Polyacrylamides Bearing Pendant α-Sialoside Groups Strongly Inhibit Agglutination of Erythrocytes by Influenza A Virus: Multivalency and Steric Stabilization of Particulate Biological Systems†

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An α–sialoside linked to acrylamide by a short connector (5-acetamido-2-*O*-(*N*-acryloyl-8-amino-5-oxaoctyl)-2,6-anhydro-3,5-dideoxy-D-glycero-D-galacto-α-nonulopyranosonoic acid, **1**) was prepared. Compound **1** formed high molecular weight copolymers with acrylamide, derivatives of acrylamide, and/or vinylpyrrolidone upon photochemically-initiated free radical polymerization. Those copolymers for which the substituents on the acrylamido nitrogen were small inhibited the agglutination of chicken erythrocytes induced by influenza virus (X-31 (H3N2); a recombinant strain of A/Aichi/2/68 (H3N2) and A/Puerto Rico/8/34 grown in chicken eggs). The inhibitory power of the polymers depended strongly on the conditions of polymerization and the sialic acid content of the polymer. The strongest inhibitors were copolymers (poly(**1**-*co*-acrylamide)) formed from mixtures of monomer containing [**1**]/([**1**] + [acrylamide]) ≈ 0.2–0.7; these copolymers inhibited hemagglutination 10^4–10^5 times more strongly than did similar concentrations of α-methyl sialoside (calculated on the basis of the *total* concentration of individual sialic acid groups in the solution, whether attached to polymer or present as monomers). Samples polymerized in the presence of low concentrations of cross-linking reagents (bis(acrylamido)methane, BIS, and 2,2′-bis(acrylamido)ethyl disulfide, BAC) also showed increased inhibition (10–10^3-fold relative to monomers), but their use was limited by their poor solubility. Sterically demanding substituents on any position of the acrylamide component (substituents attached to the vinyl group or *N*-alkyl groups that are larger than hydroxyethyl) reduced the inhibitory power of the polymer. A ^{1}H NMR assay and a fluorescence depolarization assay showed that poly(**1**-*co*-acrylamide) bound to a solubilized trimeric form of the viral receptor for sialic acid (bromelain cleaved hemagglutinin, BHA), *less* tightly than **1**, on a per sialic acid basis. A similar result was also obtained with a model system comprising lactic dehydrogenase (a tetramer) and polymeric derivatives of oxamic acid: that is, poly((**28**, **29**, **30**, or **31**)-*co*-acrylamide) had a *higher* inhibition constant for tetrameric lactic dehydrogenase than did the corresponding monomers (**28**, **29**, **30**, or **31**) on a per oxamate basis. Poly(**1**-*co*-acrylamide) is, in principle, capable of inhibiting the agglutination of erythrocytes by several mechanisms: (1) entropically enhanced binding of the polymer (acting as a polyvalent inhibitor) to the surface of the virus; (2) steric interference of the approach of the virus to the surface of the erythrocyte by a water-swollen layer of the polymer on the surface of the virus; (3) aggregation of the virus induced by the polymer. Although the most probable mechanism of inhibition is a combination of polyvalent interaction and steric interference, a mechanism based solely on polyvalent interactions cannot be ruled out.

Introduction

Influenza virus undergoes frequent antigenic variation,[1–3] and it is difficult to produce vaccines that are effective against multiple present and emerging strains of virus. In principle, a general approach to prevent infection by influenza virus (and by all other viruses and many microorganisms) would be to prevent the attachment of the virus to the surface of the cell. The binding site on the virus does not undergo a dramatic antigenic shift.[4,5] The molecular basis of the initial step in attachment of influenza virus to the cell surface is the binding of the viral membrane protein hemagglutinin (HA) to the terminal sialic acid residues of the carbohydrate portions of cell surface glycoproteins and glycolipids.[4] An effective inhibitor of this interaction might therefore prevent infection by influenza virus.

Hemagglutinin (HA) is a trimeric, membrane-bound viral glycoprotein of molecular weight approximately 225 kDa.[5–7] The external domains of the three subunits of HA contain the three binding sites for sialic acid at their tips. (It has been proposed that there is a second weaker binding site on each subunit of HA,[8] but there is no evidence that this binding site is relevant to the phenomena discussed in this work.)

The precise spatial arrangement of the hemagglutinin trimers on the surface of influenza A is unknown.[9–12] Studies using low-angle neutron scattering have implied that the hemagglutinin trimers on influenza B are 105 or 109 Å apart;[9,10] neutron scattering data for influenza A gave similar results (Figure 1).[10]

Inhibition of the interaction between virus and surface glycoprotein might be accomplished in a number of ways: (1) by sterically blocking the HA binding sites (using a ligand that competes with sialic acid on the

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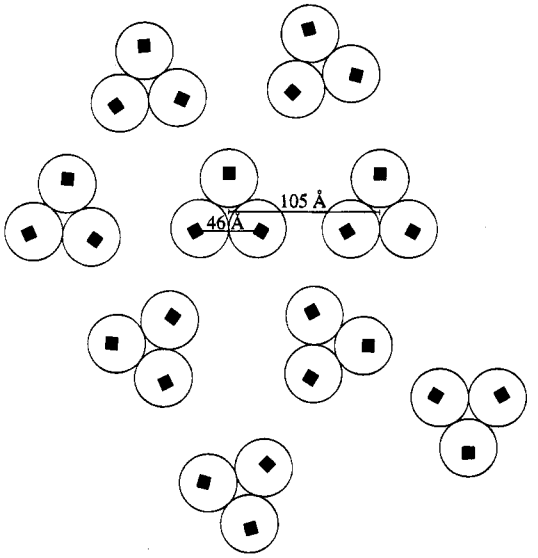
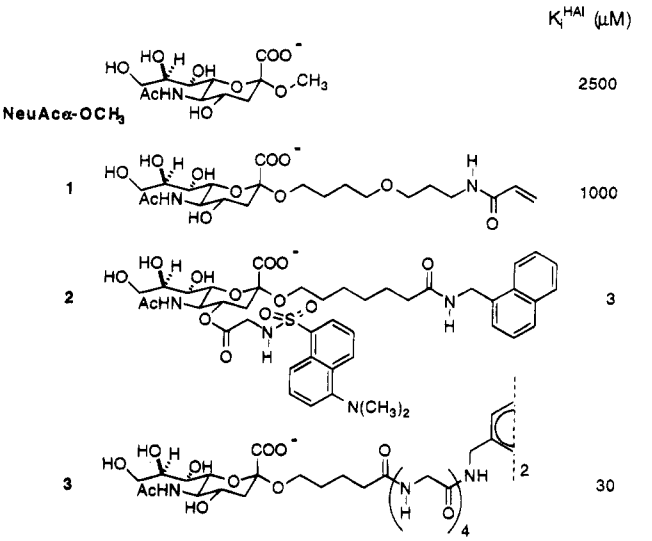


Figure 1. The HA trimers are arranged in a disordered array on the surface of the virus. The black box symbol (■) represent the binding sites for sialic acid on each HA (8 Å × 8 Å). The circles represent one subunit of HA of 40 Å in diameter. The through-space distance between the binding sites on each of the HA units within one trimer is 46 Å (anomeric carbon of one bound sialic acid to anomeric carbon of another bound sialic acid). The average through-space distance between the centers of the HA trimers, in this representation, is *ca.* 105 Å; this distance is not well defined by currently available data.

cell surface for the sialic acid binding site or that binds close to that site on the virus); (2) by forming complexes of an added ligand with sialic acid groups on the surface of the target cell that renders them inaccessible to the HA on the virion; (3) by preventing access of the HA binding sites to the sialic acid moieties by some nonspecific mechanism. Blockage of all of the sialic acid moieties on the surface of a mammalian cell is probably both impractical and unwise, since this type of complexation could interfere with other necessary functions. We have focused on inhibiting HA by blocking some sialic acid binding sites and concomitantly preventing access of the virus to the surface of the cell.

The availability of a high-resolution crystal structure of hemagglutinin[6,13] has stimulated efforts to design analogues of sialic acid that bind tightly to HA. A structure-based strategy has been claimed to be successful in generating a very tight-binding inhibitor for neuraminidase, (4-guanidino-Neu-5Ac2en has a K_i = 10^{-10} M),[14] a viral surface enzyme that cleaves the α-glycosidic linkage between a terminal sialic acid and an adjacent saccharide residue. We[15-17] and others[18-21] have tried to develop compounds that bind tightly to HA, but simple modifications of sialic acid have led to inhibitors of only modest potency (~10 μM to 1 mM). The monovalent derivative of sialic acid that binds the most tightly to bromelain cleaved hemagglutinin (BHA; a soluble form of hemagglutinin that retains the trimeric cluster of sialic acid binding sites)[5,7] is **2** (K_i = 3 μM).[19] Bivalent inhibitors can also inhibit hemagglutination effectively (~30−10^3 μM).[22-24] Compound **3** is the strongest inhibitor of hemagglutination in this class; it has been suggested that its high activity is due to binding that bridges two HA trimers on the surface of the virion.[22,23] (Because the dissociation constant of the complex between BHA and **3** was 1500 μM, it was concluded that the dimer was bridging two HA trimers and therefore binding intertrimer instead of intratrimer.) Recently, dendrimeric inhibitors, with between 2 and 16 attached sialic acid residues per molecule, have also been shown to inhibit hemagglutination (~20−10^3 μM).[24]



We and others have investigated a conceptually different approach to inhibiting the association of virus to cell based on the use of multivalent systems. Multivalent ligands have in some cases proved to bind substantially more tightly than the corresponding monomers in biological systems that display multiple copies of a complementary receptor.[7,22-49] Although the intrinsic binding of monomeric α-sialosides with HA is relatively weak (K_d ≈ 2000 μM),[4,15,20] the binding of HA reconstituted in liposomes (and, one presumes, of influenza virus) to cells appears to be strong.[50] A qualitative rationalization of this observation is that the interaction between virus and cell is polyvalent.[50,51] The influenza virion has between 200 and 1000 hemagglutinin trimers on its surface, with the estimate depending on which technique is used to obtain this number.[13,52] Because sialic acids are present in high numbers on the surface of mammalian cells,[53] multiple binding interactions between virus and the surface of the cell appear likely. This binding will be affected by the mobility of HA in the membrane (this mobility is not well understood[54]) and by the high mobility of the sialoglycoproteins in the cell membrane. The hypothesis that polyvalency is important in the interaction between virus and cell is supported by the observation that certain glycoproteins (specifically equine α_2-macroglobulin and guinea pig α_2-macroglobulin[55-58]) having a large number of sialic acid groups per protein are strong inhibitors of virus-induced agglutination of erythrocytes. The observation that other glycoproteins also having a large number of sialic acid groups per protein (human α_2-macroglobulin, fetuin) are weak inhibitors suggests that factors other than the presence of multiple sialic acids groups per protein (perhaps the arrangement of these groups in space or their steric accessibility) must also be important in determining the strength of inhibition of hemagglutination.

