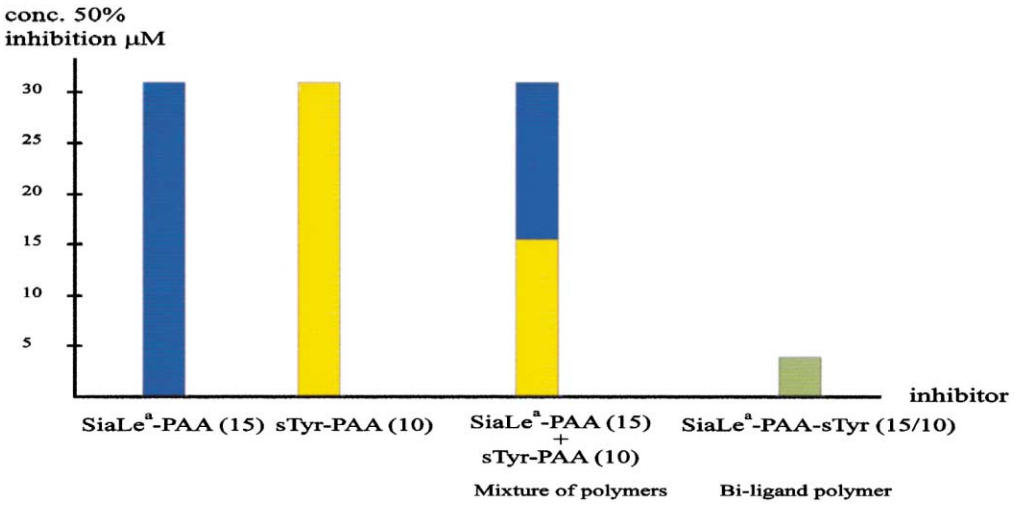

Figure 4. Synergistic Inhibitory Effect on P-Selectin of Presenting both SiaLe^a and sTyr on a Polymeric Backbone

Each of the mono-ligand-substituted polymers, SiaLe^a-PAA (15%) and sTyr-PAA (10%), inhibit P-selectin with an IC_{50} of 30 μM. A mixture of the mono-ligand PAA conjugates has an additive effect only. The bi-ligand conjugate SiaLe^a-PAA-sTyr (15/10) has a greater inhibitory potency (with respect to that of SiaLe^a), with an IC_{50} of 4 μM.

Although the polymer backbone introduces flexibility around the functional groups, it is likely that once an $L^1 + L^2$ pair has interacted with its receptor the "flexibility" of additional $L^1 + L^2$ pairs is impaired, thereby restricting the formation of truly multivalent interactions. This may be less of an issue with large polymers (e.g., >> 30 kDa). Thus, mono-ligand conjugates of LPAA may be better suited for the study of multivalent interactions than the bi-ligand polymers.

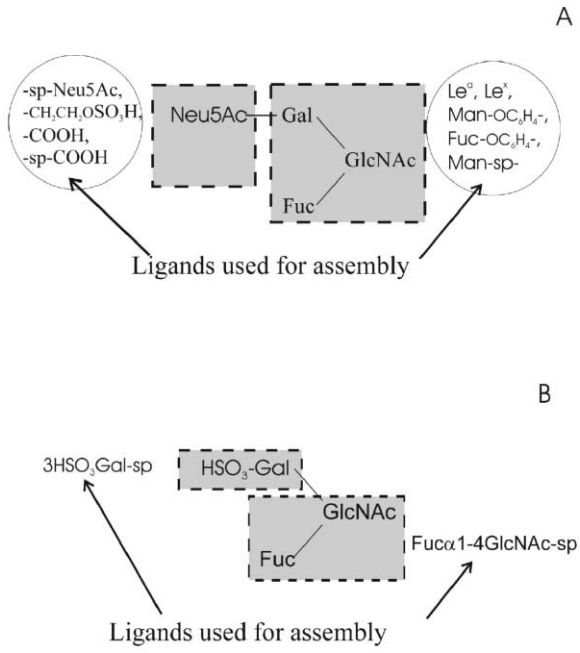

Figure 5. Dissection of Selectin Binding Oligosaccharides into Their Component Parts, which Can Be Used for Reassembly on a Polymer

(A) Tetrasaccharide SiaLe^x or SiaLe^a.
(B) Trisaccharide HSO_3Le^a.

