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Polyacrylamides Bearing Pendant α-Sialoside Groups Strongly Inhibit Agglutination of Erythrocytes by Influenza Virus: The Strong Inhibition Reflects Enhanced Binding through Cooperative Polyvalent Interactions

George B. Sigal, Mathai Mammen, Georg Dahmann, and George M. Whitesides*

Contribution from the Department of Chemistry, Harvard University, 12 Oxford Street, Cambridge, Massachusetts 02138-2902

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Abstract: An ELISA assay is described for measuring the binding of influenza virus A-X31 to α-sialoside groups that are linked to biotin-labeled polyacrylamides. The efficacy of these polymers in inhibiting the adhesion of influenza virus to erythrocytes (as measured by a hemagglutination assay) was shown to be directly related to the binding affinity of the polymers for the viral surface: the differences in inhibitory efficacy among the polymeric inhibitors and monomeric α-methyl sialoside, among fractions of a polymeric, polyvalent inhibitor with narrow molecular weight ranges, and among polymeric inhibitors prepared by copolymerization or modification of a preformed polymer chain, all correlated with differences in the affinity of the inhibitors for the surface of the virus. The polymeric inhibitors studied had affinities for the viral surface that ranged between 10^3 and $>10^6$ greater than α-methyl sialoside, on the basis of total sialic acid groups in solution. The role of steric stabilization in the mechanism by which these polymers inhibit hemagglutination was investigated. The ability of the polymeric, polyvalent inhibitors to inhibit the binding of a polyclonal antibody to the viral surface suggests that steric stabilization may also be an important effect in this system.

Introduction

The first step in the infection of a cell by the influenza A-X31 virus is the attachment of the virus to the cell membrane. The attachment occurs through the interaction of hemagglutinin (HA), a sialic acid (SA) receptor on the virus, with SA groups linked to glycoproteins and glycolipids on the surface of the cell.[1,2] In theory, one approach to preventing infection by the influenza virus might be to design analogs of SA that bind tightly to HA and prevent attachment of the virus to cells. In practice, however, the design of tight binding inhibitors of HA has been difficult because the binding pocket is small and shallow.[3,4] Solubilized HA binds only weakly ($K_d \sim 3$ mM) to α-methylsialoside (**1**).[5] The best monomeric inhibitors to date are SA derivatives linked to large hydrophobic groups at the 2 and 4 positions.[6,7] The tightest binding of these inhibitors, **2**, has a dissociation constant in the micromolar range ($K_d = 3$ μM).[7]

Although the interaction of a single HA binding site with a single SA is weak, the binding of a particle of virus to the surface of a cell is strong (i.e. stable complexes of viral particles and red blood cells can form in suspensions containing total

[⊗] Abstract published in *Advance ACS Abstracts,* April 1, 1996.

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(2) Wiley, D. C.; Skehel, J. J. *Annu. Rev. Biochem.* **1987**, *56*, 365−394.
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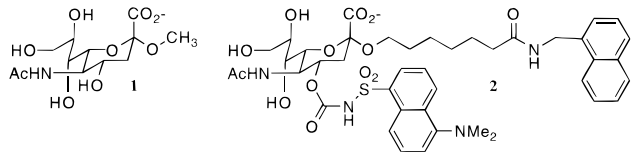
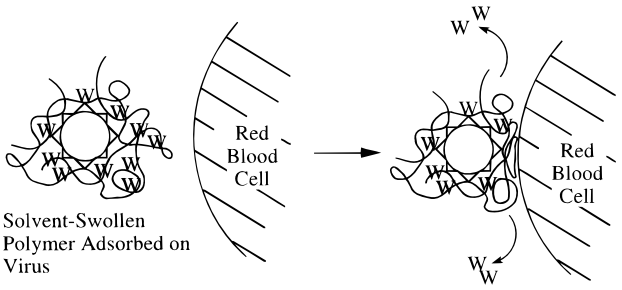


concentrations in solution of HA and SA, on the surfaces of the virus and cell, that are well under the K_d for the interaction of a single HA with a single SA).[8] We believe that this strong binding reflects the interaction of multiple copies of HA on the viral surface simultaneously with multiple SA groups on the surface of the cell.[9] Based on the idea that polyvalent interaction is required for tight binding of the cell to the virus, we and others have developed inhibitors that also present multiple copies of SA to the virus; these polyvalent inhibitors are effective at preventing the attachment of influenza virus to red blood cells at very low concentrations of inhibitor.[10−19]