On the basis of the assumption that polyvalency is important in binding virus to the surface of the cell, we set out to synthesize substances that presented multiple copies of ligands (derivatives of sialic acid and its analogues) that bound to HA in competition with α-sialosides. We hoped that these substances would

bind to the surface of the virus in a cooperative, multidentate fashion.

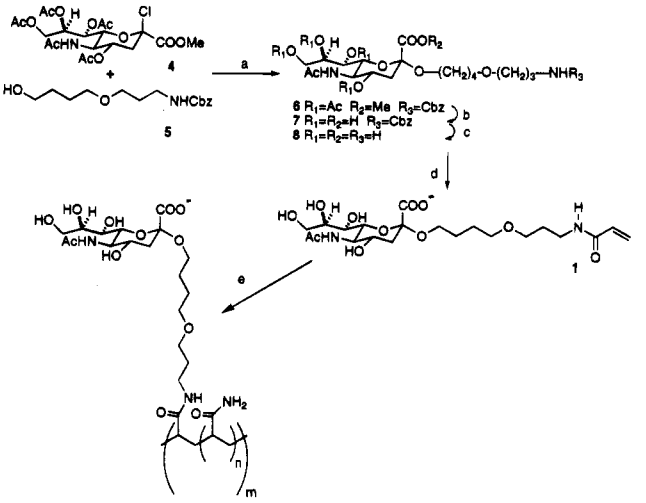
Others have synthesized multivalent inhibitors of hemagglutination chemically.[59-69] Early procedures involved the cross-linking of sialoglycoproteins that, in their monomeric forms, already displayed a capability to inhibit hemagglutination.[59-61] The approach based on cross-linking of glycoproteins is, however, limited in utility for several reasons: first, only a small number of active sialoglycoproteins are readily available; second, the synthesis of protein clusters containing more than a few monomers is difficult; third, protein oligomers are unpromising candidates for human therapies for several reasons, including antigenicity and solubility; fourth, opportunities to vary the environment around the sialic acid groups in a structurally defined way is limited in these compounds. For these reasons, we decided to pursue an approach based on the synthesis of appropriate macromolecules by free radical copolymerization of derivatives of sialic acid having attached acrylamide moieties with other vinylic monomers (especially other derivatives of acrylamide).

Polyacrylamides bearing sialic acid groups on side chains are attractive candidates for polyvalent inhibitors for several reasons. Both the substituted acrylamide monomers and the polymers derived from them have excellent solubilities in water. Polyacrylamides can be prepared readily by free radical polymerization of appropriate monomers, and this type of preparation provides an experimentally simple and flexible route to a broad range of structures interesting as potential inhibitors. We particularly wished to include secondary substituents in the polymer that would change its solubility or conformation, and that might, ultimately, include ligands that would influence delivery, targeting, and pharmacokinetics.[70] Although copolymerization is synthetically convenient, the resulting polymers are characterized by substantial geometrical randomness in the disposition of the sialic acid groups in space, both because of disorder in the sequence of monomer units along the polymer backbone and because of the conformational flexibility of the polyacrylamide backbone and the tethering groups. We have previously reported that certain polyacrylamides derivatized with pendant *O*-glycosides of sialic acid inhibited the agglutination of erythrocytes induced by influenza virus up to 10^4 times better than monomeric sialic acids, based on a comparison normalized for the total number of sialic acid moieties in solution.[62] This observation has been supported by independent work in other laboratories.[63-65] Similar inhibition of hemagglutination has subsequently been observed for polyacrylamides derivatized with pendant *C*-glycosides[66,67] and for liposomes functionalized with *O*- and *C*-glycosides.[68,69] Liposomes presenting sialic acids are even more effective than soluble polyacrylamides derivatized with pendant sialosides in preventing hemagglutination when inhibitory effectiveness is calculated based on total sialic acid groups in solution (whether present on liposomes or polymer).

Although polyacrylamides with pendant *O*- and *C*-glycosides of sialic acid, and liposomes with pendant *C*-glycosides, inhibit the hemagglutination of erythrocytes, they may or may not be active *in vivo*. When polymers containing *O*-glycosides were tested for their ability to inhibit the growth of virus in chicken cell embryos, no inhibition was observed.[65] This observation may be due to the hydrolysis of the α-glycosidic linkage between sialic acid and the polymer backbone by neuraminidase. Polymers and liposomes containing *C*-glycosides were tested for their ability to prevent infectivity in cultured Madin-Darby canine kidney (MDCK) cells using a standard plaque reduction assay.[66] A polymer containing 30% α-sialic acid residues (500 μM sialic acid moieties) and a liposome containing 1% α-sialic acid residues (3 μM sialic acid moieties) reduced the number of plaques per well due to viral lysis of infected cells by up to 80% and 96%, respectively. The activity of these liposomes in the plaque reduction assay did not correlate well with their activity in the hemagglutination inhibition assay.

This paper details the design, synthesis, and properties of a series of polymeric, multivalent inhibitors of the agglutination of chicken erythrocytes induced by influenza virus X-31 and outlines possible mechanisms of action for these polymers.

Scheme 1. Synthesis of Monomer 1 and Copolymers[a]



[a] (a) Ag salicylate, C_6H_6; (b) 1 N NaOH (43%); (c) H_2/5% Pd–C, MeOH (94%); (d) *N*-(acryloxy)succinimide, MeOH, H_2O (84%); (e) CH_2=CHCONH$_2$, 4,4′-azobis(4-cyanopentanoic acid), *hν* (quantitative).

Results and Discussion

Synthesis. Scheme 1 outlines the synthesis of the conjugate of sialic acid with acrylamide (**1**) and of the copolymers of **1** with acrylamide. The conversion of sialic acid to the fully protected glycosyl chloride **4** followed a combination of published procedures.[71,72] Coupling of **4** with alcohol **5**, using silver salicylate, afforded α-sialoside **6** in an overall yield of 56% from sialic acid.[73] Cleavage of the acetates with sodium hydroxide gave the tetrahydroxy carboxylate **7** which, upon hydrogenolytic removal of the Cbz protecting group, produced amino acid **8**. Amino acid **8** was coupled with *N*-(acryloyloxy)succinimide in phosphate-buffered methanol to generate the acrylamide **1**.

Characterization of the Polymers. We have not characterized the polymers fully, but when prepared under standard conditions (1 M in acrylamide monomers, 0.02 M in initiator) they have a molecular weight of 10^5–10^6.[74] Exhaustive dialysis of the crude reaction mixture from the polymerization reaction against water using a membrane with a molecular weight cut-off of 10 000 retained all detectable sialic acid residues[75] in the dialysis bag. This result implies that all or most of the sialic acid monomers are incorporated into the polymer. Proton NMR spectra of the crude and purified

polymers revealed broadened lines for all the protons in the polymer backbone and for some of the protons in the tether (those close to the backbone), while most of the resonances due to the sialic acid group appear as sharp lines. Few residual acrylamide resonances are detectable by proton NMR in the unpurified reaction mixture from polymerization; we conclude that incorporation of the sialic acid into polymers is complete.

Accessibility of Sialic Acid Groups to Neuraminidase. As a preliminary test for the accessibility of the sialic acid residues in the polymer, we investigated the behavior of poly(**1**-*co*-acrylamide) (1:5 molar ratio of **1** and acrylamide) toward neuraminidase (EC 3.2.1.18 from *Clostridium perfringens*; used in the form of insoluble enzyme attached to beaded agarose). Prolonged treatment (3 days at 37 °C) of a sample of polymer with immobilized neuraminidase completely abolished the hemagglutination inhibition activity and afforded, after separation on Sephadex G-10, polymer with attached tether (but without attached sialic acid groups) and free sialic acid.

Assays. *We emphasize that binding at the sialic acid binding site of hemagglutinin (whether on virus or in solution), inhibition of the agglutination of erythrocytes induced by influenza virus, and in vivo biological activity are not interchangeable.* The dissociation constant describing the first (which we superscript, for specificity, with the assay used in its determination: K_d^{NMR} for an assay based on NMR spectroscopy, K_d^F for an assay using fluorescence depolarization)[15,19] represents a well-defined molecular event, and K_d^{NMR} should equal K_d^F. The constant obtained from the hemagglutination inhibition assay (K_i^{HAI}; HAI = hemagglutination inhibition) describes the result of a more complex system.[76] The inhibition of the hemagglutination of erythrocytes by virus may be due to the occupancy of the sialic acid binding site of HA, or to any mechanism that prevented the attachment of the virion to the surface of the erythrocyte. K_i^{HAI} can be significantly different from K_d^{NMR} or K_d^F. *In vivo* activity represents a higher level of complexity, and is not discussed here.

Hemagglutination Inhibition Assay. This work relies heavily on a classical hemagglutination inhibition assay.[76] This type of assay has substantial ambiguities in its interpretation at the molecular level, and we explicitly outline the experimental procedure used. The titer of the stock solution of virus was determined for each set of experiments. A 100-μL aliquot of virus stock solution was serially diluted in half and mixed with 100 μL of a suspension of 0.5% chicken red blood cells (RBC) in PBS buffer. The lowest concentration of virus that agglutinated red blood cells after 2 h at 4 °C was determined. The lowest concentration of inhibitor that prevented the agglutination of red blood cells in the presence of virus was then assessed. The assays were run in a standard 96-well microtiter plate (250-μL wells). The inhibitor (50 μL) was serially diluted in half across 12 wells and then mixed with the titered virus solution. Standard conditions allowed the virus and inhibitor to incubate for 30 min at 4 °C before the addition of blood cells (*vide infra*). Chicken red blood cells (100 μL, 0.5% suspension) were then added, and after 2 h at 4 °C, the lowest concentration of inhibitor ($[I_L] = K_i^{HAI}$) at which the red blood cells did not agglutinate was recorded.

To investigate the kinetics of the polymer/virus interaction in the hemagglutination inhibition assay, we varied the incubation time for the virus and polymer from 1 min to 22 h (Figure 2). One minute was the shortest time that we could achieve for allowing virus and inhibitor to incubate before adding RBC; times longer than 22 h could not be investigated using this assay system because the control containing virus alone no longer agglutinated red blood cells. The amount of inhibitor needed to prevent hemagglutination decreased with increasing incubation times; at 22 h, less than 1.5% of the amount of polymer required at 30 min (standard conditions) was necessary for inhibition. *Thus, under the assay conditions used in the rest of this work, the polymer and virus have not reached thermodynamic equilibrium.*

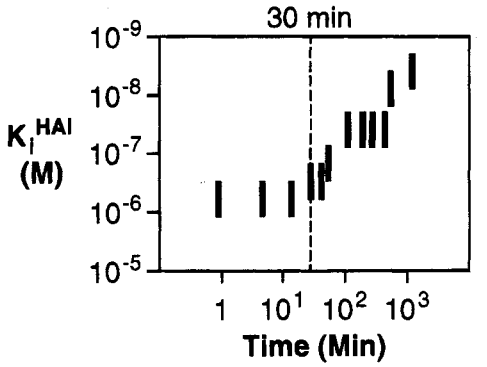


Figure 2. K_i^{HAI} of a 1:5 copolymer of **1** and acrylamide, poly(**1**-*co*-acrylamide), as a function of the preincubation time of virus and polymer in the hemagglutination assay. The error bars, a factor of 2 [representing an uncertainty of one well in the microtiter plate (± ×2)] are represented by the height of the rectangular points. The preincubation time used as standard in the rest of the work in this paper was 30 min and is represented on the plot by a dashed line.