Reconstitution of HSO_3Le^a on a Polymeric Backbone

The trisaccharide HSO_3Le^a is known to be a ligand for P-selectin [5]. Therefore, we dissected HSO_3Le^a into the fragments Fucα1–4GlcNAc and 3-HSO_3Gal (see Figure 5B) and incorporated these saccharides either individually or as bi-ligands onto the flexible polymer. The individual mono-substituted polymers containing 30% mol. of the disaccharide or 3-HSO_3Gal did not inhibit P-selectin binding. However, the bisubstituted conjugate Fucα1–4GlcNAc-PAA-HSO_3Gal, ratio 30/30 or 15/15, inhibits P-selectin with an activity that is only 3–4 times less than that of the parent HSO_3Le^a-PAA (our unpublished data). Similar results were recently described for the conjugate HSO_3GalβOC_6H_4- with an α-fucose attached to polyacrylamide, which was an inhibitor of P- and L-selectin [11].

Table 1. Effect of Incorporation of either Le^x or Le^a and an Acidic Motif into Neoglyconjugates of a "Virtual" SiaLe^x or SiaLe^a Structure, Respectively

Compound	Glycoconjugate	IC_{50}, μg/ml
1	Le^a-polyacrylic acid (20)	30
2	Le^a-PAA (20)	N.I.
3	Polyacrylic acid	150
4	Neu5Acα-PAA (20)	N.I.
5	Le^x-PAA-$(CH_2)_5$COOH (10/10 or 20/20)	N.I.
6	Le^x-PAA-Neu5Acα (20/20)	>500
7	Le^x-PAA-$CH_2CH_2OSO_3H$ (10/10)	>500
8	SiaLe^a-polyacrylic acid (20)	2
9	SiaLe^x-PAA (20)	40

Values indicate the inhibitory potency (IC_{50}, μg/ml) of glycoconjugates in a P-selectin assay. The number in parentheses denotes the molar percent of the ligand or ligands. N.I., no inhibition.

Table 2. Incorporation of Mannose and an Acidic Motif into a "Virtual" Selectin Ligand

Compound	Glycoconjugate[a]	IC_{50}, μg/ml (μM)
10	αMan-OC_6H_4-polyacrylic acid (20)	0.5 (0.7)
11	αFuc-OC_6H_4-polyacrylic acid (20)	20 (30)
1	Le^a-polyacrylic acid (20)	15 (15)
12	αMan-$OCH_2CH_2CH_2$-polyacrylic acid (20)	4 (6)
3	polyacrylic acid[b]	150 (1500)
13	αMan-$OCH_2CH_2CH_2$-PAA-COOH[c] (20/20)	>500 (>1000)
14	αMan-OC_6H_4-PAA-sTyr (20/10)	30 (50)
15	**αMan-OC_6H_4-PAA (20)**	**250 (400)**
16	**αMan-$OCH_2CH_2CH_2$-PAA (20)**	**100 (150)**
17	**PAA**	**N.I.**
18	**Man**	**N.I. (0.3 M)**
8	$SiaLe^a$-polyacrylic acid (20)	2 (1.3)
19	$SiaLe^a$-PAA (20)	60 (40)
20	$SiaLe^a$-PAA-sTyr (15/20)	0.4 (0.25)

The inhibitory potency of glycoconjugates was determined in the P-selectin assay. Glycoconjugates differing in the nature of the sugar, spacer, and acidic group on polymer are compared in the upper part of the table. The data for the corresponding uncharged polyacrylamide derivatives and negative controls are given in bold in the center of the table, whereas the activities of several most active conjugates described in this paper are shown in the lower part of the table.

