Effective Inhibitors of Hemagglutination by Influenza Virus Synthesized from Polymers Having Active Ester Groups. Insight into Mechanism of Inhibition

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Highly effective sialic acid-containing inhibitors of influenza virus X-31 were synthesized using poly[N-(acryoyloxy)succinimide] (pNAS), a polymer preactivated by incorporation of active ester groups. Polymers containing two and three different components were prepared by sequential reaction of pNAS with two and three amines, respectively. This preparation of co- and terpolymers was synthetically more efficient than methods involving copolymerization of different monomers and gave polymers that were more easily compared than those generated by copolymerization. Polymers in this study (prepared from a single batch of pNAS) had a constant degree of polymerization (DP $\approx$ 2000) and probably had a distribution of components that was more random than analogous polymers prepared by copolymerization. Use of C-glycosides of sialic acid made it possible to investigate inhibition by different polymers at temperatures ranging from 4 to 36 °C without artifacts due to the hydrolytic action of neuraminidase. The inhibitors were, in general, more effective at 36 °C than at 4 °C. The hemagglutination (HAI) assay was used to measure the value of the inhibition constant K_i^{HAI} each polymer. The value of K_i^{HAI} for the two-component polymer containing 20% sialic acid on a polyacrylamide backbone at 4 °C was 4 nM (in terms of the sialic acid moieties present in solution) and was approximately 50-fold more effective than the best inhibitors previously described and 25-fold more effective than the best naturally occurring inhibitor. The most effective inhibitor synthesized in this work contained 10% benzyl amine and 20% sialic acid on a polyacrylamide backbone, and its value of K_i^{HAI} was 600 pM at 36 °C. Approximately 100 polymers that differed in one or two components were assayed to distinguish between two limiting mechanisms for inhibition of the interaction between the surfaces of virus and erythrocytes: high-affinity binding through polyvalency, and steric stabilization. The results suggest that *both* mechanisms play an important role. The system comprising polyvalent inhibitors of agglutination of erythrocytes by influenza provides a system that may be useful as a model for inhibitors of other pathogen–host interactions, a large number of which are themselves polyvalent.

Introduction

We describe a series of polymeric inhibitors designed to inhibit the attachment of influenza virus X-31[1-4] to mammalian cells. The inhibitory potency of the best inhibitor described here (K_i^{HAI} = 0.6 nM; calculated on the basis of sialic acid groups present in solution) is about 2 orders of magnitude better than that of other synthetic inhibitors (of which the best are liposomes presenting sialic acid on their exterior surface, K_i^{HAI} = 200 nM)[5] or natural inhibitors (of which the best is equine α_2-macroglobulin, K_i^{HAI} = 100 nM)[6] (Figure 1).

Nature uses polyvalency—the cooperative association of a receptor (or aggregate of receptors) containing multiple recognition sites with a molecule containing multiple complementary ligands—in a number of biological contexts, including many interactions between pathogen and host.[7,8] The attachment of influenza virus to its target cell is a model system for such polyvalent interactions; this attachment is mediated by multiple simultaneous interactions between viral hemagglutinin[9-11] (HA) and cellular sialic acid[2,12,13] (Neu5Ac,[14] SA). HA is a glycoprotein that occurs with high density

(2–3 copies/100 nm²; or ~600 copies per virus particle) on the surface of influenza virus.[12] SA is a nine-carbon sugar that occurs with high density (22–25 molecules/100 nm²) on the surface of the mammalian cell.[15-17] SA terminates many of the extracellular saccharidic portions of mammalian glycoproteins and glycolipids.

We assayed our inhibitors using the hemagglutination inhibition (HAI) assay.[18] Interaction between virus and erythrocytes in solution leads to the formation of an extended, cross-linked gel (hemagglutination). The HAI assay enabled us to measure the ability of an inhibitor to prevent hemagglutination by preventing the interaction between the surfaces of virus and erythrocyte; we quantitated the effectiveness of the inhibitor with an inhibition constant, K_i^{HAI}. We define K_i^{HAI} as the lowest concentration of ligand that successfully inhibits hemagglutination. All values of K_i^{HAI} reported here are in terms of the concentration of SA groups in solution (whether free in solution as monomeric units, attached covalently to the backbone of a soluble polymer, or associated noncovalently with a structure such as a liposome).

We used SA-containing polymeric inhibitors (glycopolymers)[19] that are based on polyacrylamide (pA) or poly(acrylic acid) (pAA). We have described this strat-

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egy previously,[5,20−22] and it has also been used explicitly by others including Matrosovich,[23,24] Roy,[25−27] Paulson,[28] Bednarski,[29] and Lee.[30] The monomeric α-2-methyl-*N*-NeuAc (α-MeSA)[14] inhibits virally induced hemagglutination at 2 mM (K_i^{HAI} = 2 mM). The value of K_i^{HAI} for the most effective polymer reported in the present work is 600 pM. This highly efficient inhibition of hemagglutination reflects the ability of these polymers to prevent interaction of two biological surfaces: those of the virus and the erythrocyte. We believe that this mechanism of action reflects a pharmacologically relevant property,[31] but we have not assayed these polymers for activity *in vivo*.

Polymeric versus Monomeric Inhibitors. A significant body of work has developed around the synthetic modification of *monomeric* SA in order to improve its ability to inhibit virally induced hemagglutination.[20,32−34] The most successful of these attempts gave derivatives of SA with K_i^{HAI} equal to 3.7 μM,[32] 3 orders of magnitude better than α-MeSA.

Both natural and synthetic *polymeric* inhibitors (inhibitors containing *multiple* copies of SA) are much more successful at inhibiting hemagglutination than are their monomeric counterparts. A number of polymeric systems have yielded values of K_i^{HAI} less than 1 μM.[20,22,29,31] The mechanism by which a polymer acts as an inhibitor of hemagglutination, and the reasons that it is more successful than its monomeric counterpart, is understood in broad outline,[21] but not in detail. The monomeric inhibitor must compete with SA groups on the surface of the erythrocytes for SA-binding sites of HA on the surface of the virus; it is at an intrinsic disadvantage in this competition since the interaction between virus and erythrocyte is polyvalent.

The polymer can inhibit hemagglutination by at least two conceptually independent mechanisms (Figure 2).[21] First, it may act by high-affinity interactions based on the possible entropic advantage of many SA groups on the same polymer binding to the surface of the virus; these potentially cooperative interactions may lead to efficient competitive inhibition of viral attachment by competing with the polyvalent cooperative interactions between virus and cell. Second, the polymers may act by steric stabilization:[35] SA groups on the same polymer may bind noncooperatively (with similar affinity to that of monomeric SA) to the surface of the virus, but bring with them a large, water-swollen polymer that may make sites on the surface of the virus adjacent to those bound to SA *physically inaccessible* to SA on the surface of the cell. This second type of inhibition is a type of mixed inhibition (a combination of competitive and noncompetitive inhibition). The relative importance and detailed mechanisms of these two types of inhibition are currently not understood in sufficient detail to design new polymers that are more effective inhibitors than those already known.

We had four goals in this work: (i) to develop a synthesis for polymeric inhibitors based on pA and pAA that is of more use than that used previously (copolymerization of a mixture of acrylamide monomers); (ii) to explore the role of temperature in inhibition of hemagglutination by SA-containing polymers; (iii) to understand the relative importance of the two major mechanisms of inhibition for these polymers through a guided choice of different side chains on the pA backbone; and

Scheme 1. Two strategies: (A) Copolymerization[a] and (B) Preactivation[b]
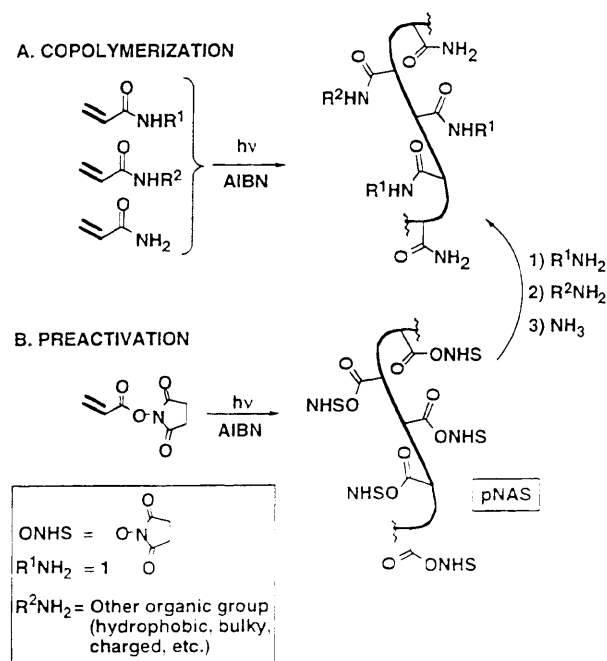
[a] Irradiation (medium-pressure Hg arc lamp through a quartz vessel for 6 h) of a mixture of substituted acrylamides and a radical initiator (AIBN) yields a substituted polyacrylamide. [b] Heating a solution of *N*-(acryloyloxy)succinimide (NAS) and AIBN in benzene to 60 °C yields the preactivated "parent" polymer pNAS; subsequent sequential treatment with different amines yields an *N*-substituted polyacrylamide.