The hemagglutination inhibition assay cannot distinguish between interactions of an inhibitor with virus that result in coating individual virions, aggregating virus (with the inhibitor acting to crosslink virus particles), and other possible mechanisms that deactivate the virus. We have observed, qualitatively, that prolonged incubation of virus with polymers containing sialic acid residues, at much higher concentrations of virus than those used in the hemagglutination inhibition assay, results in the formation of a visible precipitate (which we presume to be an insoluble aggregate of virus and polymer). This observation supports the hypothesis that an earlier stage of coating of virus by polymer might be a contributor to inhibition of hemagglutination.

The Influence of the Conditions Used in Polymerization on K_i^{HAI}. The procedure used for the copolymerization reaction influences several important physical properties of the polyvalent inhibitors. These properties include their size, the extent to which they are branched or cross-linked (if at all), and, perhaps, the distribution of the monomers along the polymer backbone. The conformation of the polymer in solution may also be important and may be a function of molecular weight. We conducted a series of survey experiments that examined the influence on K_i^{HAI} of experimental conditions used in the polymerization reactions and found that the observed inhibitory potency of the copolymers varied significantly with the conditions used in the polymerization reaction. We varied the initiation systems used, the concentrations of monomers, and addition of chain-transfer agents.

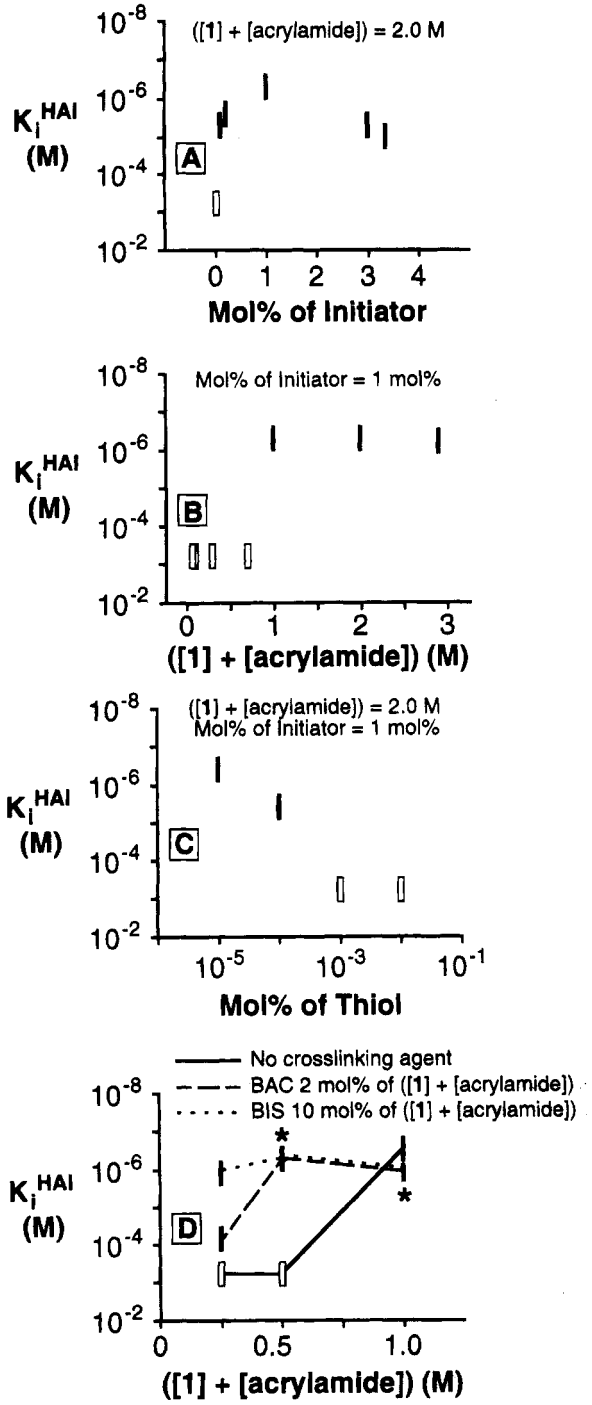


Figure 3. Inhibitory properties of 1:5 copolymers as a function of conditions used in their preparation: (A) varying the mol % of free radical initiator (mol% of ([1] + [acrylamide])) while keeping ([1] + [acrylamide]) at 2.0 M; (B) varying ([1] + [acrylamide]) while keeping [initiator] at 1 mol % of ([1] + [acrylamide]); (C) varying the mol % of mercaptoethanol (mol % of ([1] + [acrylamide])), a chain-transfer agent, while keeping ([1] + [acrylamide]) at 2.0 M and [initiator] at 0.02 M; (D) different cross-linking agents (a, none; b, BAC; c, BIS) at different concentrations of ([1] + [acrylamide]) ([initiator] = 0.02 M). Polymers giving results marked with an asterisk (*) were only partially soluble; BAC at 1.0 M ([1] + [acrylamide]), and BIS at 0.5 and 1.0 M ([1] + [acrylamide]). All polymerization reactions were performed at pH 7 in water under a UV lamp in borosilicate glass tubes. In all cases, the highest concentration of sialic acid groups attached to polymer was 600 μM, and therefore any value of K_i^{HAI} greater than 600 μM is represented by an open rectangle at 600 μM. The error bars, a factor of ±2 (i.e., ±1 well in a 96-well assay), are represented by the height of the rectangular points.

We investigated two methods of initiating polymerization: charge-transfer initiation (persulfate/TEMED, an initiation system used previously by Roy and Laferriere[64]), and UV/ABCV [4,4′-azobis(4-cyanovaleric acid)] promoted initiation. Both systems gave polymers showing similar general trends in the dependence of K_i^{HAI} on the concentration of initiator, monomer, or additives (Figure 3), but the photoinitiation scheme was more reproducible and more amenable to small-scale reactions. We therefore abandoned the charge-transfer initiation system at an early stage in favor of the more convenient and practical photoinitiation system.

Figure 3 demonstrates how polymer preparation conditions affect values of K_i^{HAI}. The value of K_i^{HAI} of the polymers depends strongly on the concentration of free radical initiator and on the initial concentration of monomer. Figure 3A indicates that K_i^{HAI} is lowest when the initial quantity of initiator is ca. 1% that of monomer (mol:mol). Figure 3B indicates that a total concentration of monomers greater than approximately 0.5 M is required to achieve optimum inhibition. Over the range 1–2.8 M ([1] + [acrylamide]), K_i^{HAI} is approximately constant (the upper limit in these experiments is set by solubility of the monomer).

Both decreasing initiator concentration (above the point required to remove adventitious impurities that act as chain terminators) and increasing the monomer concentration are predicted to increase the molecular weight of the polymers (see below). Following this analogy, a high molecular weight polymer gives lower values of K_i^{HAI}. To explore the opposite effect—intentionally shortening the polymer chain—we added increasing amounts of the chain-transfer agent mercaptoethanol to the polymerization mixture.[77] Above thiol concentrations of 10^{-5} mol% of vinylic monomer groups ([1] + [acrylamide]), we observe a significant decrease in inhibitory potency (Figure 3C).

In order to investigate larger polymers and polymers with branching, we added bifunctional cross-linking agents (BAC, 2,2′-bis(acrylamido)ethyl disulfide and BIS, bis(acrylamido)methane) to the polymerization reactions at concentrations of 2 and 10% of vinylic monomer groups, respectively. Generally, adding cross-linker to a copolymer decreased K_i^{HAI}. At low concentrations of monomer, where the reference copolymer shows no detectable inhibition, the cross-linked samples show values of K_i^{HAI} in the micromolar range. Even more tightly binding materials appear to result from cross-linking the polymers obtained from more highly concentrated monomer mixtures, although the poor solubility in water of these polymers prevented detailed analysis. The inclusion of cross-linking agents in these experiments will increase the molecular weight and change the structure of the polymer: it may also generate cross-linked gel particles. We do not have enough information to tell which of these effects might be the most important in decreasing K_i^{HAI}.

Equation 1 relates the degree of polymerization in the absence of chain-transfer

$$DP_o = \frac{k[M]}{[I]^{0.5}} \quad (1)$$

$$\frac{DP}{DP_o} = \frac{1}{1 + C_S DP_o \frac{[S]}{[M]}} \quad (2)$$

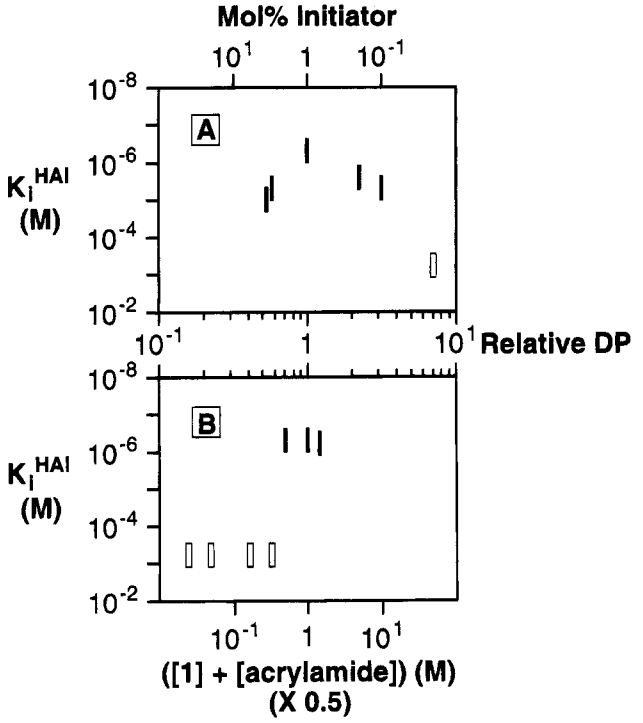


Figure 4. K_i^{HAI} as a function of calculated relative molecular weight. (A) The relative molecular weight is predicted to be proportional to [initiator]$^{-0.5}$. (B) The relative molecular weight is predicted to be proportional to ([1] + [acrylamide])$^{-0.5}$ since the mol % of initiator (mol % of ([1] + [acrylamide])) in the reaction mixture was kept constant. The ([1] + [acrylamide]) was divided by 2 to account for the fact that the ([1] + [acrylamide]) = 2 M in the initiator experiments. In all cases, the highest concentration of sialic acid groups attached to polymer was 600 μM, and therefore any value of K_i^{HAI} greater than 600 μM is represented on the graphs as an open rectangle at 600 μM. The error bars, ± a factor of 2, are represented by the height of the rectangular points. The points in A were obtained from Figure 3A, using eq 1; those in B were obtained from Figure 3C using eq 2.

agents, DP_o, to the concentration of monomer(s) [M] and initiator [I].[78] Equation 2 relates DP_o to the degree of polymerization, DP, in the presence of a chain-transfer agent at concentration [S].[79] In eq 1, k is a constant characteristic of the system of initiator and monomer; it reflects the efficiency of initiating and propagating chains. In eq 2, C_S is a similar constant reflecting the efficiencies of chain termination and transfer.