These studies determined the effectiveness of the inhibitors by an assay measuring the inhibition of the agglutination of red blood cells (hemagglutination inhibition, HAI). The inhibition constant (K_i^{HAI}) is the minimum concentration of inhibitor required to inhibit hemagglutination by the virus in a 96-well assay format.[20] Here and throughout this paper, K_i^{HAI} for polyvalent inhibitors is calculated on the basis of the total concentration of SA groups in the system (whether as monomers attached to polymers, or linked to the surface of liposomes or cells) and not on the basis of the concentration of polymer molecules. Liposomes incorporating lipids derivatized with SA analogs prevent hemagglutination with inhibition constants as low as $K_i^{HAI} = 10$ nM .[10,11] Polyacrylamide chains bearing pendant α-sialoside groups are also effective inhibitors of hemagglutination,[12−19] and viral replication.[12b] Polymers formed by the copolymerization of acrylamide with acrylamide derivatives bearing SA analogs have inhibition constants as low as $K_i^{HAI} = 200$ nM.[12−18] More recently, we have prepared polyacrylamides presenting SA groups—using the reaction of preformed polymers of *N*-acryloyloxysuccinimide with an amine-functionalized SA derivative and ammonia—that are the most effective known inhibitors of hemagglutination by influenza virus ($K_i^{HAI} = 1$ nM).[19]

Scheme 1. Cartoon Showing Volume Restriction and Loss of Water (W) from a Sterically Stabilized Viral Particle on Approach of a Red Blood Cell[a]



[a] Both changes are associated with unfavorable energy terms due to losses of both conformational entropy and favorable water−polymer interactions.

A balance of three factors—two favorable and one unfavorable—are believed to contribute to the effectiveness of these polyvalent inhibitors in preventing viral attachment. First, there is an increase in the affinity of the polyvalent inhibitor for the surface of the virus, relative to a polymer having only one (or a few) copies of the ligand, due to the binding of multiple SA groups per inhibitor molecule. Second, the large effective molecular volume of these high molecular weight inhibitors prevents the virus from coming close enough to a cell for the viral receptors and cell-surface ligands to interact. Third (and unfavorable), a single ligand attached to a polymer binds less tightly to the receptor than a ligand in a low molecular weight form.[18]

While inhibition of viral attachment by a monomeric inhibitor requires a concentration of monomer high enough to block at least half of the SA binding sites on the virus, high molecular weight inhibitors might only need to make enough attachments to achieve a critical amount of excluded volume around the virus and, in theory, could target receptors other than those involved directly in viral attachment. For inhibitors having large structures with a defined shape, such as liposomes whose surfaces are decorated with ligands, the structures physically block the approach of the red blood cell to the virus.[21] For inhibitors with no defined shape, such as the copolymers of acrylamide, the blocking effect is believed to be more like the well-known ability of adsorbed polymers to prevent the aggregation (flocculation) of colloidal dispersions (steric stabilization).[22−25] Scheme 1 is a cartoon representing the approach of a red blood cell to a viral particle coated with a well-solvated layer of polymer. As illustrated in the cartoon, the approach of the virus to the surface of the red blood cell results in compression of the polymer chains. This compression results in two, potentially large, repulsive energy terms. The first—volume restriction—is due to loss of conformational entropy as

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(25) Sato, T.; Richard, R. *Stabilization of Colloidal Dispersions by Polymer Adsorption*; Marcel Dekker: New York, 1980.

the polymer strands are restricted into a smaller volume. The second—osmotic repulsion—results from the expulsion of solvent (water) on compression; this second term may have both entropic and enthalpic components.