(iv) to find inhibitors of hemagglutination that are more effective than those already known.

Using Activated Polymers to Construct Copolymers. Various physical properties of the polymer (conformation in solution, conformation on the surface of the virus, conformational flexibility, net charge, size, degree of branching, degree of hydration, and hydrophobicity) might, in principle, influence the success of inhibition by polymers. We have previously attempted to correlate some physical characteristics of the polymer—especially the structures of the side chains of the polymer and of the group linking the SA to the backbone—with their effectiveness in inhibiting hemagglutination.[22] This previous work was based on a copolymer of two or more differently *N*-substituted acrylamides: one was *N*-substituted with a spacer-group terminated with SA, **2**; the other was *N*-substituted with the test-group, R[2] (Scheme 1A). We define
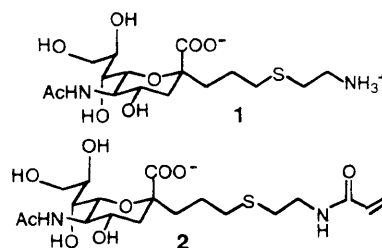
K_i^{HAI} as the ratio of side chains containing R[i] to the total number of side chains in the polymer (eq 1); for example, $χ^{SA}$ is the proportion of side chains with linkers terminated in SA. The "standard polymer" is one in which $χ^{SA}$ = 0.20. All comparisons between polymers are ultimately referenced to the standard polymer.

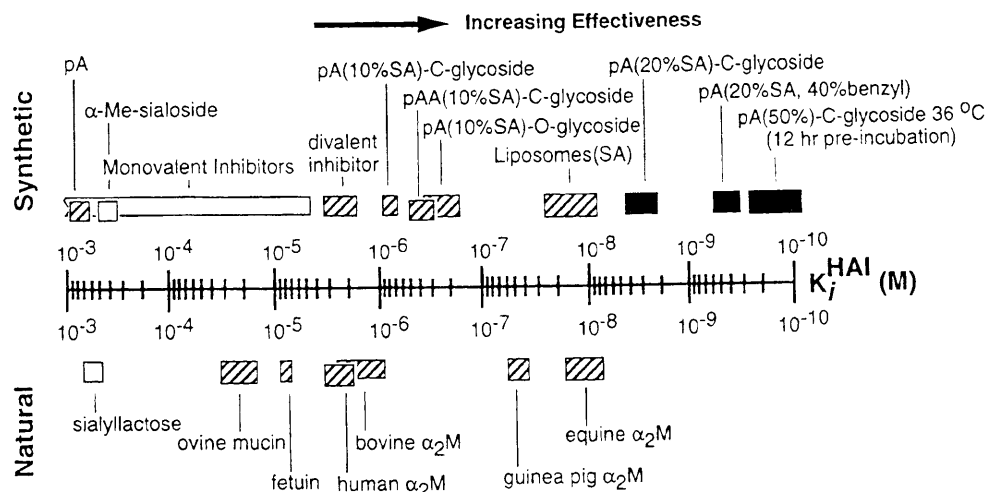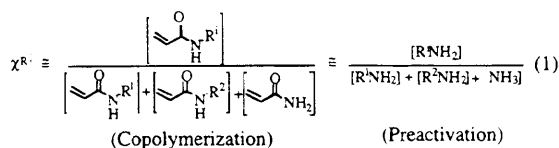Increasing Effectiveness

Synthetic

pA

α-Me-sialoside

Monovalent Inhibitors

divalent inhibitor

pA(10%SA)-C-glycoside

pAA(10%SA)-C-glycoside

pA(10%SA)-O-glycoside

Liposomes(SA)

pA(20%SA)-C-glycoside

pA(20%SA, 40%benzyl)

pA(50%)-C-glycoside 36 °C
(12 hr pre-incubation)

10^{-3} 10^{-4} 10^{-5} 10^{-6} 10^{-7} 10^{-8} 10^{-9} 10^{-10}

K_i^{HAI} (M)

10^{-3} 10^{-4} 10^{-5} 10^{-6} 10^{-7} 10^{-8} 10^{-9} 10^{-10}

Natural

sialyllactose

ovine mucin

fetuin   human α_2M

bovine α_2M

guinea pig α_2M

equine α_2M

Figure 1. Values of K_i^{HAI} for natural and synthetic inhibitors of hemagglutination based on SA. Monomeric inhibitors (open boxes), previously studied polymeric inhibitors (hatched boxes), and the best polymeric inhibitors from the present study (solid boxes) are shown. Synthetic polymeric inhibitors are based on liposomes, poly(acrylic acid) (pAA) or polyacrylamide (pA). The width of the boxes represent the uncertainty in the values (generally ± a factor of 2; that is, ± 1 well in serial dilution).

$$\chi^{R'} \equiv \frac{\left[\overset{O}{\underset{H}{\overset{\|}{\diagdown}}N^{R^1}}\right]}{\left[\underset{H}{\overset{O}{\overset{\|}{\diagdown}}N^{R^1}}\right] + \left[\underset{H}{\overset{O}{\overset{\|}{\diagdown}}N^{R^2}}\right] + \left[\overset{O}{\overset{\|}{\diagdown}}NH_2\right]} \equiv \frac{[R^1NH_2]}{[R^1NH_2] + [R^2NH_2] + NH_3} \quad (1)$$

(Copolymerization)   (Preactivation)

Copolymerization (Scheme 1A) possibly introduces two uncontrolled variables into the experiment (Figure 3): (i) unknown differences in rate constants for copolymerization among differently *N*-substituted acrylamides might result in differences in the distribution of SA along the backbone of the polymer; (ii) the length, polydispersity, and tacticity of the polymer might change as the side chain was altered, due to differences in rates and stereochemistry of polymerization.

In the present study, we used a different strategy to prepare polymeric inhibitors based on addition of amines to polymers having activated esters of carboxylic acid groups as side chains (Scheme 1B); this strategy has been used previously,[36,37] but with different motivations. We refer to the synthesis of different polymers by this strategy as "preactivation". Polymerizing *N*-(acryloyloxy)succinimide yielded the preactivated polymer poly-[*N*-(acryloyloxy)succinimide] (pNAS). We then converted the activated esters on pNAS to amides by sequential reaction with two amines: first with **1**, a molecule containing a SA group connected to a flexible spacer (**1** = R^1NH_2, Scheme 1); then with R^2NH_2, where we varied R^2 depending on the hypothesis being tested. Preactivation was synthetically and mechanistically more attractive than copolymerization for several reasons: (i) the characteristics of the polymer backbone—length, polydispersity, tacticity—were kept constant (the same batch of pNAS was used for all experiments); (ii) the distribution of SA groups along the backbone, although not controlled, was the same for all polymers, since introduction of the SA moiety was the first step in modifying pNAS; (iii) the distribution of SA groups along the backbone was probably more uniform than from copolymerization (addition of the sterically bulky molecule **1** to adjacent sites along the backbone of the polymer was less likely than addition to nonadjacent sites); (iv) preactivation was more convenient synthetically than copolymerization, since constructing many different polymers did not require synthesizing and

characterizing different acrylamide monomers (most of the amines used were commercially available).

Exploring the Role of Temperature Using C-Glycosides. Changes in the temperature at which the HAI assay is performed might, in principle, change the measured value of K_i^{HAI}. Temperature influences several variables of the HAI assay: the preferred distribution of conformations of the polymer in solution; the rate of adsorption of the polymer to the surface of the virus; the rate of structure formation and the preferred distribution of conformations of the polymer on the surface of the virus; and the ability of a layer of polymer on the surface of a particle to stabilize that particle sterically. It has been difficult to examine the influence of temperature on hemagglutination using substrates containing O-glycosides of SA, because neuraminidase[38-40] (NA) cleaves these groups rapidly. Assays of inhibitors that contain SA connected to the backbone of the polymer by O-glycosidic linkages could only be performed at 4 °C (degradation of the inhibitor occurred acceptably slowly at 4 °C). The physiologically more relevant temperature is approximately 36–37 °C. In this study we used C-glycosides of SA (**1**) because they are resistant to the action of NA. We collected parallel data at 4, 19, and 36 °C for each polymer.