Figure 4A (for initiator) and 4B (for monomer) are in qualitative agreement in indicating a threshold in DP below which inhibition of hemagglutination is ineffective. Since the proportionality constant in eq 2, ($C_S DP_o$), is different from that in eq 1 (k), the DP obtained from eq 1 cannot be directly compared with the DP obtained from eq 2. We conclude from these data that the DP of the polymer is important in determining K_i^{HAI}, for a given ratio of **1** to acrylamide.

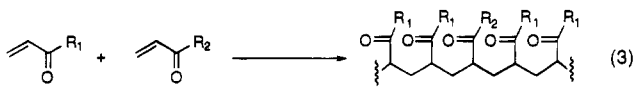
On the basis of the data in Figure 3, we settled on standard polymerization conditions as: [1] + [acrylamide] = 1 M and [concentration of initiator] = 0.02 M.

The Influence of Comonomers on K_i^{HAI}. To test the influences of other groups incorporated as side chains into the polymer on K_i^{HAI}, we copolymerized **1** (R_2 = $NH(CH_2)_3O(CH_2)_4O$-α-sialic acid) with a variety of comonomers (eq 3, Table 1). Small substituents appear to be tolerated well; large substituents (e.g. Tris, tris[(hydroxymethyl)amino]methane) and charged substituents lead to polymers that do not inhibit hemagglutination. The sensitivity of K_i^{HAI} to the structure of the comonomer is striking; we hypothesize that K_i^{HAI} is sensitive to the conformation of the polymer, which, in turn depends on the properties of the monomer such as charge and size. Larger substituents would tend to force the polymer into an extended rod conformation. A change in DP is, however, also possible for polymers with large substituents, since the DP should decrease as the monomers become more sterically hindered. We were not able to test hydrophobic substituents, since the resulting polymers were insoluble in water. Entries 18–20 in Table 1 serve as controls to exclude nonspecific carbohydrate binding or effects of charge as the origin of the inhibition of hemagglutination.

Table 1. Inhibition of Hemagglutination by Various Copolymers (eq 3; R_1:R_2 = 1:5)

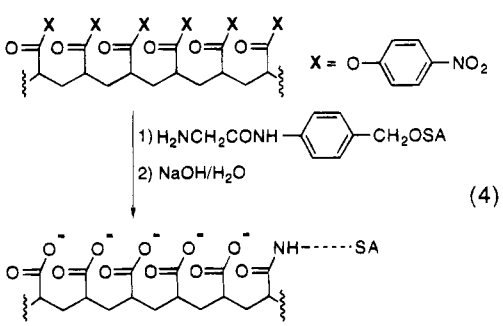
Entry	R_1 or monomer	R_2	K_i^{HAI} (μM)
1	NH_2	$NH(CH_2)_3O(CH_2)_4O$-α-sialic acid, **1**	0.30
2	$NHCH_2OH$, **9**		(<0.30[a])
3	$NHCH_2CH_2OH$, **10**		(<150[a])
4	$NHCH_3$, **11**		10
5	$NH(CH_2)_2O(CH_2)_2OCH_3$, **12**		no inhibition[b]
6	$NH(CH_2)_2O(CH_2)_2O(CH_2)_2OCH_3$, **13**		no inhibition[b]
7	$NH(CH_2)_3O(CH_2)_4OH$, **14**		no inhibition[b]
8	$N(CH_3)_2$, **15**		2.5
9	$NHC(CH_2OH)_3$, **16**		no inhibition[b]
10	$NH(CH_2)_2O$-β-glucose, **17**		no inhibition[b]
11	$NH(CH_2)_3O(CH_2)_4O$-β-glucose, **18**		no inhibition[b]
12	$NH(CH_2)_3O(CH_2)_3COO^-Na^+$, **19**		no inhibition[b]
13	$NH(CH_2)_6NH_3^+$, **20**		no inhibition[b]
14	O^-Na^+, **21**		no inhibition[b]
15	crotylamide, **22**		no inhibition[b]
16	methacrylamide, **23**		300
17	*N*-vinylpyrrolidone, **24**		5.0
18	NH_2	$NH(CH_2)_3O(CH_2)_4O$-β-glucose, **18**	no inhibition[b]
19	NH_2	$NH(CH_2)_3O(CH_2)_3COO^-Na^+$, **19**	no inhibition[b]
20	NH_2	NH_2	no inhibition[b]

[a] This polymer was only partially soluble and this may affect the value of K_i^{HAI}. [b] No inhibition of hemagglutination was observed at the highest concentration of copolymer tested: 600 μM in sialic acid groups (or glucose, or carboxylate, for entries 18 and 19), in the HAI assay.



(3)

Although the polymers with composition poly(**1**-*co*-acrylic acid) that we prepared did not inhibit the agglutination of erythrocytes, the related polymer shown in eq 4 synthesized by Matrosovich *et al.*[63] was strongly



(4)

inhibiting in the HAI assay (K_i^{HAI} = 1.8 μM) and in a fetuin binding assay (FBA, K_i^{FBA} = 0.23 μM).[82]

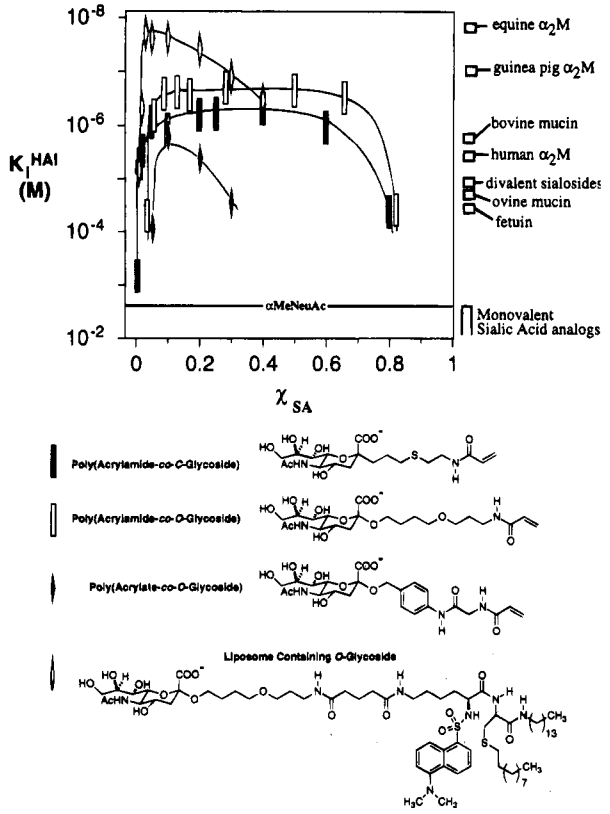


Figure 5. Hemagglutination inhibition as a function of the mole fraction of sialic acid linked groups in the polymer. Filled rectangles represent the results for *O*-glycosides tethered to polyacrylamide obtained in this work; open rectangles represent the results for *C*-glycosides tethered to polyacrylamide obtained by Sparks *et al.*[67]; open diamonds represent the results for liposomes obtained by Kingery-Wood *et al.*;[68] filled diamonds represent the results for *O*-glycosides tethered to polyacrylate obtained by Matrosovich *et al.*[63] The error bars, a factor of ±2 (i.e., ±1 well), are represented by the height of the symbols. The data were obtained by a protocol that involved preincubating virus with the inhibitor for 30 min; the effectiveness of inhibitor increases (the value of K_i^{HAI} decreases) for longer preincubation times (see Figure 2). The values of K_i^{HAI} for a few reference compounds, on a per sialic acid basis, are represented on the right hand side of the graph with a box.

Inhibition as a Function of Content of Sialic Acid. Figure 5 summarizes the values of K_i^{HAI} of a number of derivatized polyacrylamides and of a second class of multivalent inhibitors we have investigated, liposomes, as a function of their sialic acid content. Theplot is in terms of the mole fraction of the sialic acid-containing monomer (RSA) in the polymer or liposome (eq 5).

$$\chi_{SA} = \frac{[RSA]}{[RSA] + [\text{acrylamide or acrylic acid or lipid}]} \quad (5)$$

The total concentration of monomers (e.g., [**1**] + [acrylamide] = 1 M) was the same in all the solutions used to prepare polymer. We presume that the DP is approximately the same for all the polymers (this presumption may be incorrect at high values of χ_{SA}). We also assume that **1** and acrylamide copolymerized randomly and that the composition of the copolymer was that of the solution from which it was made. (The monomer reactivity ratios for acrylamide and *N*-isopropylacrylamide are 1.0 and 0.5, respectively: pH 6.5; 1.0 M in vinyl residues; 0.13 mM $K_2S_2O_8$; 25 °C; water.[80,83]

Table 2. Inhibition of the Agglutination of Red Blood Cells by Poly(**25**-*co*-acrylamide)

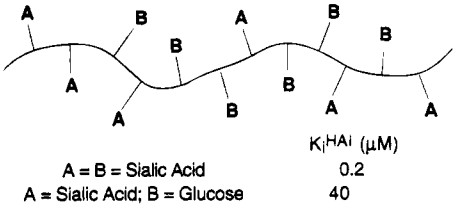
χ_{SA} of **25**	K_i^{HAI} (μM)	χ_{SA} of **25**	K_i^{HAI} (μM)
0.07	>600[a]	0.17	320
0.09	320	0.29	600
0.12	160		

[a] The assay did not detect activity at concentrations that corresponded to values of K_i^{HAI} > 600 μM.

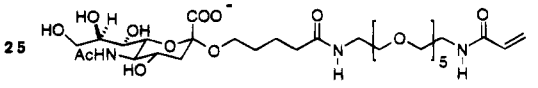
The monomer reactivity ratios for acrylamide and *N*-(hydroxymethyl)acrylamide are 0.9 ± 0.2 and 2.9 ± 0.4, respectively: pH 7.0; 10 wt % vinyl residues; 0.01 wt % $K_2S_2O_8$; 70 °C; water.[80,84])

One remarkable feature of the plot for polymers containing *O*-glycosides is its abruptness. Changes of a factor of 2 in χ_{SA} (from 0.05 to 0.1 and from 0.4 to 0.8) result in changes of 10^2 in K_i^{HAI}. Copolymers containing low levels of sialic acid ($\chi_{SA} \leq 0.05$) do not inhibit hemagglutination significantly more strongly than unpolymerized **1** when calculated on the basis of total sialic acid groups in solution. At intermediate values of χ_{SA} ($\chi_{SA} = 0.2-0.6$), the polymers containing *O*-glycosides are potent inhibitors of hemagglutination. The plateau observed between these values is also observed by Mastrosovich *et al.*,[63] and a similar form is observed for structurally analogous *C*-glycosides.[67] At even higher sialic acid content (including polymers obtained by homopolymerization of **1**) the effectiveness of inhibition calculated on a per sialic acid basis decreases sharply.