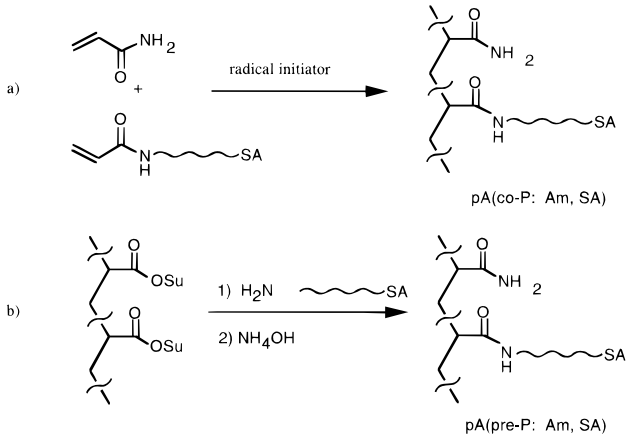
The relative importance of enhanced affinity and steric stabilization in the inhibitory ability of SA-containing polymeric inhibitors is unknown because the two effects cannot be distinguished by the HAI assay. One way to separate these two effects is to measure *directly* the affinity of SA-containing polymers for the viral surface and to compare the binding ability with the effectiveness in preventing hemagglutination. In this paper we describe an ELISA assay for quantifying the binding to the influenza virus of polymers containing both SA groups and biotin. By comparison of the values of K_i^{HAI} with the results of the ELISA assay, we show that the potency of the polymeric inhibitors in inhibiting hemagglutination correlates with (and, we presume, reflects) their enhanced affinity for the viral surface. In particular, we describe experiments that demonstrate the following: (i) the effective dissociation constants of the polymeric inhibitors for the viral surface as determined by the ELISA assay are roughly equal to the measured values of K_i^{HAI}; (ii) the differences in the ability of SA-containing polymers prepared by two different synthetic routes—copolymerization and modification of a preformed polymer—to inhibit hemagglutination correlates with the relative affinities of the two types of polymers for the viral surface; and (iii) the ability of SA-containing polymers to inhibit agglutination increases with increasing molecular weight, and this dependence correlates with increases in the affinity of the polymer for the virus with increasing molecular weight.

Steric stabilization is more difficult to measure directly than enhancements in affinity. One approach for demonstrating a role for steric stabilization is to show that the polymeric inhibitors inhibit the binding to the virus of receptors directed at sites other than the binding pocket of HA. We show that the polymeric inhibitors inhibit the binding to the virus of a polyclonal antibody generated against the viral surface and are, therefore, capable of steric stabilization of the viral surface.

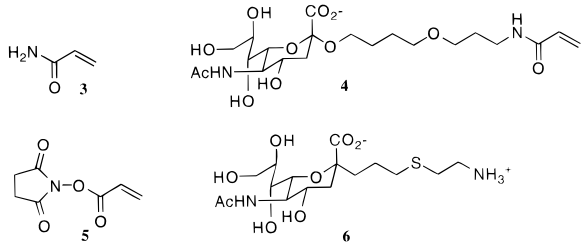
Results

Preparation of Polyacrylamides Presenting SA Groups. Polymers were prepared by two routes: copolymerization (Scheme 2a) or modification of a preformed, activated, polymer chain (Scheme 2b).[12,18,19] The following nomenclature is used in this paper to distinguish between the different polymer preparations: pA(co-P: Am, SA) refers to a polyacrylamide chain (pA) prepared by copolymerization (co-P) of acrylamide and an acrylamide derivative bearing an SA group. In this nomenclature, Am (for primary amide) and SA refer to the groups presented on the polymer side chains. Similarly, pA-(pre-P: Am, SA) refers to a polymer also presenting primary amides and SA groups on the side chains, but prepared by modification of a preformed polymer chain (pre-P). Inclusion of biotin (BT) or fluorescein (F) derivatives in the polymer chain by either of the two synthetic methods is indicated by including the abbreviation for the label within the parentheses, for example, pA(pre-P: Am, SA, F). We use the symbol χ to represent the mole fraction of monomeric units in a polymer chain that present a particular group (i.e. χ_{SA} = the number of monomeric units containing SA divided by the total number of monomeric units in the polymer chain).