The Hypotheses Guiding the Survey of Side Chains. There are at least two independent mechanisms that might contribute to the inhibitory action of these polymers: high affinity polyvalent binding and steric stabilization (Figure 2). We synthesized polymers incorporating different side chains from different chemical classes (charged, polar-uncharged, and nonpolar), to try to distinguish between these two mechanisms of inhibition. No *single* structural modification allowed us to identify clearly which of the two mechanisms is more important for inhibition of hemagglutination by polymers. We expected that the results in *aggregate* would, however, be informative. A guided survey of side chains allowed us both to explore mechanisms of inhibition by polymers and to determine the side chain (R^2NH_2, Scheme 1) on a pA backbone that promoted the strongest inhibition.

We had five hypotheses: (i) Incorporating a number of negatively charged groups along the backbone might
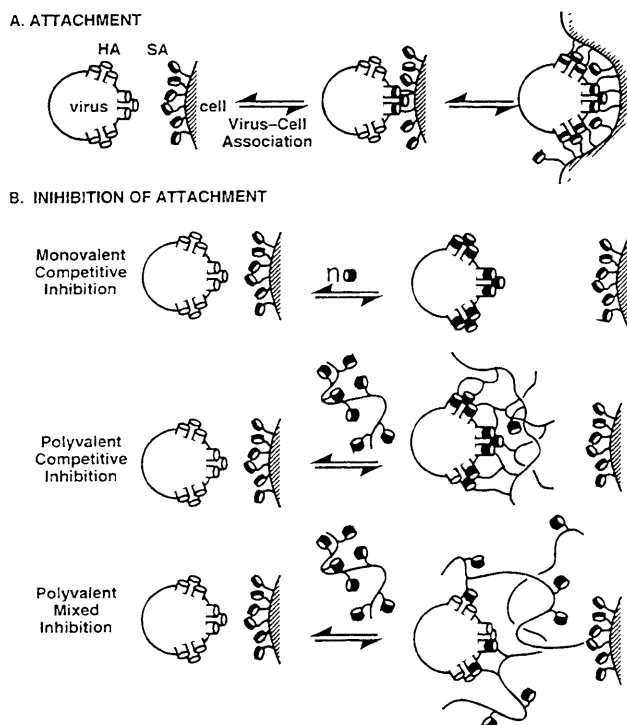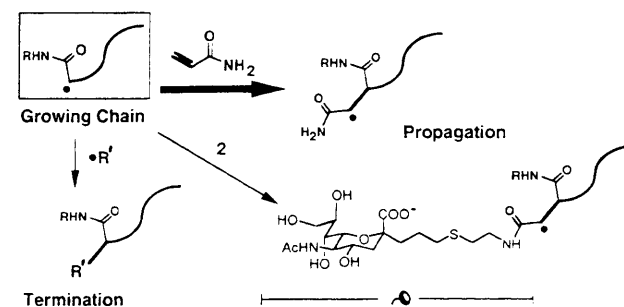
A. ATTACHMENT

B. INIHIBITION OF ATTACHMENT

Figure 2. (A) Influenza virus attaches to its host cell through polyvalent interaction of HA with SA. (B) Inhibition: monomeric molecules containing SA inhibit attachment by competitive inhibition, whereas polymeric molecules inhibit attachment by either (i) high-affinity binding through polyvalency (competitive inhibition); and/or (ii) steric stabilization of a surface (mixed inhibition).

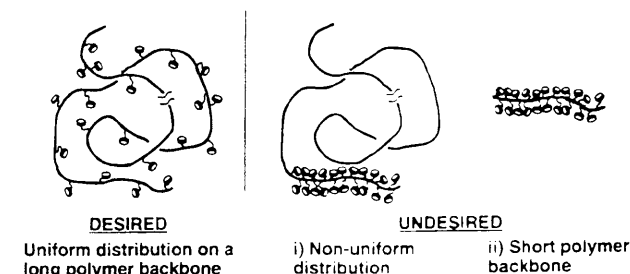A. DIFFERENT RATES OF CHAIN PROPAGATION

B. POSSIBLE CONSEQUENCES

Figure 3. (A) Acrylamide substituted with a sterically bulky molecule (for example, 2) might add more slowly to the growing polymeric chain that does unsubstituted acrylamide. (B) Consequently, at least two deviations from the *desired* polymer (a long one, with SA distributed evenly along the backbone) are possible: (i) the bulky substituted acrylamides may polymerize into blocks after all the unsubstituted acrylamide is reacted; (ii) the bulky substituted acrylamides may form short polymers when polymerized in the absence of the unsubstituted acrylamides.

change the conformation of the polymers by favoring long persistance lengths (thus favoring an extended, rigid-rod geometry).[35] We expected extended polymers to be less effective than random-coil polymers in steric stabilization because these polymers have less conformational entropy. (ii) Placing bulky substituents on or near the backbone might also lead to longer persistance lengths for the polymer and decrease steric stabilization.[35] In addition, bulky substituents on sites adjacent to SA might "crowd" the sialic acid and thus hinder access to the HA on the viral surface. (iii) Adsorbing a negatively charged polymer onto the surface of a virus might result in increased stabilization by electrostatic repulsion of the negatively charged surface of the erythrocyte. Conversely, adsorbing a positively charged inhibitor on the surface of the virus might promote electrostatic attraction between the polymer-coated virus and the erythrocyte. (iv) Adding hydrophobic side chains might increase the affinity of the polymer for the virus by inducing secondary hydrophobic binding interactions. (v) Using cross-linking agents in small proportions might increase steric stabilization. These agents might lead to branching of the polymer, or gel formation, and thus increase the effective degree of polymerization, DP. We have recently found that increasing DP generally increases the effectiveness of the polymers.[41]

Results and Discussion

Synthesis of Polymers. We prepared all polymers used in this study from a single batch of pNAS (Scheme 1B). We characterized the molecular weight of this "parent" polymer by complete hydrolysis to pAA followed by gel filtration chromatography (GFC): the average

molecular weight of the hydrolyzed sample was 146 kD (M_w) (DP $\approx$ 2000). The distribution of molecular weights was narrow (M_N = 75 kD and M_z = 279 kD).[42]

Sequential addition of two different amines to pNAS yielded the final functionalized polymer (Scheme 1B). We first added 0.20 equiv per activated ester of 1. In most instances, we added the second amine. R^2NH_2, in excess. In some instances, R^2NH_2 was not added in excess, and we quenched the residual. unreacted active esters of the polymer with ammonia.

The number of equivalents of 1 determined χ^{SA} in the final polymer. To establish the relationship between the number of equivalent of 1 and χ^{SA}, we performed a set of experiments in which pNAS was reacted with quantities of 1 that varied from 0.05 to 1.2 equiv per activated ester, then was quenched with ammonia. We used two sorts of analysis on the resulting polymers (Figure 4): (i) ^{1}H NMR spectroscopy compared the integrated area under the peaks characteristic of the SA (e.g., the protons of the acetylated nitrogen) to the area under the peaks characteristic of the polymer (e.g., the $-(CHCH_2)-$ protons); and (ii) combustion analysis provided estimates of relative values of χ^{SA} directly from analysis of sulfur.[43] For a small number of equivalents, χ^{SA} was generally smaller than predicted theoretically; as the number of equivalents was increased, there was stronger agreement between the measured value of χ^{SA} and that predicted theoretically. For most of the polymers used in this study, we used 0.20 equiv per activated ester; our analyses suggested that χ^{SA} was equal to ~0.17–0.18 in these polymers.
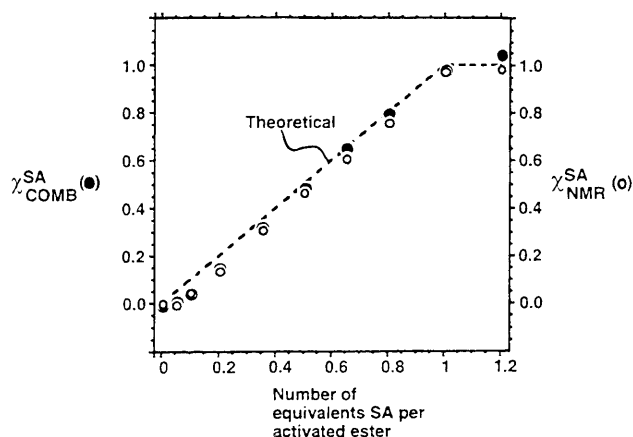
Figure 4. We estimated χ^{SA} in the polymers using ^{1}H NMR analysis (O) and combustion analysis for sulfur (●) as a function of the number of equivalents of SA added per activated ester in the polymer (see text for details).
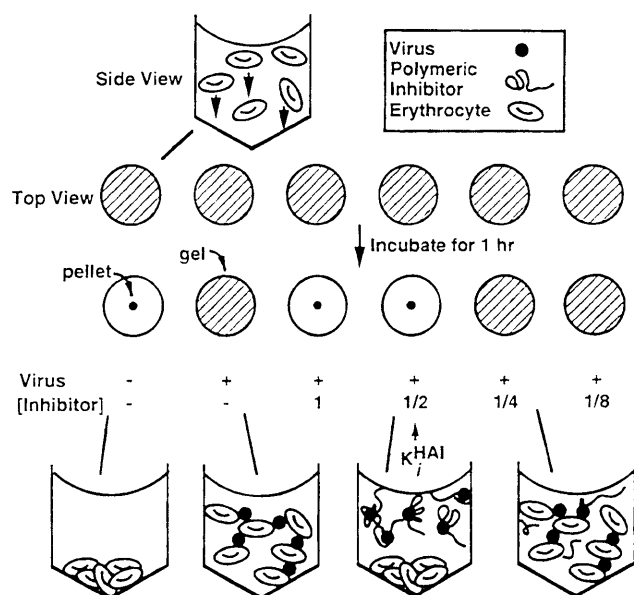