The sensitivity of this system to the detailed structure of the polymer and to the mole fraction of sialic acid in the polymer is illustrated in another type of experiment. A copolymer prepared from a 1:5:1 mixture of **1**, acrylamide and **18** (glucose tethered to acrylamide) (χ_{SA} =0.14) has K_i^{HAI} = 40 μM. This level of inhibition is about 100 times weaker than would be expected for the copolymer prepared from a 1:6 mixture of **1** and acrylamide (χ_{SA} = 0.14; K_i^{HAI} = 0.5 μM), or from a 2:5 mixture (χ_{SA} = 0.28; K_i^{HAI} = 0.2 μM). Thus, the replacement of half of the sialic acid groups of this latter polymer by glucose residues results in a decrease in the effectiveness of each sialic acid group in inhibiting hemagglutination of approximately 10^2. We can only speculate about the origin of this remarkable sensitivity of K_i^{HAI} to the details of the sugars present on side chains in the polymer.

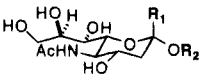


We prepared a monomer with an extended linker to SA, **25**, and determined the inhibition constants of copolymers derived from **25** (Table 2). These experi-



ments were qualitative, and the intermediates leading up to compound **25** were not characterized completely; they were intended primarily to test the influence of the length of the linker between the sialic acid and the polymer backbone on K_i^{HAI}. The result of these experi-

Table 3. Values of K_i^{HAI}, K_d^F, and K_d^{NMR} for Monomeric Derivatives of Sialic Acid and for Copolymers Prepared from 1:5 Molar Mixtures of Carboxy-Modified Sialic Acids and Acrylamide



R_1	R_2	K_i^{HAI} (μM)	K_d^F or K_d^{NMR} (μM)
	α-Alkyl Glycosides		
COO^-Na^+	CH_3	2500	2800 (K_d^{NMR}) 2900 (K_d^F)
$CONH_2$	CH_3		$>10^5$ (K_d^{NMR})
CO_2CH_3	CH_3	14000	
COO^-Na^+, **1**	$(CH_2)_4O(CH_2)_3NHCOCH=CH_2$	1000	600 (K_d^F)
	Poly(X-*co*-acrylamide)[a]		
COO^-Na^+, **1**	$(CH_2)_4O(CH_2)_3NHCOCH=CH_2$	0.3	>2000[b] (K_d^{NMR}) ≥1700 (K_d^F)
$CONH_2$, **26**	$(CH_2)_4O(CH_2)_3NHCOCH=CH_2$	>600[c]	
$COOCH_3$, **27**	$(CH_2)_4O(CH_2)_3NHCOCH=CH_2$	>600[c]	
COO^-Na^+ [d]	$(CH_2)_4O(CH_2)_3NHCOCH=CH_2$	0.3	

[a] X is the sialic acid moiety having the indicated groups R_1 and R_2. [b] The assay did not detect binding at this concentration. [c] The assay did not detect activity at concentrations that corresponded to values of K_i^{HAI} > 600 μM. [d] This value was measured for the polymer generated upon basic hydrolysis of poly(**27**-*co*-acrylamide).

ments was that poly(**25**-*co*-acrylamide) (1:5) had K_i^{HAI} ~ 300 μM (the corresponding value for poly(**1**-*co*-acrylamide) (1:5) was K_i^{HAI} ~ 0.3 μM); most other copolymers with **25** were also weaker inhibitors than the corresponding copolymers with **1**. (The results of these experiments are summarized in the supplementary material.) Extending the sialic acid groups *farther* away from the polymer backbone would be expected to increase the binding of SA to HA if the steric effect between HA and the polymer backbone is not offset by a loss in entropy due to the longer sidearm. We infer that steric effects involving the surface of the HA and the backbone of the polymer are not, therefore, of primary importance in determining K_i^{HAI}.

Copolymers Modified at the Sialic Acid Residues. In an effort to clarify the relationship between the strength of the interaction of HA with individual sialic acid groups (and analogues) and the effectiveness of a polymer having multiple copies of these groups in inhibiting hemagglutination, we synthesized and tested polymers presenting ligands that bind *less* tightly to HA than does sialic acid itself. We constructed two acrylamide-linked analogues of **1** for this purpose: amide **26** and methyl ester **27** (Table 3). The binding of the methyl ester of α-methyl sialoside is roughly 10 times weaker than that of α-methyl sialoside itself[20] and NMR data for the analogous monomeric amide indicated that its binding was at least 30 times weaker than that of α-methyl sialoside.[21] Monomers **26** and **27** were copolymerized with acrylamide under the standard conditions at a molar ratio of 1:5. Neither polymer inhibited hemagglutination below concentrations of the sialic acid derivatives of 600 μM (Table 3). These polymers are less effective than structurally analogous polymers containing sialic acid by at least a factor of 10^3. We have independently confirmed that this result is not due to a difference in behavior of the monomers on polymerization. Poly(**1**-*co*-acrylamide) and the polymer obtained after hydrolysis of the methyl ester groups of poly(**27**-*co*-acrylamide) were indistinguishable in their ability to inhibit hemagglutination, provided the two copolymers were synthesized using the same procedure (Table 3). These results demonstrate that inhibition of hemagglutination by polyvalent systems is sharply dependent on the affinity of their ligands for the hemagglutinins.

Interaction of Poly(1-*co*-acrylamide) with Bromelain-Cleaved Hemagglutinin (BHA). Treatment of influenza virus with the protease bromelain (EC 3.4.22.4) results in the release of a soluble form of HA (BHA) containing the sialic acid binding site in active form; the trimeric nature of the molecule is maintained.[5] This soluble trimer thus presents three sialic acid binding sites in approximately the same geometry relative to one another that they have on the viral surface, and with the same opportunity for mono-, di-, and trivalent interactions with a polymer having sialic acid side chains.

The binding of the sialic acid groups present in the polymer with BHA can be measured directly using a fluorescence assay developed by Weinhold and Knowles.[19] This assay is based on the decrease in fluorescence polarization that occurs when **2** dissociates from the sialic acid binding sites of BHA. By measuring the decrease in fluorescence polarization on adding poly(**1**-*co*-acrylamide) to a solution of BHA and **2**, it is straightforward to estimate the dissociation constants of the polymer-bound sialic acid relative to the known dissociation constant of **2** (K_d = 3.7 ± 0.6 μM).[19] (This assay is not applicable to a suspension of intact virus, because the virus strongly scatters light at the concentrations required to achieve a measurable change in the fluorescence polarization.) Using this assay, the dissociation constant of monomer **1** is 0.6 mM, and the dissociation constant *per sialic acid* of poly(**1**-*co*-acrylamide) is ≥1.7 mM; that is, the individual sialic acid groups of the polymer bind *less* tightly to BHA than do structurally analogous monomeric derivatives of sialic acid.

Application of the fluorescence assay to the polymer was technically complicated and the value of K_d^F for it may have a large uncertainty. Initial results from application of the fluorescence assay to poly(**1**-*co*-acrylamide) indicated that there was no interaction between BHA and the polymer at concentrations of up to 12 mM in sialic acid groups linked to polymer, but after correcting for the unusual background of highly polarized fluorescence from the polymer, we were able to estimate a dissociation constant of 1.7 mM. Incubation of the polymer at its highest concentration (12 mM in SA) with **2** and BHA for 2 h did not alter the fluores-

cence. The validity of this value of K_d^F was, however, reinforced by obtaining a similar result using the recently developed competitive proton NMR assay: the dissociation constant *per sialic acid* of the poly(**1**-*co*-acrylamide)-BHA complex was established to be >5.0 mM;[85] that is, when the concentration of sialic acid moieties attached to poly(**1**-*co*-acrylamide) was 5 mM, there was no observable change in the NMR spectrum of the competing ligand (See experimental section). This assay is not applicable to a suspension of intact virus, because the virus aggregates and precipitates at the concentrations of poly(**1**-*co*-acrylamide) required to achieve a measurable change in the NMR spectrum.

These results from independent assays also indicate that sialic acid groups attached to the polymer bind *less* tightly to BHA on a per monomer basis than does the monomer **1** from which the polymers were made, despite the ability of the polymer to make multiple attachments to BHA. They do not, however, resolve the importance of multivalency in binding of the polymer to BHA. In particular, they do not establish whether a sialic acid group attached to polymer having one such sialic acid group would bind less strongly than a sialic acid on a chain having *multiple* attached sialic acid groups. It is possible that binding of poly(**1**-*co*-acrylamide) to BHA or to virus would show enhancement due to polyvalency relative to a hypothetical polymer of the same DP with only one pendant sialic acid group. It is technically impractical to study the system necessary to resolve this issue, since the very low values of χ_{SA} required in the copolymer would require impractically high concentrations of this polymer in solution to observe significant binding.

In summary, the aggregated results in this section argue against entropically enhanced binding of the sialic acid groups attached to the polymer to different subunits within one HA trimer as the *sole* mechanism of hemagglutination inhibition. Whether this conclusion also applies to the binding of sialic groups on the polymer to whole virus particles, which could exhibit inter HA binding as well as intra HA binding, remains to be determined. A virion has approximately 10^3 sialic acid binding sites available, distributed over $\sim 4 \times 10^6$ Å^2 of surface;[10] the importance of cooperative binding and polyvalency depends upon the interaction of these systems. A level of cooperativity in binding too small to be detected in these studies with soluble BHA might become important with larger numbers of interacting sialic acid groups and hemagglutinins in the viral membrane.

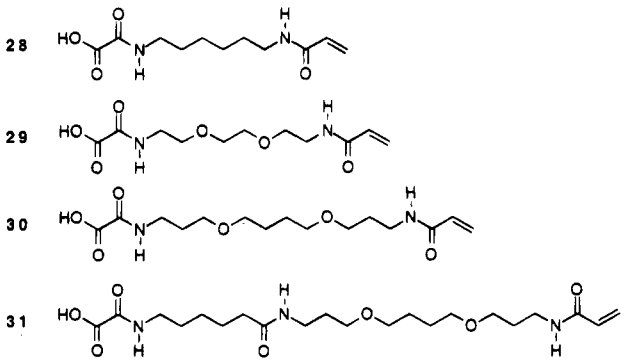
Binding of Polyoxamates to Lactic Dehydrogenase: A Model System for Interactions of a Polyvalent Polymer with a Multisite Protein. To check our hypothesis that presentation of a ligand on a polymer to a protein with multiple binding sites increased its dissociation constant, we examined a second system: lactic dehydrogenase (LDH) and a polyacrylamide having side chains terminating in oxamate moieties. We chose this system for a number of reasons:[86-88] (i) LDH is a tetramer, with four independent binding sites; (ii) LDH is well characterized, both kinetically and structurally; (iii) the reduction of pyruvate provides a reliable and sensitive assay for enzymatic activity (directly related to the occupancy of the lactic binding site); (iv) monoalkyl oxamates ($RNHCOCO_2^-$) are inhibitors of LDH, with values of K_i similar to the values of K_d for sialic acid from HA; and (v) oxamates are easily synthesized and easily incorporated in polyacrylamides.