Scheme 2. Polyacrylamides Bearing Pendant α-Sialoside Groups (SA) Prepared (a) by Radical Copolymerization of Acrylamide with an Acrylamide Derivative Containing an SA Group or (b) by Modification of a Preformed, Activated Polymer—Poly(*N*-acryloyloxysuccinimide), pNAS—with an Amine Derivative Containing an SA Group and then with Concentrated Ammonium Hydroxide



The polymer pA(co-P: Am, SA) was prepared by the free-radical copolymerization in water of a mixture of 10 equiv of acrylamide **3** with 1 equiv of the SA-containing acrylamide derivative **4**[18] (an *O*-glycoside of SA) under UV light in the presence of azobis(cyanovaleric acid) as a radical initiator.[12,19] Under these conditions all of **4** was consumed over the course of the polymerization; the proportion of monomer units linked to SA in the resulting polymer was $\chi_{SA} \approx 0.09$ (as determined by a colorimetric assay for *O*-glycoside of SA[31]). The polymer pA(pre-P: Am, SA) was synthesized by the reaction of pNAS (a polymer of *N*-acryloyloxysuccinimide **5** prepared by free-radical polymerization in benzene) with 0.2 equiv of SA derivative **6** (a *C*-glycoside of SA) per equiv of succinimide ester groups in DMF, followed by aminolysis of the remaining active ester groups in aqueous concentrated ammonium hydroxide.[19] The SA content of this polymer was determined by elemental analysis and ^{1}H NMR spectroscopy to be $\chi_{SA} \approx 0.17-0.18$.[19] The proportion of monomers that were $-CONH_2$ was ~0.8 based on elemental analysis (i.e., the proportion of activated esters that hydrolyzed to COOH was negligible).



Molecular Weight Distribution of Polyacrylamides Presenting SA Groups. Polymers prepared by free-radical polymerization usually have a broad molecular weight distribution.[26] The molecular weight distribution of the SA-containing polymers was determined by gel-filtration chromatography (GFC). To ensure accurate calibration of the GFC column, we hydrolyzed the polymers to polyacrylic acid under acidic conditions,[27] and compared the GFC elution profile of the

(26) Ishige, T.; Hamielec, A. E. *J. Appl. Polym. Sci.* **1974**, *175*, 3117.
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(29) For narrow molecular weight fractions, Mp ~ $(M_n \times M_w)^{0.5}$, see ref 28.
(30) Skoza, L.; Mohos, S. *Biochem. J.* **1976**, *159*, 457−462.
(31) Roy, R.; Tropper, F. D.; Romanowska, A. *J. Chem. Soc., Chem. Commun.* **1992**, 1611−1613.

Table 1. Hemagglutination Inhibition Constant (K_i^{HAI}), Weight Average Molecular Weight (M_w), Number Average Molecular Weight (M_n), and Molecular Weight at the GFC Peak Retention Time (M_p), for Unfractionated pA(co-P: Am, SA) and pA(pre-P: Am, SA)

polymer	K_i^{HAI} (nM)	M_p (kD)	M_n (kD)	M_w (kD)
pA(co-P: Am, SA)	300	490	210	450
pA(pre-P: Am, SA)	4	100	70	140

hydrolyzate to standards of polyacrylic acid of known molecular weight. Figure 1a shows the GF chromatograms, after acid hydrolysis, for pA(co-P: Am, SA) and pA(pre-P: Am, SA). By calibrating the GFC column using the peak retention time of the standards (Figure 1a inset), the molecular weight distributions of the polymers were calculated (Figure 1b).[28] Table 1 shows that although pA(pre-P: Am, SA) inhibits hemagglutination more strongly than does pA(co-P: Am, SA), it has a smaller chain length, as indicated by the weight and number average molecular weights and the molecular weight at the peak maximum (M_w, M_n, and M_p, respectively) for the hydrolyzed polymer backbones.