Figure 5. Hemagglutination inhibition assay (HAI). See text for detailed explanation. The 250−μL microtiter well is viewed both from the side and the top. Hemagglutination (formation of a gel) is represented by hashed marks; a pellet is shown as a dark spot. The concentration of the inhibitor is 1 unit in the third well. The inhibitor is sequentially diluted by a factor of two in the wells to the right of the third one. The value for K_i^{HAI} is defined as the lowest concentration of inhibitor that inhibits hemagglutination; in this case $K_i^{HAI} = \frac{1}{2}$ unit.

The HAI Assay. We assayed the effectiveness of the polymers in inhibiting virally induced hemagglutination using the hemagglutination inhibition (HAI) assay.[18,44] Obtaining accurate and reproducible values for K_i^{HAI} required attention to certain details of the assay and an understanding of the principles that underlie its operations. Influenza virus agglutinates erythrocytes (hemagglutination). The lowest concentration of inhibitor that prevents hemagglutination is defined as K_i^{HAI} (Figure 5). The dissociation constants (K_d) for the complexes between different *monomeric* inhibitors and hemagglutinin have been measured using ^{1}H NMR spectroscopy and fluorescence and were found to equal their corresponding values of K_i^{HAI}. Since half the receptors are occupied when a ligand is at a concentration equal to K_d, we infer that monomeric inhibitors of
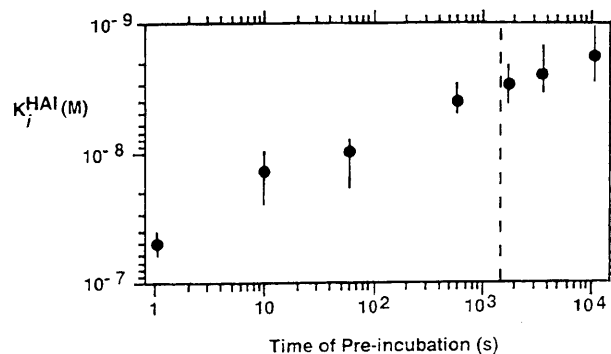

Figure 6. Using pA with $\chi^{SA} = 0.2$, the value for K_i^{HAI} (●), decreased with increasing time of preincubation (the time for which virus and inhibitor mix, prior to addition of erythrocytes). The time of preincubation used in this study (shown by a dashed line) was 1800 s (30 min).

SA need to bind approximately half the HA on the surface of the virus in order to inhibit hemagglutination.

Measuring K_i^{HAI} requires three components in PBS buffer: erythrocytes, virus, and inhibitor (at varying concentrations). As we performed the assay, virus and inhibitor were mixed and allowed to preincubate for 30 min at a specified temperature. Erythrocytes were then added and mixed, and the system was allowed to incubate for 1 h. The order and timing of addition of the three components strongly influenced the results: if we added the inhibitor last, all inhibitors were ineffective; if we added the erythrocytes last, the time that the polymer and virus were allowed to preincubate strongly influenced K_i^{HAI} (Figure 6). We have not examined protocols in which the virus was added last.

The erythrocytes usually required at least 30 min of incubation at 36 °C (or 1 h at 4 °C) to form stable pellets or gels; we allowed experiments at all temperatures to incubate for 1 h before reading the plates. At 4 °C, the patterns of inhibition did not change as we varied the time of incubation from 1−12 h. At higher temperatures, however, the values for K_i^{HAI} generally decreased over 12 h: at 19 °C, the values decreased by a factor of two, and at 36 °C, the values decreased by varying amounts (from a factor of 2 to 8). We have not defined the origin of these decreases, but we emphasize that it was important to keep details of the experiment constant—especially the time of preincubation of polymer and virus and the time of incubation after the erythrocytes are added—to allow comparison of different polymers.

The precision (reproducibility) in our experiments was generally good (K_i^{HAI} multiple repetitions allowed precision of *less* than a factor of 2) so long as we used a single batch of blood, a single preparation of polymer and virus, and followed the timing of the various additions to within a few minutes. If we repeated an experiment with different preparations of all three components of the assay, we could reproduce the value for K_i^{HAI} to within a factor of 3−4.

Poly(acrylic acids) That Do Not Contain SA Inhibit Hemagglutination. We assayed, as controls, a number of polymers that do not contain SA (Table 1). The values of K_i^{HAI} are given arbitrarily in terms of concentration of monomeric equivalents contained in the polymers. These values have no clear meaning, but they allow comparison with values of K_i^{HAI} obtained using

Table 1. Inhibition of Hemagglutination by Polymers That Do Not Contain SA

polymer	K_i^{HAI} $(\mu M)^a$
pAA 450[b]	100
pAA 750[b]	210
pAA 1200[b]	230
pAA 3000[b]	400
pAA 4000[b]	1000
pA 200[b]	>13000
pA 200,[b] 10% acid	300
MeO-PEG(2000)-OH[c]	>100000
MeO-PEG(5000)-OH[c]	>100000
MeO-PEG(5000)-NH₂[c]	>100000

^{a} K_i^{HAI} is given in terms of total concentration of monomeric units. ^{b} MW (kD); pAA = poly(acrylic acid); pA = polyacrylamide. ^{c} Number of monomeric units shown in parantheses; MeO-PEG = ω-methoxypoly(ethylene glycol).
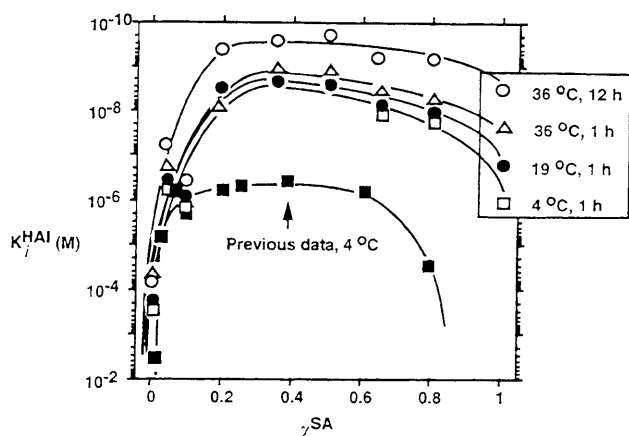
Figure 7. K_i^{HAI} is plotted a function of the proportion of SA in the polymers, χ^{SA}. Data were collected for polymers (prepared by preactivation; Scheme 1) at three different temperatures: 4 °C (□), 19 °C (●), and 36 °C (△). Data from previous studies for polymers (prepared by copolymerization) at 4 °C are also shown (■). Incubation times were generally 1 h. Longer incubation times (12 h, O) yielded lower values of K_i^{HAI}.

SA-containing polymers (see below). We found, as have others,[22] that pA does not inhibit hemagglutination at any concentration up to 100 mM. The pAA, perhaps surprisingly, inhibits hemagglutination: pAA with lower molecular weights were more effective than pAA with higher molecular weights. These polymers may interact nonspecifically with the surface of both the virus and the erythrocyte. They may then inhibit hemagglutination mainly through steric stabilization.