N.I., no inhibition.

[a] Number in parentheses denotes molar percent of the ligand or ligands.

[b] For polyacrylic acid, molar concentration is calculated on the COOH group; for glycoconjugates it is calculated on the saccharide moiety.

[c] "COOH" is a dipeptide or ω-amino acid moiety.

Significance

The design of inhibitors of complex receptor-ligand interactions is a difficult task. The current work demonstrates that the assembly of a complex epitope from two ligands on a flexible polymer is possible under situations either when there are two distinct binding epitopes separated in space (e.g., sTyr plus $SiaLe^a$, first section of Results and Discussion) or when a complex epitope such as $SiaLe^x$ or HSO_3Le^a can be broken down into simpler components that are then displayed on the polymer (second through fourth sections of Results and Discussion). A similar strategy has been described in the search for influenza virus hemagglutinin inhibitors [12, 13]. Now that the basic principle of the proposed strategy for ligand assembly has been established, the concept can be extended to broaden the types of functional groups incorporated onto the polymer. A combinatorial approach with a multimeric template has been described for the discovery of a synthetic receptor for sialic acid [14], and the concept of "virtual combinatorial libraries" has been discussed recently [15, 16, 17]. These approaches will expand the range of ligand mimetics capable of interacting with a receptor or enzyme. Ultimately, this strategy will facilitate the discovery of novel inhibitors of receptors or enzymes.

Experimental Procedures

Materials

Fucoidan, 2-ethanolamine, triethylamine, Sephadex LH-20, and dimethylformamide were from Sigma (USA). All other chemicals were analytical grade from Fluka (Switzerland). Nitrophenyl glycosides were a gift from Dr. Y. Vozney (Zelinsky Institute, Moscow). The P-selectin assay system has been described in detail in [2, 18]. It is based on inhibition of the binding of the neoglycoconjugate HSO_3Le^a-PAA-biot to recombinant P-selectin-ZZ immobilized on 96-well polystyrene plates via an IgG bridge.

Synthesis of N-Substituted Polyacrylamide-Based Glycoconjugates: $SiaLe^a$-PAA-sTyr as an Example

A solution of 1.5 μmol of 3-aminopropyl glycoside of tetrasaccharide $SiaLe^a$ [19] in 0.5 ml DMF and 10 ml triethylamine was added to a solution of 1.93 mg (10 μg-Eq.) poly(4-nitrophenylacrylate) [20] in 0.5 ml DMF, and the resulting solution was kept at 25°C for 16 hr. Then a solution of 2 μmol tyrosine sulfate [21] in 0.5 ml DMF was added, and the solution was kept again as above. A 30-fold molar excess of 2-ethanolamine was added and left for 16 hr. The solution was applied to a Sephadex LH-20 column, and the conjugate (compound 20, Table 2) was eluted with a mixture of acetonitrile/water 1:1 with RI control of fractions. The yield of lyophilized conjugate was more than 90%, with a molecular weight of 30–40 kDa.

Synthesis of Polyacrylic Acid-Based Glycoconjugates: αMan Derivative as an Example

A solution of 7 μmol 3-aminopropyl glycoside of α-D-mannose (Syntesome GmbH, Munich) in 0.2 ml DMF was mixed with 0.07 ml of 10% solution of poly(4-nitrophenylacrylate) [20] (35 μg-Eq.) in DMF, 0.02 ml of triethylamine was than added, and the mixture was kept at 20°C for 48 hr. The resulting conjugate was further modified by the addition of 2 ml 0.1 N aqueous NaOH. The solution was applied to the column with Sephadex LH-20 (1 × 25 cm), and the conjugate was eluted with the mixture of acetonitrile/water 1:1. The polymer with 20% Man molar content and 80% carboxylic groups content (compound 13, Table 2) was thus obtained.

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