Dependence of K_i^{HAI} on the Molecular Weight of the Polymeric Inhibitors. To test the dependence of inhibitory ability on molecular weight, we prepared narrow molecular weight fractions of the polymers by preparatory GFC. Figures 2a and 2b show the GF chromatograms for the individual fractions obtained from the two polymers. Figures 3a and 3b show the chain length and the concentration of the polymers in each fraction. We calculated the polymer chain length (N), given as the number of monomeric units per chain, from the value of M_p measured for each fraction after acid hydrolysis.[29] Integration of the HPLC peak for the hydrolyzed polymers gave the concentration of the polymer in each fraction. For the pA(co-P: Am, SA) fractions, a colorimetric assay gave directly the concentration of the *O*-glycosides of SA.[30] Figure 3a shows that the concentration of SA was proportional to the concentration of polymer and indicates that polymers of all lengths had similar ratios of the two monomeric components. This result is evidence that the SA groups in pA(co-P: Am, SA) were distributed evenly among polymers of different lengths (the distribution of SA groups along the length of individual polymer chains, however, remains unknown and could be nonrandom). There is no convenient assay with sufficient sensitivity to determine the concentration of *C*-glycoside SA groups in pA(pre-P: Am, SA) fractions, so the concentration of SA groups was calculated directly from the polymer concentration by assuming an even distribution of SA groups.

We tested the narrow molecular weight fractions for their ability to inhibit agglutination of chicken red blood cells by the X-31 strain of influenza A virus (H3N2). Figure 4 gives K_i^{HAI} as a function of molecular weight and chain length. The log−log plot for pA(co-P: Am, SA) gives a linear least-squares fit with a slope of −1.1 over a range of N covering close to two orders of magnitude. The measured slope indicates that for this range of molecular weights, K_i^{HAI} was roughly inversely proportional to chain length (i.e. the inhibitory potency was roughly linearly proportional to chain length). For pA(pre-P: Am, SA), K_i^{HAI} was also inversely related to chain length, but the dependence was weaker than linear (slope of log−log plot = −0.68). The slope calculated for the pre-P polymers may be a lower bound; we will demonstrate later in this paper that the values of K_i^{HAI} measured for the pre-P polymers approach the limits of the HAI assay and that very small values of K_i^{HAI} (that is, very tight binding) may be inaccurate, in the sense that the assay *underestimates* the effectiveness of the most effective inhibitors.

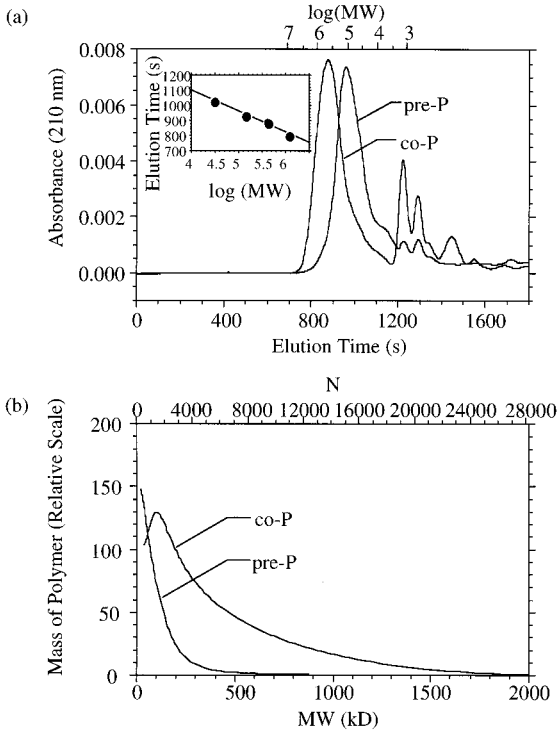


Figure 1. Characterization of acid-hydrolyzed pA(co-P: Am, SA) and pA(pre-P: Am, SA) by gel filtration chromatography (GFC). (a) GFC chromatograms for the two polymer samples. The complex peaks at elution times greater than 1200 s are due to air and low molecular weight salts in the sample; these peaks also occur after injection of samples containing no polymer. The inset shows the molecular weight calibration curve determined for the GFC column with polyacrylic acid molecular weight standards. (b) Molecular weight distributions for the two polymer samples. Distributions are given as a function of molecular weight (MW) or chain length (N) calculated as the number of monomeric units per chain.

Due to the complexity of the interactions occurring in the HAI assay, it is difficult to explain the molecular weight dependence just on the basis of these data. The longer polymers, because they contained more SA groups per polymer chain, may have bound to the virus at a lower concentration of SA groups due to increased polyvalent binding. Another possibility is that the longer polymer chains, once adsorbed on the surface of the virus, provided more steric stabilization. Some combination of the two factors is also possible. To distinguish between these possibilities, we developed a direct binding assay for the interaction between virus and polymer.