Dependence of K_i^{HAI} on χ^{SA}: Qualitative Differences from Previous Studies. We found previously that SA-containing polymers varied in effectiveness (K_i^{HAI}) as a function of χ^{SA} in the polymer (Figure 7). The shape of the curve that describes the relationship between χ^{SA} and K_i^{HAI} is characteristic of many of the polymeric inhibitors of hemagglutination that we have previously examined: as χ^{SA} increases from 0, the effectiveness increases rapidly, forms a plateau, then decreases rapidly as χ^{SA} exceeded ~0.5. In this work, we varied the value of χ^{SA} from 0.0 to 1.0 (Figure 7). The pattern of inhibition was qualitatively different than before: the initial increase in effectiveness with increasing χ^{SA} was not as sharp; the plateau was less pronounced; and the decrease in effectiveness occurred at higher values of χ^{SA} and was less pronounced (in some cases, it did not occur at all). Although we attribute

these differences to the different methods of preparing the polymer, we can only guess the precise mechanistic origin of these results: the highly effective inhibition by polymers prepared by preactivation (for all values of χ^{SA}) *may* have been because of a more uniform distribution of SA along the backbone of the polymer (that is, fewer wasted SA moieties) than was possible using copolymerization; the rapid decrease in effectiveness of the polymers prepared by copolymerization at high values of χ^{SA} *may* have been because the DP for these polymers decreased with increasing χ^{SA} (Figure 3).

The polymers prepared previously by copolymerization had values of K_i^{HAI} that decreased linearly with DP;[41] a doubling of DP resulted in a halving of the value of K_i^{HAI}. The average value of DP for these copolymers was 4000,[21,22] where the average value of DP for the polymers in the present work was 2000. If all else was constant (especially the temperature and the value of χ^{SA}), we expected the polymers from the present study to have values of K_i^{HAI} 2-fold *higher* (worse) than the polymers prepared previously. All data indicate significantly *more* effective inhibition (values of K_i^{HAI} were 100-fold lower) than obtained previously.

The differences between the values of K_i^{HAI} obtained at different temperatures increased with increasing χ^{SA}. The lowest value of K_i^{HAI} we observed in this study was 200 pM (at χ^{SA} = 0.50 at 36 °C with a 12-h incubation). These two observations suggest that kinetically slow processes of some sort (probably equilibration of the polymer over the biological surfaces involved) increase effectiveness of the polymers in inhibiting hemagglutination.

Effectiveness of the Polymers Decreases with Increasing Charge. We performed a number of experiments in which we changed the side chains of SA-containing polymers (in all cases, χ^{SA} equals 0.20) and correlated these changes to differences in K_i^{HAI}. The standard polymer (χ^{SA} = 0.20, R²NH₂ = NH₃) provided a point of reference. We divided the changes into groups based on the chemical nature of the side chain and the hypothesis we were testing. In the first experiments, we varied the charge of the side chain group (R²NH₂, Scheme 1) from +1 to −3 using the molecules shown in Figure 8. As the charge changed from 0 to −3, the effectiveness of the polymers decreased; a change from 0 to +1 resulted in an even sharper decrease. Since the groups that introduced charge were relatively large, the effects of charge and size on K_i^{HAI} were not cleanly decoupled by this one experiment. The different influences of the +1 and −1 side groups, which are of similar size, suggested, however, that size alone is not *fully* responsible for the observed differences between these differently charged polymers.

In an attempt to distinguish the effects of steric bulk from those of charge, we synthesized three sets of polymers in which the charge on the side chain was varied relatively independently of the size of the side chain: (i) Using 0.10 and 0.20 equiv of charged side chain per activated ester, we obtained polymers that were of similar size to the standard polymer, but still substantially charged. (ii) Hydrolyzing the polymer rather than quenching it with ammonia (Scheme 1A) yielded polymers with carboxylates as side chains
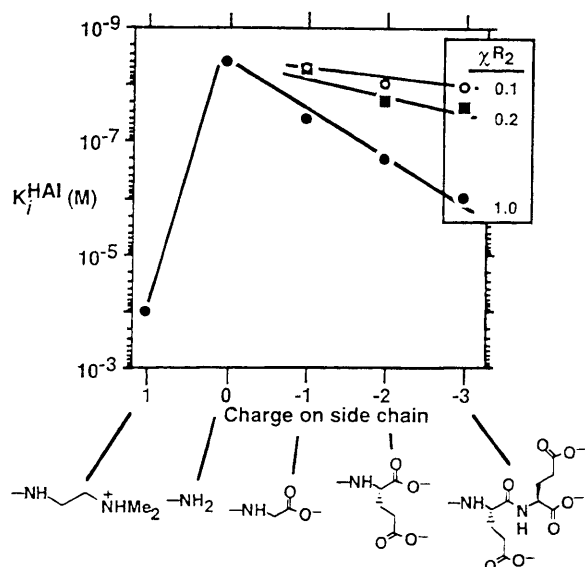
Figure 8. Charged groups as side chains ($CONHR^2$, where R^2 is charged) (Scheme 1). Various proportions of charged side chains, χ^{R^2}, were assayed: 0.1 (O), 0.2 (■), 1.0 (●).
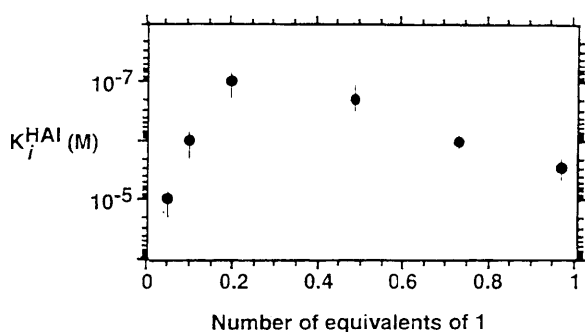

Figure 9. EDC and NHS are used to couple 1 to pAA (MW 750 kD). EDC, NHS and 1 were used in a 1:1:1 ratio. The value for K_i^{HAI} (●), is plotted a function of the number of equivalents of 1 per free carboxylic acid of pAA.

(primary amides and carboxylates are approximately isosteric). Alternatively, coupling pAA to 1 using EDC and NHS yielded similar SA-containing polymers with carboxylates as side chains. (iii) Incorporating crown ethers yielded polymers whose charge could be titrated by varying the concentration of the cation that the side chain could specifically complex. The results of these three experiments follow.

Incorporation of lower proportions of the charged side chains resulted in a less pronounced but similar relationship between the charge and the value of K_i^{HAI} (Figure 9).

The parent polymer pNAS was reacted with 0.20 equiv of 1 and then hydrolyzed to give a negatively charged, isosteric analog of the standard polymer. The value of K_i^{HAI} of this charged analog was 900 nM, which was approximately 300-fold less effective than the standard polymer. In an analogous experiment, we coupled 1 to pAA (MW 750 kD) using EDC and *N*-hydroxysuccinimide. We did not characterize the actual extent of incorporation of 1 into the polymer. We varied the equiv of 1 from 0.0 to 1.0 (Figure 9); the form of the plot relating the number of equiv of 1 and the value of K_i^{HAI} was similar to that observed for the pA polymers (Figure 8). In this experiment, the introduction of charge introduced *no* increase in size of the side chains
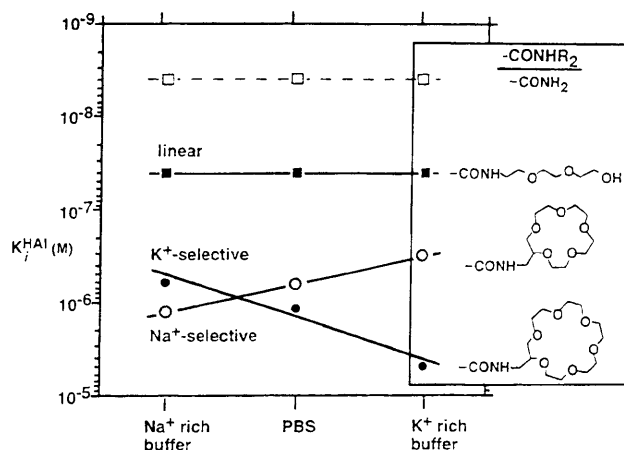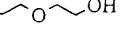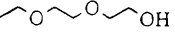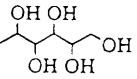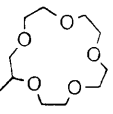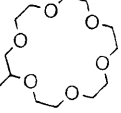