Table 4. Inhibition Constants (K_i, mM) of the Oxamate Monomers and Copolymers

monomer or $\chi_{oxamate}$ of poly([**28**, **29**, **30**, or **31**]-*co*-acrylamide)	linker			
	hexyl (**28**)	$(EG)_3$ (**29**)	dioxadodecane (**30**)	triamide (**31**)
monomer (**28**, **29**, **30**, or **31**)	2	17	5	6
copolymer				
0.00	>10[a]		>10[a]	>10[a]
0.10	>10[b]	>10[b]	>10[b]	>10[b]
0.20	>10[b]	>10[b]	>10[b]	>10[b]
0.30	>10[b]			
0.50	>10[b]			

[a] The acrylamide concentration in the polymer was 500 mM. It is reported as >10 so that it can be compared with the $\chi_{oxamate}$ = 0.10 polymer. [b] Little or no inhibition of LDH was detected at this concentration. The K_i is therefore greater than this value. The value of K_i for oxamate itself is 0.02 mM.

Synthesis and Analysis of LDH Inhibitors. The four monomers **28**–**31** were copolymerized with acrylamide using conditions similar to those employed in making polymers having sialic acid groups under UV light, using ABCV, and the same total concentration of acrylamide moieties (1 M). The ratio of oxamate-derived



monomer to acrylamide in the polymerization reaction was varied from 1:9 to 1:1 (10% to 50%). These copolymers and their corresponding monomers were then assayed for inhibition of LDH (Table 4). The concentration of monomer (**28**, **29**, **30**, or **31**) in the polymerization reaction was assumed to be the concentration of oxamate in the resulting copolymer: As in our studies of poly(**1**-*co*-acrylamide), we assume complete incorporation of monomer into polymer. The inhibition constants, K_i, of the copolymers were analyzed by an Eadie–Hofstee plot[89,90] (see the supplementary material).

We conclude from these studies that presenting oxamate moieties to LDH as side chains on a polyacrylamide backbone substantially decreases their inhibitory capabilities, relative to unpolymerized monomer. This result parallels that observed with BHA and poly(**1**-*co*-acrylamide): it suggests that not only is there no intrinsic entropic *enhancement* of binding to the small number of binding sites present on LDH (or BHA) that results from this type of polyvalent presentation, but that there is a *decrease* in binding (possibly due to undefined interactions between the polymer backbone and the protein). Similar effects also appear in affinity electrophoresis using cross-linked polyacrylamide gels,

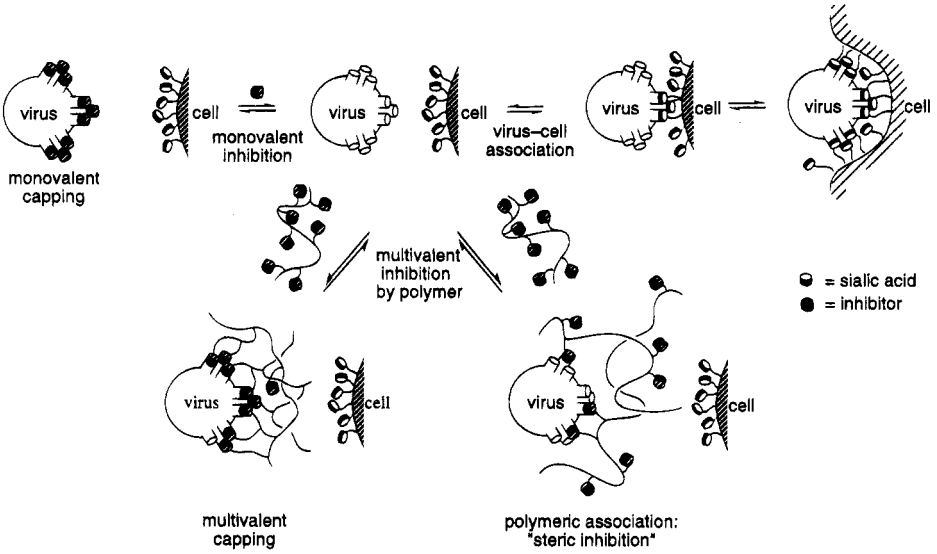


Figure 6. Possible explanations for the inhibition of attachment of influenza particles to sialic acid groups on the surface of a cell by poly(**1**-*co*-acrylamide). For simplicity, only a few of the hemagglutinin trimeric clusters are shown on the surface of the virion. Dimensions are not to scale.

most noticeably when the arm linking the affinity ligand to the polymer is short.[91]

Conclusions

Three key observations emerge from this work: first, incorporation of sialic acid groups into the side chain of a polyacrylamide polymer strongly increases their ability to inhibit the agglutination of erythrocytes by influenza virus X-31; second, this increase in the inhibitory strength of polymeric, polyvalent sialic acids does *not* correlate with the observed increase in the dissociation constant of the complex between an individual polymer-linked sialic acid and the binding site of BHA: in an NMR spectroscopic assay, the polymeric sialic acids bind *less* tightly than the corresponding monomeric sialic acids; third, the enhancement of inhibition observed on incorporating sialic acids as part of the side chains on a polymer is sensitive to the details of the composition of the polymer to χ_{SA}, to the incorporation of other functional groups, and to the linker connecting the sialic acid moiety to the polymer backbone.

A priori, there are at least three possible explanations for the high effectiveness of polyvalent derivatives of sialic acid in inhibiting hemagglutination (Figure 6):

(1) The inhibition is a result of entropically-enhanced binding of sialic acid groups at the sialic acid binding sites of hemagglutinins that are present on the surface of the virion. This explanation attributes the enhancement in inhibition of hemagglutination to an increased probability of occupying the HA binding sites by sialic acid groups attached to polymer, relative to the same number of sialic acid groups homogeneously distributed as monomers in solution. This effect would be the equivalent of the chelate effect: once one HA binding site was occupied by a sialic acid group on a molecule of polymer, the effective concentration of SA on other HA binding sites would be increased by their connection through a common polymer backbone.

(2) The inhibition is due to a "steric" stabilization of the virion by attached polymers. Adsorbed polymers can stabilize colloids by a mechanism that depends on the resistance of the polymer to compression and displacement of solvent.[92] Hemagglutination requires contact between the surfaces of the virion and the erythrocyte. The presence of a solvent-swollen polymer on the surface of the virion would hinder the contact of the virion and erythrocyte for two reasons. First, compression of polymer requires displacement of water and is unfavorable osmotically. Second, compression of polymers restricts the volume of space that is open to it conformationally and is unfavorable entropically. This steric stabilization does not require a high occupancy of sialic acid binding sites on the surface of the virus: it requires only enough points of attachment of polymer and virus to form a continuous, adherent layer of adsorbed polymer on the surface.

(3) The inhibition might be due to other effects, such as the viscosity of the solution or the aggregation and precipitation of virus by the cross-linking of virus particles by the polymer. Experimentally we observe formation of a turbidity or a precipitate on incubation of virus with polymer at concentrations of virus much higher (1000 fold) than those used in the HAI assay.

Solutions of low-χ_{SA} polymers ($\chi_{SA} < 0.05$, [sialic acid] $\approx$ 1 mM) are noticeably more viscous than water and flow and mix more slowly than polymer-free buffer, during the pipetting processes required in the HAI assay. For high-χ_{SA} polymers in buffer ($\chi_{SA} > 0.10$, [sialic acid] < 600 μM (the highest concentration of sialic acid that was normally used in the HAI assay)), the solution of polymer is not noticeably more viscous than water.[94] These observations may be due to the fact that there is 4 times more polymer present (mg/mL) in a solution of polymer with $\chi_{SA} = 0.05$ and a concentration of sialic acid residues of 100 μM than in a solution with $\chi_{SA} = 0.20$ and the same concentration of sialic acid moieties. The increased viscosity might slow collisions of the virus with the erythrocytes and affect the kinetics of the interaction of polymer with virus; the decrease in the collision frequency could affect K_i^{HAI}, since the hemagglutination inhibition assay is partially a kinetic assay (Figure 2). Other types of binding might also be important. For example, a polymer containing *O*-sialosides may bind (at least transitorily) to the neuraminidase on the surface of the virus [K_m = 1–10 mM for sialoglycosides with influenza A_2 (H2N2)];[95] this binding might also contribute to K_i^{HAI} by adding additional points of attachment between polymer and the surface of the virion. There might be interactions of

polymer that are either nonspecific or have a specificity different from that of hemagglutinin with either sialic acid or erythrocytes.

Mechanism of Inhibition of Hemagglutination by Poly(1-*co*-acrylamide). There appear to be at least two processes by which poly(1-*co*-acrylamide) interferes with adhesion of virus to the surface of the erythrocyte and permits formation of the observable pellet that is the basis of the hemagglutination inhibition assay. Our present hypothesis is that *attachment* of the polymer to the surface of this virus depends on multivalency, but that the *prevention of hemagglutination* depends largely on "steric stabilization"[92] rather than on occupying a high fraction of the sialic acid binding sites of the hemagglutinins. Formation of a layer of polymeric hydrogel on the surface of the virion and stabilization of the virion against adhesion to the cell by this layer both appear to be processes crucial to performance in the hemagglutination assay, and the nonspecific physical–chemical aspects of the polymer chemistry appear to be as critical to the prevention of adhesion as is the specific recognition of the sialic acid group by hemagglutinin. The two following sections outline the arguments for a contribution to binding of polymer to virion from multivalency and for steric stabilization.

Multivalency. In the absence of both positive and negative cooperativity in binding, the binding of polymer to virion (as measured by the fraction, Θ_p, of HA sites occupied by polymer-attached sialic acid) is the same as the corresponding fraction Θ_M for monomeric sialic acid groups of the same concentration in solution. That is, if the sialic acid groups and HA molecules are considered to be independent and noninteracting, it makes no difference whether they are joined by polymer, attached on the viral surface, or free in solution.

Assuming complete independence of sialic acid and HA subunits leads to a physical picture that does not, in our opinion, rationalize the observed results. For example, at the concentration of sialic acid groups effective in inhibiting hemagglutination, 10^{-6} M, the fraction of HA sites occupied by polymer-attached sialic acids is 0.001 ($\Theta_p \approx 10^{-3}$), assuming a K_d of 1 mM. If there are $\sim 10^3$ HA subunits on a virion, each virus particle would be bound to an average of one sialic acid group on one polymer. This level of attachment seems unlikely to be able to account for the stabilization provided by the copolymer. At 10^{-7} M in sialic groups, each virus particle would be bound to an average of only 0.1 sialic acid group, and the polymer would therefore probably provide little or no steric stabilization: this inference is incompatible with the observation that the poly(1-*co*-acrylamide) with χ_{SA}=0.2 is still effective when the sialic acid concentration is 10^{-8} M, provided that it is allowed to preincubate for a sufficiently long time with virus (Figure 2).