Figure 10. Polymers containing 18-crown-6 (selective for K⁺, ●), 15-crown-5 (selective for Na⁺, O), or linear triethylene glycol (■), and the standard polymer (□) were assayed in phosphate buffered saline (PBS) or in buffers enriched in either Na⁺ or K⁺. PBS contained 8 g of NaCl, 0.2 g of KCl, 1.1 g of Na_2HPO_4, and 0.2 g of KH_2PO_4. Na⁺-rich buffer was made by replacing all K⁺ in PBS with Na⁺; K⁺-rich buffer was made by replacing all Na⁺ in PBS with K⁺.
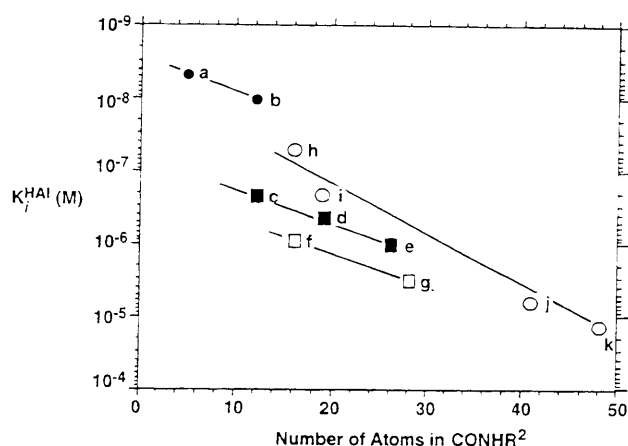
over pA; the negatively charged, isosteric polymers were less effective than the standard polymer.

In another experiment relating charge to K_i^{HAI}, we incorporated Na⁺- and K⁺-selective crown ethers in the side chains (Figure 10), and compared the values of K_i^{HAI} to that of a polymer containing linear triethylene glycol (not selective for a specific cation). We used buffers enriched in either Na⁺ or K⁺. These buffers were equivalent to phosphate-buffered saline (PBS) in ionic strength and pH, but only a single type of cation was used: Na⁺-rich buffer contained only Na⁺, and K⁺-rich buffer contained only K⁺. By examining the values of K_i^{HAI} for these polymers in the three buffers (PBS, Na⁺-rich, and K⁺-rich), we hoped to detect a change in the inhibition that could be correlated with the selectivity of the crown ether. We observed that in all instances in which the polymer selective for Na⁺ or K⁺ was assayed in Na⁺-rich or K⁺-rich buffer, respectively (conditions in which the polymer presumably had a net positive charge), the polymers became less effective than when assayed in PBS. Furthermore, when the polymer selective for Na⁺ or K⁺ was assayed in K⁺-rich or Na⁺-rich buffer, respectively, the polymer became more effective than when assayed in PBS . Since we do not know the dissociation constants for the complexes between the crown ethers and the cations, we do not know the magnitude of the positive charge on the polymer in the different buffers. These observations are consistent qualitatively, however, with those from experiments in which positively charged side chains are incorporated into the polymer (Figure 10): positively charged polymers are much less effective inhibitors of hemagglutination than neutral polymers.

Effectiveness of the Polymers Decreases with Increasing Size of the Side Chain. We varied the size of uncharged side chains using four different families of molecules: primary amides, linear ethylene glycols, linear polyols, and cyclic ethers (Table 2). The trends for all four families were the same: the effectiveness of the polymers decreased with increasing size of the side chain (Figure 11). We represent the size of the side chain by the number of atoms (C, H, N, O)

Table 2. Inhibition of Hemagglutination by Polymers with Polar Side Chains, $CONHR^2$, that Differ in Size

Molecule	Number of Atoms[a] (Figure 12)	Identification
$-CONH_2$	5	a
$-CONH \diagdown \overset{O}{\diagup} NH_2$	12	b
$-CONH \diagdown \diagup OH$	12	c
$-CONH \diagdown_O \diagdown OH$	19	d
$-CONH \diagdown_O \diagdown^O \diagup OH$	26	e
$-CONH \diagdown \overset{OH}{\diagup} OH$	16	f
$-CONH \diagdown \overset{OH\ OH}{\diagup \diagdown} OH$ (OH OH)	28	g
$-CON \diagdown O$ (ring)	16	h
$-CONH \diagdown O$ (ring)	19	i
$-CONH$ (crown ether)	41	j
$-CONH$ (crown ether)	48	k

[a] The total number of atoms in $-CONHR_2$.

Figure 11. As the size of the side chain $CONHR^2$ ($CON(R^2)_2$) was increased the value of K_i^{HAI} increased. See Table 2 for identification of the labels. Four families of neutral side chains are shown: primary amides ($\bullet$), cyclic ethers (O), polyethylene glycols ($\blacksquare$), and linear polyols ($\square$). With all families of side chains, the size is roughly proportional to the number of atoms in $CONHR^2$.

contained in $CONHR^2$: we did not calculate molecular volumes. As the number of atoms in the side chain was increased, the value of K_i^{HAI} decreased. The backbone of polymers containing bulky side chains may be less flexible (fewer available conformations) than those with small side chains. Furthermore, such polymers may adopt more extended (rigid-rod) conformations. In either case, the ability of such polymers to stabilize a surface sterically probably decreases. Since in these
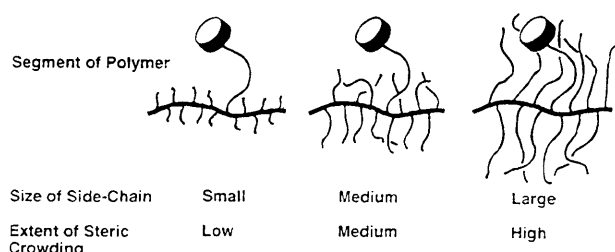
Figure 12. Extent of steric crowding around the sialic acid (cylinders) increases with size of the side chain (represented by curves of different length). Large side chains may introduce sufficient steric crowding to hinder access to the HA receptors on the surface of the virus.
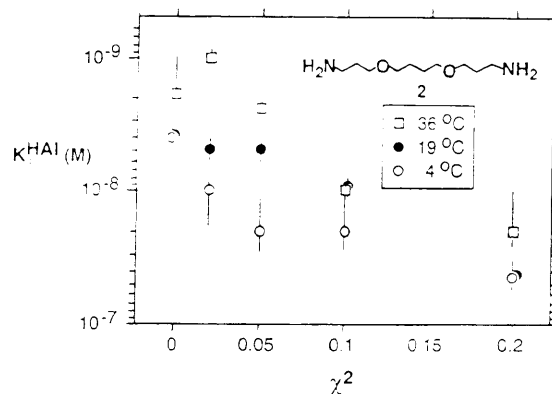
Figure 13. The value of K_i^{HAI} increased as the proportion of cross-linking agent **2** (χ^2) was increased. We assayed the polymers at various temperatures: 4 °C (O), 19 °C ($\bullet$), 36 °C ($\square$).
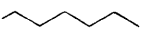
experiments, the effectiveness of the polymers decreased as the size of the side chain increased, we tentatively conclude that steric stabilization plays a role in inhibition of hemagglutination by polymers.

Another possibly relevant consequence of increasing the size of the side chains was to crowd the sialic acid sterically and thereby hinder access to the HA receptor (Figure 12). We do not know if steric crowding was the major mechanism by which the effectiveness of the polymers was decreased as a function of the size of the side chain. In an experiment reported previously,[22] the length of the linker connecting the SA to the backbone of the polymer was increased. This experiment attempted to decrease any effect of steric crowding. The observation was, however, that the polymers containing long linkers were *less* effective than polymers with linkers of intermediate length.

Low Levels of Cross-Linking Increase the Effectiveness of Polymers. We added various proportions (0.02–0.20) of diamine **2** to cross-link the esters within the same polymer and between polymers (Figure 13). While low levels of cross-linking increased the effectiveness of the polymers, high levels had the opposite effect. We reported previously that the effectiveness of polymers correlates positively with DP. Introducing low levels of cross-linking may be equivalent to increasing the effective DP. Interestingly, as the temperature increased, the differences between polymers differing in χ^2 also increased. At this time, we do not know the precise mechanistic origin of these results.

Hydrophobic Side Chains Increase the Effectiveness of the Polymers. Different nonpolar (hy-

Table 3. Inhibition of Hemagglutination by Polymers with Hydrophobic Side Chains, $CONHR_2$

Hydrophobic side chain -CONHR2	Equiv[a]	K_i^{HAI} (nM) 4 °C	19 °C	36 °C
-CONH2	0.8	3.8	3.8	1.9
-CONH⌇⌇ (hexyl)	0.1	16	8	8
-CONH-⟨phenyl⟩	0.1	10	10	5
-CONHCH2-⟨phenyl⟩	0.05	10	2	2
	0.1	10	1	0.6
	0.2	10	4	2
	0.4	10	6	4
-CONH-⟨adamantyl⟩	0.1	10	5	2
-CONHCH2-⟨adamantyl⟩	0.1	10	5	2

[a] Number of equivalents of R2NH2 per activated ester used in the synthesis (Scheme 1).

drophobic) side chains were incorporated into the polymer in an attempt to increase the affinity of the polymer for the virus.[32,34,45] We expected that interactions between the hydrophobic groups and hydrophobic sites on the surface of the virus (including the lipid membrane) would enhance binding of the polymer to the viral surface. Due to decreasing solubility with increasing extent of incorporation, we were limited generally (except in the case where R^2NH_2 = benzyl amine, BA) to values of K_i^{HAI} less than ~0.1. In the synthesis of the polymers (Scheme 1), addition of 0.1 equiv of the hydrophobic R^2NH_2 was followed by quenching with ammonia to convert all unreacted activated esters to primary amides.

Adding certain hydrophobic side chains yielded polymers that were more effective than the standard polymer (Table 3). With BA the solubility of the polymer remained satisfactorily high with extents of incorporation as high as 0.4: we varied χ^{BA} from 0.05 to 0.40 and found that as χ^{BA} increased, the effectiveness of the polymer increased. Even polymers with χ^{BA} = 0.05 improved the effectiveness of the polymer by a factor of 2 at room temperature. This increase was low but reproducible. The hydrophobic groups added bulk to the polymer and, in the absence of a balancing effect, were expected to decrease the effectiveness of the polymer. The case of the BA group provided an example of a positive effect that more than balanced the negative effect of bulk.

The effectiveness of the polymers increased by a factor of 4−20 as the temperature was increased from 4 °C to 36 °C. In contrast, the effectiveness of the standard polymer increased by a factor of 2. As temperature increases, the importance of hydrophobic interactions (which are often entropically driven) generally increases: that the effectiveness of the polymers increased significantly with increasing temperature was therefore reasonable.[46]

Measurements of K_i^{HAI} at Different Temperatures. We took parallel data for all side chains at 4, 19, and 36 °C (the entire assay—preparation of virus, blood and buffer, preincubation, and incubation—was performed at a single temperature). As the temperature of the assay was increased, the effectiveness of the polymer generally increased to varying extents. These observations can be rationalized in two ways: (i) If the HAI assay measured equilibria between the virus, polymer and erythrocyte (that is, the system was at thermodynamic equilibrium), then the dependence on temperature suggests that there is a positive change in entropy during the process of inhibition. The value of K_i^{HAI} varied, however, as a function of time of incubation and time of preincubation, suggesting that the system was *not* at thermodynamic equilibrium during the assay. (ii) If the system was not at thermodynamic equilibrium, then we expected that the effectiveness of inhibition should increase with increasing temperature (the rate of most processes increase with increasing temperature). The dependence of the value of K_i^{HAI} on temperature did not follow similar trends with all polymers. For example, the value of K_i^{HAI} decreased by a factor of two for polymer containing the 20% SA (χ^{SA} = 0.2) as the temperature of the assay was increased from 4 °C to 36 °C, compared with a factor of 60 for the polymer containing 100% SA (χ^{SA} = 1.0). These differences in the temperature dependence of the values of K_i^{HAI} for polymers containing different side chains imply that there are differences in the enthalpy of activation for inhibition using different polymers.

Conclusions

This work confirms that incorporation of SA groups into the side chain of polyacrylamide strongly enhances its ability to inhibit hemagglutination mediated by Influenza A X-31. Polymers prepared using the strategy of preactivation reproducibly gave values of K_i^{HAI} that were more than 2 orders of magnitude better than polymers prepared previously by copolymerization. We have suggested possible reasons for this enhancement (Figure 3), but we have not explored these hypotheses further in this work.

The effectiveness of the polymers generally increased with increasing temperature. Since the system was probably not at thermodynamic equilibrium (the periods of preincubation and incubation both influenced the measured value of K_i^{HAI}), this observation is consistent with the increase in rates that are expected with increasing temperature. Differences in the relationships between temperature and the value of K_i^{HAI} for different polymers imply that there are differences in the enthalpy of activation for inhibition by these polymers.

Steric Stabilization Is an Important Mechanism in Preventing Interaction of Surfaces. Side chains that we expected would increase the likelihood of steric stabilization generally increased the effectiveness of the polymer; side chains that we expected would decrease the likelihood of steric stabilization generally decreased the effectiveness of the polymer. Taking clues from the area of steric stabilization (also known as polymeric stabilization) of colloidal suspensions,[35] two characteristics may be responsible for changing the effectiveness of a polymer in stabilizing a surface sterically (Figure 14). First, if the conformation that a polymer adopts in solution becomes less random-coil and more rigid-rod (that is, if the ratio of its persistance length to contour length approaches 1), then the polymer is less
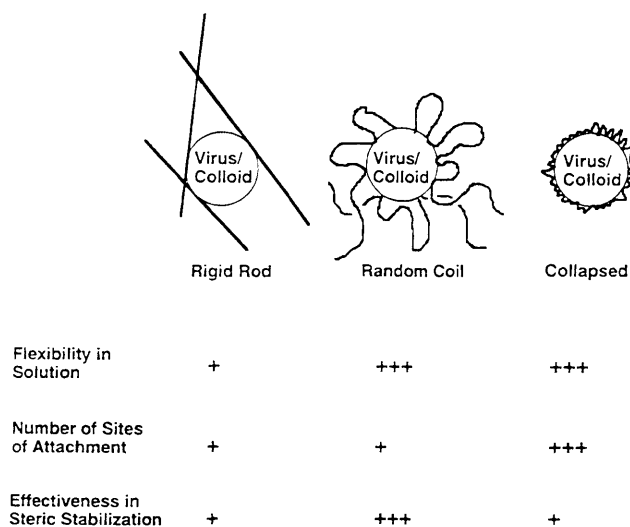
	Rigid Rod	Random Coil	Collapsed
Flexibility in Solution	+	+++	+++
Number of Sites of Attachment	+	+	+++
Effectiveness in Steric Stabilization	+	+++	+

Figure 14. The conformation of the polymer on the surface of the colloidal particle (the virus is modeled here as a colloidal particle) partly determines how effectively the polymer stabilizes a suspension of these particles. The polymers shown here have equal length. Relative to a random-coil polymer with an intermediate number of sites of attachment, changing either the conformation to a rigid rod (and keeping the sites of attachment constant), or increasing the number of sites of attachment (and keeping flexibility constant), decreases the effectiveness of the polymer in stabilizing a colloidal suspension.

able to prevent a colloidal suspension from flocculating. Second, if the polymer binds to the surface of the colloid by a very large number of attachment points, then the polymer is collapsed onto the surface and is again not able to prevent effectively a colloidal suspension from flocculating. The polymers that are effective at steric stabilization of a colloidal suspension are long (a high value of DP), well solvated, have a large number of available conformations, and are random-coils.

High-Affinity Binding Is an Important Mechanism in Preventing Interaction of Surfaces. Side chains that we expected would decrease the affinity of the polymer for the surface of the virus generally decreased the effectiveness of the polymers; side chains that we expected would increase the affinity of the polymer for the surface of the virus generally increased the effectiveness. As discussed in the previous section, we expect that increasing the affinity of the polymer for the surface of the virus by increasing the number of attachment points may, at some critical number of attachment points, becomes an ineffective strategy in building better inhibitors of hemagglutination. We suspect that between two polymers of the same length and having the same affinity for the surface of the virus, the one with a lower proportion of tighter binding monomers will be more effective; that is we suspect that incorporating small proportions of very tight binding analogs of SA will be an effective strategy of building highly effective inhibitors of hemagglutination.

Some of the polymers from this study are the best inhibitors of hemagglutination that have been reported. Of the polymers whose side chains are 20% SA, our most effective inhibitor at 36 °C is one where 10% of the side chains are benzyl groups (R^i = CH$_2$Ph, Scheme 1; K_i^{HAI} = 600 pM). In conclusion, we suspect that polymers that interact strongly with the surface of the virus, or those that effectively stabilize that surface, will be

effective at inhibiting interaction between virus and cell. Methods of increasing the affinity of the polymer for the surface of the virus beyond that of the standard polymer may include: increasing the affinity of a single SA–HA interaction by using tighter binding derivatives of SA; incorporating hydrophobic groups that bind adventitiously, or by design, to secondary sites on the surface of the virus; incorporating tight-binding inhibitors of NA. Methods of increasing the ability of the polymer to stabilize the surface of the virus sterically may include: increasing DP, and incorporating a *few* very large, flexible and water-swollen side chains. Polyvalent inhibitors of the interaction between influenza and erythrocyte provide a system that may be useful as a model for inhibitors of other pathogen–host interactions.