Several experimental data support the inference of cooperativity in binding of copolymer to virus. First, the sensitivity of K_i^{HAI} to the dissociation constant of the complex between monomeric units and virus (Table 3) and to steric hindrance by comonomers (Table 1) is much greater than would be expected from independent binding of sialic acid groups and their derivatives. Second, the inhibition increases rapidly with the number of sialic acid groups in the polymer (as reflected in the strong dependence of K_i^{HAI} on χ_{SA} at values of χ_{SA} between 0.0 and 0.1 and by the data in Figure 5).

Steric Stabilization. Several data indicate an important role for steric stabilization of the virus by copolymer on its surface as a mechanism for inhibiting hemagglutination. First, and most importantly, the competitive occupancy of hemagglutinin binding sites does *not* seem to be sufficient to account for the stabilization; direct measurements for copolymer and BHA indicate a *decrease* in the strength of binding of individual sialic acid groups in going from monomers to polymers. This conclusion is reinforced with model studies using LDH. Therefore, while we argue for multipoint binding of polymer to the surface of the virus, we do not expect that every available sialic acid binding site will be occupied. Second, the enhanced stabilization of the virus against adhesion to the erythrocyte depends, as expected, on the use of high molecular weight polymer. Third, the ability of a polymer to inhibit hemagglutination is sensitive to the specific details of the composition and structure of the polymer; this sensitivity is probably reflected in the conformation of the polymer in solution and around a virus particle to which it adsorbs. Fourth, there is extensive precedent for stabilization of colloids against flocculation[92] and for modification of other nonbiological surfaces by the adsorption of polymers.

Unresolved Issues. A number of issues concerning the inhibition of attachment of a virus particle to the surface of a cell by adsorbed polymer are unresolved by these studies: (i) The most important of these is the structural features of the polymer that will give the greatest stabilization. Qualitatively, it is clear that the polymer should be large and strongly solvated by water. Interaction with the surface of the virus that is too strong will collapse the polymer onto the surface of the virion, and decrease steric stabilization. (ii) The optimum strength of binding of the ligand is not defined so far. For a strongly binding inhibitor, a *low* value of χ_{SA} may be necessary. (iii) Including charge in the polymer (negative charges may help repel the negatively charged surface of the erythrocyte) may also be beneficial. The electrical neutrality of copolymers incorporating the amide of sialic acid, **26**, and the methyl ester of sialic acid, **27**, may explain their lack of activity.[6] The inclusion of too many negative charges on the polymer may, however, affect the conformation of the polymer. This effect may contribute to the decrease in K_i^{HAI} for poly(1-*co*-acrylic acid) and for poly(1-*co*-acrylamide) with $\chi_{SA} > 0.6$. In the latter polymer, for higher values of χ_{SA}, both charge–charge repulsion and steric repulsion between the large side-chain groups would tend to favor an extended rod conformation for the polymer; it is difficult to separate these two effects with the available data. Matrosovich *et al.*[63] have reported a polymer with a similar overall composition (eq 4) that is a good inhibitor ($K_i^{HAI} \approx 1.8$ μM) of hemagglutination. These data are not directly comparable with ours, but do indicate both that a high density of negative charges along the polymer backbone is not sufficient to abolish the activity in inhibition of hemagglutination and that efficient inhibition of hemagglutination depends sensitively on details of structure of these polymers.

These considerations may also underlie the differences in the extent of inhibition of hemagglutination observed with sialoglycoproteins and with other sialic acid-containing structures. For glycoproteins, the structure and physical properties of the polypeptide backbone

could be important in mechanisms involving steric stabilization. Discussions of these systems to date have focused on their content of sialic acid groups, and this parameter may be less important than other details of their structures. Liposomes may simply occlude the surface of the virus, and their size may be as important as the density of sialic groups on their surface, provided that there are sufficient sialic acids to effect attachment to the virus.

Experimental Section

Materials. Reagents, solvents, and starting materials were purchased from Aldrich, from Fluka, or from Polysciences. Fetuin (fetal calf serum), bovine mucin (type II from porcine stomach; type I from bovine submaxillary glands; Type I-S from bovine submaxillary glands), colominic acid (*E. coli*), neuraminidase (EC 3.2.1.18; insoluble enzyme from *Clostridium perfringens* attached to beaded agarose), L-lactic dehydrogenase (EC 1.1.1.27) type VII-S from porcine heart), and NADH were from Sigma Chemicals. Sialic acid was synthesized according to published procedures.[96] Chicken erythrocytes were from Spafas Inc. Influenza virus X-31 was obtained from Professor J. J. Skehel. Thin-layer chromatography was performed on silica gel precoated glass plates (E. Merck, Darmstadt). Flash column chromatography was performed on silica gel 60 (230–400 mesh, E. Merck). Ion exchange chromatography was performed on Dowex 50W-X8 (H^+ form) cation-exchange resin. Deoxygenated water was prepared by bubbling argon through doubly distilled deionized water for 2 h. Phosphate-buffered saline, PBS, is a solution of NaCl (80 g), KCl (2 g), Na_2HPO_4 (11.5 g), and KH_2PO_4 (2 g) in H_2O (10 L). The ultraviolet lamp was a Spectroline Model XX-15A long-wave UV-365 nm lamp from Spectronics Corp. Proton and carbon NMR spectra were measured on a Bruker spectrometer. Chemical shifts are reported in ppm relative to the solvent; $CHCl_3$ in $CDCl_3$ at 7.24 ppm, HOD in D_2O at 4.80 ppm, CD_2HOD in CD_3OD at 3.30 ppm, and CHD_2SOCD_3 in CD_3SOCD_3 at 2.49 ppm. For carbon spectra the references are 77.0 ppm for $CDCl_3$, 49.9 ppm for CD_3OD, and 39.5 ppm for CD_3SOCD_3. High-resolution mass spectroscopy was performed on a JEOL SX-102 at the Harvard University Chemistry Department Mass Spectroscopy Facility. Elemental analyses were performed by Oneida Research Services, Spang Microanalytical Laboratories, or Robertson Microlit Laboratories.

Methyl 5-Acetamido-4,7,8,9-tetra-*O*-acetyl-2,6-anhydro-2-*O*-(*N*-(carbobenzyloxy)-8-amino-5-oxaoctyl)-3,5-dideoxy-D-glycero-D-*galacto*-α-nonulopyranosonate, 6. A suspension of 4.5 g (14.6 mmol) of sialic acid and 25 g of Dowex W50 (H^+ form) in 500 mL of methanol was stirred at 25 °C for 3 h. The resulting clear solution was decanted, and 350 mL of methanol was added. After 2 h at 25 °C, the product was eluted from the resin with 600 mL of methanol, and the combined organic solutions were evaporated *in vacuo* to afford the methyl ester as a white solid (4.5 g).[71] A portion of this material was saved and 4.0 g (a maximum of 12.4 mmol if the solid were pure) was dissolved in 300 mL of acetyl chloride and stirred at 25 °C for 24 h. The volatiles were removed *in vacuo*, and residual acetyl chloride was co-evaporated five times with chloroform to yield crude halide, **4**, as a white foam (6.6 g).[72] A small sample (50 mg) was saved, and the remaining material was dissolved in 35 mL of benzene. Molecular sieves (4 Å, *ca.* 2 g), Cbz protected amino alcohol, **5**, (5.0 g, 17.8 mmol), and silver salicylate (3.68 g, 15 mmol) were added, and the resulting mixture was stirred in the dark for 4 days at 25 °C. Filtration through Celite, rinsing of the residue with methanol, and evaporation of the volatiles *in vacuo* afforded a brown solid, which was partitioned between CH_2Cl_2 and saturated aqueous $NaHCO_3$.[73] Extraction of the aqueous layer with CH_2Cl_2, drying of the combined organic extracts over $MgSO_4$, and chromatography on silica gel (ether/methanol, 200:1, then ethyl acetate) yielded the glycoside **6** as a white foam (5.04 g, 56% over three steps). NMR data for the intermediate methyl ester and **4** agree with the literature values.[72] Compound **6**: ^1H NMR (400 MHz, $CDCl_3$) δ 7.33–7.25 (m, 5H, CBz), 5.35 (ddd, J = 8.3, 5.7, 2.6 Hz, H-8), 5.29 (dd, J = 8.3, 1.6 Hz, H-7), 5.29–5.23 (br peak, 2H), 5.05 (s, 2H, CBz), 4.79 (ddd, J = 12.4, 9.7, 4.6 Hz, H-4), 4.27 (dd, J = 12.4, 2.6 Hz, H-9a), 4.08–3.99 (m, H-5, H-6, H-9b), 3.77–3.72 (m, linker, 1H), 3.73 (s, Me ester, 3H), 3.44 (t, J = 5.8 Hz, linker, 2H), 3.37 (br t, linker, 2H), 3.26 (q, J = 6.2 Hz, linker, 2H), 3.18 (dt, J = 9.3, 6.0 Hz, linker, 1H), 2.53 (dd, J = 12.8, 4.6 Hz, H-3a), 2.10 (s, 6H), 1.99 (s, 3H), 1.99 (s, 3H), 1.90 (t, J = 12.6 Hz, H-3b), 1.84 (s, 3H), 1.72 (pentet, J = 6.1 Hz, linker, 2H), 1.60–1.53 (m, linker, 4H). ^{13}C NMR (100 MHz, $CDCl_3$) 170.96, 170.62, 170.18, 170.10, 170.03, 168.43, 156.38, 136.71, 128.43, 128.00, 127.96, 98.66, 72.34, 70.48, 69.10, 68.95, 68.51, 49.33, 39.18, 38.01, 29.56, 26.12, 26.08, 23.14, 21.06, 20.80, 20.72 ppm; HRMS-FAB $[M + Na]^+$ calcd for $C_{35}H_{50}O_{16}N_2$ 777.3058, found 777.3076.

5-Acetamido-2,6-anhydro-2-*O*-(*N*-(carbobenzyloxy-8-amino-5-oxaoctyl)-3,5-dideoxy-D-*glycero*-D-*galacto*-α-nonulopyranosonic Acid, 7. A solution of 3.0 g (4.0 mmol) of glycoside **6** in 20 mL of 2 N NaOH was stirred at 25 °C for 2 h and the resulting mixture was extracted with ether. The pH of the aqueous layer was adjusted to pH 4.0 with 3 N HCl, and the volatiles were removed *in vacuo*. Chromatography on silica gel (*n*-BuOH/acetic acid/water, 5:4:1) afforded compound **7** (0.90 g, 43%) as a yellow foam. A second fraction of lower purity (~80%, NMR analysis) was also isolated (0.60 g); ^1H NMR (500 MHz, D_2O) δ 7.40 (m, 5H, CBz), 5.11 (s, 2H, CBz), 3.90–3.46 (m, 13H), 3.19 (t, J = 7 Hz, linker, 2H), 2.71 (dd, J = 12, 5 Hz, H-3a), 2.02 (s, 3H), 1.70 (t, J = 12 Hz, H-3b), 1.53 (m, linker, 6H); ^{13}C NMR (100 MHz, D_2O) 174.48, 173.02, 157.68, 135.95, 128.16, 127.73, 127.03, 100.01, 71.95, 71.15, 69.65, 67.66, 67.56, 67.20, 66.82, 63.85, 61.91, 51.32, 39.82, 36.95, 28.11, 25.08, 24.61, 21.43 ppm; HRMS-FAB $[M - H]^-$ calcd for $C_{26}H_{40}O_{12}N_2$ 571.2503, found 571.2512.

5-Acetamido-2,6-anhydro-2-*O*-(8-amino-5-oxaoctyl)-3,5-dideoxy-D-*glycero*-D-*galacto*-α-nonulopyranosonic Acid, 8. A 100-mL flask was charged with 0.90 g (1.7 mmol) of compound **7**, 0.1 g of 5% palladium on carbon, and 20 mL of methanol and was purged twice with hydrogen. The suspension was stirred under a slight positive hydrogen pressure for 2 h at 25 °C, filtered through a plug of Celite, and evaporated *in vacuo* to give **8** as a colorless oil (0.70 g, 94%): ^1H NMR (300 MHz, D_2O) δ 3.98–3.45 (m, 13H), 3.14 (t, J = 7 Hz, linker, 2H), 2.77 (dd, J = 7, 6 Hz, H-3a), 2.08 (s, 3H), 1.98 (m, linker and H-3b, 3H), 1.66 (m, linker, 4H); ^{13}C NMR (100 MHz, D_2O) 174.44, 173.00, 100.00, 71.94, 71.71, 69.62, 67.64, 67.56, 63.84, 61.94, 51.28, 39.81, 37.06, 29.36, 25.07, 24.56, 21.39 ppm; HRMS-FAB $[M - H]^-$ calcd for $C_{18}H_{34}O_{10}N_2$ 437.2135, found 437.2131.

5-Acetamido-2-*O*-(*N*-acryloyl-8-amino-5-oxaoctyl)-2,6-anhydro-3,5-dideoxy-D-*glycero*-D-*galacto*-α-nonulopyranosonoic Acid, 1. A solution of 0.175 g (0.4 mmol) of compound **8** in 1 mL of methanol, 2 mL of water, and 1 mL of 0.1 M phosphate buffer (pH 7.0) was cooled to 0 °C, and 0.167 g (1 mmol) of *N*-acryloyloxysuccinimide was added. After 5 h, a fresh portion (0.1 g, 0.6 mmol) of *N*-(acryloyloxy)-succinimide was added and the temperature was allowed to reach 25 °C. After 12 h, the mixture was loaded at 4 °C onto a Dowex AG1 X8 anion-exchange column (formate form) and eluted with a formic acid gradient (0 to 1.2 M) to give 0.167 g (84%) of compound **1**: ^1H NMR (400 MHz, D_2O) δ 6.21 (dd, J = 17.1, 10.0 Hz, 1H), 6.12 (dd, J = 17.1, 1.6 Hz, 1H), 5.70 (dd, J = 10.0, 1.6 Hz, 1H), 3.83–3.40 (m, 13H), 3.28 (t, J = 6.8 Hz, 2H), 2.69 (dd, J = 12.4, 4.6 Hz, 1H), 1.98 (s, 3H), 1.77 (pentet, J = 6.6 Hz, 2H), 1.63–1.54 (m, 5H); ^{13}C NMR (100 MHz, D_2O) 175.87, 174.44, 169.29, 130.84, 127.91, 101.47, 73.42, 72.64, 71.12, 69.14, 69.04, 68.77, 65.29, 63.40, 52.78, (49.69 CH_3OH), 41.34, 37.39, 29.05, 26.53, 26.09, 22.84 ppm. Anal. ($C_{21}H_{35}O_{11}N_2Na$): C, H, N. HRMS-FAB $[M - Na]^-$ Calcd 491.2241, found 491.2242.

Standard Conditions for the Polymerization Reactions. Stock solutions were adjusted to pH 7 with 1 N NaOH or 1 N HCl and deoxygenated for at least 2 h under a stream of argon. Solutions were transferred under argon with Hamilton syringes. Polymerizations were conducted at ambient temperature using a long-wave ultraviolet light (365 nm). Reactions were carried out in borosilicate glass tubes, fitted with a septum, and purged with argon. The tubes were

charged with deoxygenated aqueous solutions of acrylamide and monomer (e.g., **1**, **26**, **27**) in the desired ratios. The appropriate amounts of 0.2 M 4,4'-azobis(4-cyanovaleric acid) (the photoinitiator) and doubly distilled water were added to reach a final concentration of 1.0 M of vinyl residues (e.g., [**1**] + [acrylamide]) and 0.02 M of photoinitiator. The tubes were placed 15 cm from the ultraviolet lamp and irradiated at 365 nm for 4–5 h until no monomeric acrylamides were detectable by TLC and/or NMR spectroscopic analysis. The results from any specific polymer preparation were reproducible in subsequent batches, and we therefore did not investigate the sensitivity of the resulting polymers to the purity of the components of the systems, to traces of O_2, and to the details of the polymerization reactions. The polymers containing sialic acid were diluted to a final concentration of sialic acid residues of 5 mM with phosphate-buffered saline (PBS) and directly assayed for their ability to inhibit the influenza virus-induced hemagglutination of chicken erythrocytes. Control experiments carried out after exhaustive dialysis or gel filtration of the polyacrylamides revealed no detectable difference in hemagglutination inhibition titers between crude and purified samples. The polymers containing oxamate were diluted to a final concentration of oxamate residues of 50 mM with 0.2 M pH 7.3 Tris.

Measurement of the Binding of Poly(1-*co*-acrylamide) (χ_{SA} = 0.2) to Bromelain-Cleaved Hemagglutinin (BHA) by the Fluorescence Depolarization Assay and the Proton NMR Assay. The fluorescence assay was performed according to the protocol of Weinhold *et al.*[19] A quantity of poly(**1**-*co*-acrylamide) (χ_{SA} = 0.2) containing 9.8 μmol of **1** was dissolved in 100 μL of PBS to generate a stock solution of polymer (98 mM in sialic acid). The monomeric sialic acid (**1**, 8.7 mg, 16.9 mmol) was dissolved in 211 μL of PBS to generate a stock solution of monomer (80 mM in sialic acid). The K_d was calculated as a function of total sialic acid concentration in solution (that is, *not* as a function of polymer concentration) for both the monomeric and polymeric sialic acid solutions ($K_d^{monomer}$ = 0.5 mM, $K_d^{polymer} \geq$ 1.7 mM). A correction was applied to all the solutions containing polymer due to the unusually high background of highly polarized fluorescence from the polymer. This correction was applied linearly with polymer concentration and was determined from the fluorescence of a solution of polymer containing 9.8 mM sialic acid (stock solution of polymer diluted 10 fold with PBS). The stock polymeric sialic acid solution inhibited the hemagglutination of chicken erythrocytes by influenza X-31 virus (whole virus) at 0.7 μM in sialic acid under the standard assay conditions (K_i^{HAI} = 0.7 μM).

The proton NMR assay was performed in a competitive fashion according to the protocol of Sauter *et al.*[15] A solution of poly(**1**-*co*-acrylamide) (χ_{SA} = 0.2) similar to that above was lyophilized from 1.0 mL of D_2O three times and dissolved in 1.0 mL of buffer (pD 7.0, 100 mM phosphate, 150 mM NaCl, 0.1% NaN_3 in D_2O) to generate a stock solution of polymer (9.8 mM in equivalents of sialic acid). No binding of sialic acid to BHA was observed at 5 mM sialic acid in the proton NMR assay; therefore K_d > 5 mM. Again, the K_d was calculated as a function of the concentration of sialic acid groups in solution *not* as a function of polymer concentration. The stock solution was assayed for sialic acid content using the Boehringer Mannheim neuraminic acid assay kit: [sialic acid] = 5.2 mM. The inhibition by this stock solution of the hemagglutination of chicken erythrocytes by influenza whole virus was determined by the HAI assay (K_i^{HAI} = 0.7 μM, presuming a 10 mM stock solution).

Hemagglutination Inhibition Assay. Polymers were assayed using a protocol similar to published procedures.[76] The titer of a stock solution of influenza virus (X-31), provided to us by J. J. Skehel, in PBS (~15 mg/mL) was determined by serial dilution of 100 μL of the virus solution through 12 microtiter plate wells of a 96-well microtiter plate, each containing 100 μL of PBS. A suspension of chicken erythrocytes (0.5%) in PBS (100 μL) was then added to each well. After 1 h at 4 °C, the lowest concentration of virus that agglutinated the erythrocytes was determined by visual inspection [virus in this well] = V_a. The lowest concentration of inhibitor that prevented the agglutination of red blood cells in the *presence of virus* was then determined: A stock solution of inhibitor in PBS (50 μL) was serial diluted through 12 microtiter plate wells each containing 50 μL of PBS. A suspension of X-31 (50 μL) virus at a concentration of $4V_a$ (see above) was then added to each well. After 30 min at 4 °C, 100 μL of a suspension of chicken erythrocytes (0.5%) in PBS was added to each well with mixing. After 2 h at 4 °C, the lowest concentration of inhibitor, ([I_L] = K_i^{HAI}) that inhibited agglutination of the erythrocytes was determined. The α-methyl glycoside of sialic acid (K_d determined by NMR) was used as a reference compound.

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Supplementary Material Available: Experimental details of the synthesis of compounds **5**, **10**, **12–14**, **16–19**, **25**, **27**, and **28–31**, NMR data for compounds, and a table of the hemagglutination inhibition constants of various poly(**25**-*co*-acrylamide derivatives) polymers (76 pages). Ordering information is given on any current masthead page.

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(80)

$$\sim M_1^* + M_1 \rightarrow \sim M_1M_1^*\ k_{11}$$

$$\sim M_1^* + M_2 \rightarrow \sim M_1M_2^*\ k_{12}$$

$$\sim M_2^* + M_2 \rightarrow \sim M_2M_2^*\ k_{22} \qquad (6)$$

$$\sim M_2^* + M_1 \rightarrow \sim M_2M_1^*\ k_{21}$$

$$r_1 = k_{11}/k_{11}\ r_2 = k_{22}/k_{21}$$

In eq 6, $\sim M_1^*$ represents a polymer chain ending in a radical derived from monomer 1, $\sim M_2^*$ represents a polymer chain ending in a radical derived from monomer 2, M_1 represents monomer 1, M_2 represents monomer 2, k_{11}, k_{12}, k_{22}, and k_{21} are rate constants for the respective reactions, and r_1 and r_2 are the monomer reactivity ratios.

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