Experimental Section

Materials and Methods. All solvents and reagents were purchased from Aldrich and used without further purification unless otherwise noted. Reaction mixtures were stirred magnetically and monitored by thin-layer chromatography on silica gel precoated glass plates (Merck). Flash column chromatography was performed on silica gel 60$_{F254}$ (230–400 mesh, E. Merck) using the solvents indicated. Size-exclusion chromatography was performed on Biogel P2 resin or Sephadex G10 using distilled water as the eluent. Ion-exchange chromatography was performed on Dowex 50W-X8 (H$^+$ form) cation-exchange resin. Vortexing of copolymer samples was performed with a Fisher vortex Genie II. Dialysis was performed using Spectra/Por Molecularporous Membrane (28.6-mm cylinder diameter, 2-mL volume, molecualr weight cutoff ~12 kD). The protected dipeptide Glu(OtBu)Glu(OtBu)-OtBu[47] and compound 1[20] (mp 240 °C) were prepared as previously described.

All melting points were obtained using a Mel-temp apparatus and were uncorrected. Proton (^{1}H) and carbon (^{13}C) NMR spectra were measured on a Bruker AM-400 MHz or AM-500 MHz NMR spectrometer. Chemical shifts are reported in ppm relative to TMS for the ^{1}H NMR spectra, and relative to DMSO-d_6 at 39.5 ppm for the ^{13}C spectra.

N-Acetylneuraminic acid was obtained from extraction of edible Chinese swiftlet's nest. Erythrocytes from 2-week-old chickens were purchased from Spafas Inc., and used within 48 h of shipment. Influenza virus (X-31) was obtained from the laboratory of Professor John Skehel. The PBS used in the HAI assays was prepared from 80 g of NaCl, 2 g of KCl, 11 g of Na$_2$HPO$_4$, and 2 g of KH$_2$PO$_4$ in 1 L of distilled H$_2$O. This 10× stock solution was diluted as needed with distilled H$_2$O and then adjusted to pH 7.2 with 1 N NaOH.

***N*-(Acryloyloxy)succinimide.** Acryloyl chloride (33.4 g, 30 mL, 369 mmol) was added dropwise to a stirred solution of *N*-hydroxysuccinimide (42.5 g, 369 mmol) and triethylamine (41.0 g, 56.5 mL, 409 mmol, 1.1 equiv) in CHCl$_3$ (300 mL, 1.23 M) at 0 °C. The solution was allowed to stir for 3 h at 0 °C, then washed with water (2 × 300 mL) and brine (300 mL), then dried over MgSO$_4$, and recrystalized from a solution of ethyl acetate/hexane (1:1) to give 46.1 g (273 mmol) colorless crystals in 72% yield (mp 69 °C; lit.[36] mp 69.5 °C). ^{1}H NMR (400 MHz, CDCl$_3$): δ 6.78 (=CH$_2$, dd, 1H, $J^2$$_{gem}$ = 17.2 Hz, $J^3$$_{trans}$ = 0.78 Hz), 6.40 (=CH$_2$, dd, 1H, $J^3$$_{gem}$ = 17.2 Hz, $J^3$$_{cis}$ = 10.88 Hz), 6.24 (CH=, dd, 1H, $J^3$$_{cis}$ = 10.88 Hz, $J^3$$_{trans}$= 0.78 Hz), 2.94 (CH$_2CH_2$, s, 4H).

Poly[*N*-(acryloyloxy)succinimide] (pNAS). A mixture of *N*-(acryloyloxy)succinimide (3.15 g, 18.6 mmol) and AIBN (20 mg, 0.007 equiv) in benzene (150 mL) was heated at 60 °C for 24 h. After the solution was cooled to room temperature (rt), a white precipitate formed. This precipitate was filtered and washed four times with tetrahydrofuran (THF, 30 mL). Drying *in vacuo* afforded poly[*N*-(acryloyloxy)succinimide] (3.08 g, 18.2 mmol, 98%) as a white fluffy solid. The polymer was taken up in dry THF (300 mL), vigorously stirred for 3

days, filtered, and dried *in vacuo*. IR[36] (Nujol mull): 3340, 3200, 1730, 1660, 1210, 1070 cm^{-1}.

Determination of Molecular Weight of pNAS. A solution of pNAS (2 mg) in 6 N HCl (aq, 1 mL) was heated at 105 °C for 24 h in a sealed tube. After cooling to rt, the pH was adjusted to 7.2 with 1 N NaOH and water (1 mL) was added. The solution was exhaustively dialyzed against buffer (pH 7.2: 150 mM Na$_2$SO$_4$, 10 mM Na$_2$HPO$_4$). The solution of pAA thus obtained was analyzed by HPLC (Waters Ultrahydrogel Linear) using the following standards: pAA of MW 130 kD, 390 kD, and 1100 kD. Found: M_P = 129 kD, M_N = 75 kD, M_W = 146 kD, M_Z = 279 kD.

Preparation of Mixed Polymers Containing SA. A solution of **1** (243 μmol) in triethylamine (TEA, 0.5 mL) was added to a stirred solution of pNAS (6.78 g, 1.22 mmol NHS ester) in dimethylformamide (DMF, 20 mL). The solution was stirred at rt for 20 h, heated at 65 °C for 6 h, and then stirred at rt for an additional 48 h. This procedure yielded the *stock solution* of preactivated polymer with χ^{SA} = 0.20 (the stock solution contained 2.0 μmol SA/mL and 8.0 μmol NHS/mL). All other polymers that contained different proportions of SA were prepared analogously by changing the amount of **1**. The next step varied depending on the polymer.

For Polymers Containing SA on a pA Backbone (R^{2}NH$_2$ = NH$_3$). The stock solution (600 μL, 4.8 μmol NHS) was added dropwise to NH$_4$OH (concentrated aqueous, 1.5 mL) and stirred at rt for 12 h.

For Polymers Containing SA and One Other Component (R^{2}NH$_2$ ≠ NH$_3$). R^{2}NH$_2$ (48 μmol, 10 equiv) and TEA (48 μmol, 10 equiv) were added to a stirred stock solution (600 μL, 4.8 μmol NHS, 1 equiv). The resulting solution was heated at 65 °C for 6 h. Any remaining NHS ester was quenched by addition of NH$_4$OH (concentrated aqueous, 1.0 mL) follwed by stirring at rt for 12 h.

For Polymers Containing SA and Two Other Components. R^{2}NH$_2$ (0.48 μmol for χ^{R2}, χ^{R2} = 0.1) and TEA (0.48 μmol) were added to a stirred stock solution (600 μL, 4.8 μmol NHS). The resulting solution was heated at 65 °C for 6 h. NH$_4$OH (concentrated aqueous, 2 mL) was added and the solution heated at 65 °C for 6 h and then stirred at rt for an additional 12 h.

For all three classes of polymers above, the resulting mixture was then dialyzed exhaustively against distilled H$_2$O, then NH$_4$Cl (1 M), again with distilled H$_2$O, and then lyophilized to yield a white powder. Typical recovery of SA was > 80%.

Supporting Information Available: A table of values of K_i^{HAI} at 4, 19, and 36 °C for all 100 polymers is available on request (6 pages). Ordering information is given on any current masthead page.

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(43) The amount of water in the sample was calculated from the ratios of carbon to hydrogen and nitrogen. Using the corrected mass for the polymers, we established a relation between the percentage of sulfur and χ^{SA}. A similar relationship between the number of equiv of 1 and χ^{SA} was obtained using the *ratio* of S to N from combustion analysis.

(44) In the HAI assay a dilute suspension of chicken erythrocytes is added to the wells of a 96-well microtiter plate. These wells have a volume of 250 μL and a conically shaped base: the erythrocytes settle by gravity and concentrate to a point (a "pellet", easily identified visually). If influenza virus is present in suspension with the erythrocytes, the virus cross-links the erythrocytes by interaction of viral HA with cellular SA groups and forms a loose gel. The erythrocytes in such a gel do not settle to a pellet: they are "hemagglutinated" and appear homogeneously red. An inhibitor that binds to the virus prevents formation of the gel; it inhibits hemagglutination.

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