Measurement of the Affinity of the Polymeric Inhibitors for the Surface of Influenza Virus: Binding Studies Using Biotin-Labeled Polymers. Roy et al. have reported an ELISA method for measuring the interaction of polyacrylamide copolymers bearing sugar and biotin (BT) groups with lectins and streptavidin.[31] In their procedure, the polymer was adsorbed on a polystyrene surface, labeled with either a lectin−enzyme or a streptavidin−enzyme conjugate, and quantified using a standard ELISA protocol. We used a similar approach in our assay for measuring the binding of SA-containing polymers to the influenza virus. For our binding assay we prepared polyacrylamides containing both SA and BT groups both by the copolymerization protocol (pA(co-P: Am, SA, BT)) and by reaction with prepolymerized pNAS (pA(pre-P: Am, SA, BT). The pA(co-P: Am, SA, BT) was prepared by copolymerizing acrylamide (**3**), the SA-containing acrylamide (**4**), and the biotin-containing acrylamide (**7**) at a ratio of 100:10:1. The

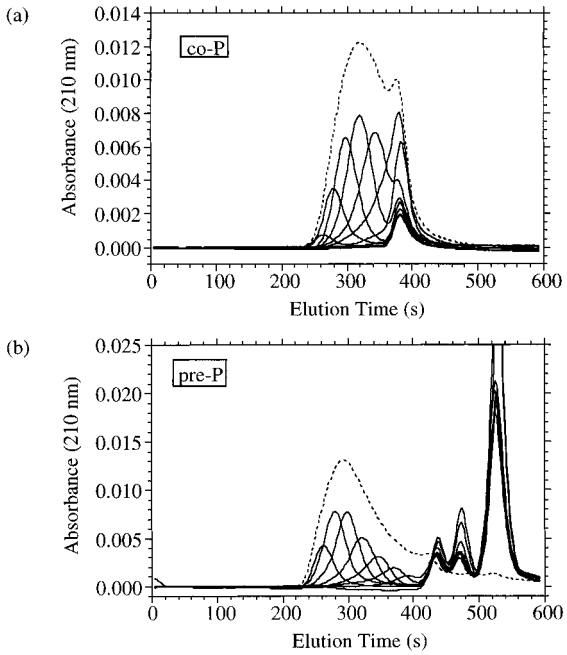
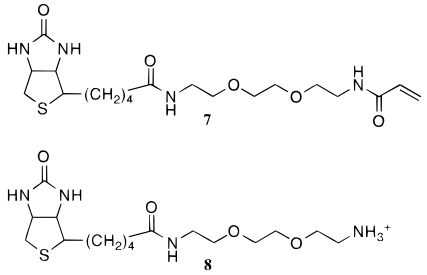


Figure 2. Fractionation of SA-containing polyacrylamides by GFC. GFC chromatograms from fractionation of (*a*) pA(co-P: Am, SA) using a 4000 Å pore size column and (b) pA(pre-P: Am, SA) using a 300 Å pore size column. Plots show chromatograms from unfractionated polymer (dashed line) and narrow molecular weight fractions (solid lines). The sharp peak at an elution time of 380 s in (a) and the complex peaks at elution times greater than 400 s in (b) are due to air and low molecular salts in the sample; these peaks also occur after injection of samples containing no polymer.

pA(pre-P: Am, SA, BT) was prepared by sequentially reacting pNAS with 0.2 equiv of the SA derivative (**6**) and then 0.01 equiv of the biotin derivative (**8**) in DMF, followed by aminolysis of the remaining active esters in aqueous ammonium hydroxide. Both preparations led to polymers containing a proportion of BT-linked monomer units, $\chi_{BT} = 0.01$ (as determined by a colorimetric assay for BT),[32] and proportions of SA-linked monomer units, χ^{SA}, roughly equal to the corresponding polymers prepared without BT. The incorporation of BT groups at low mole fractions into the polyacrylamides presenting SA groups had no apparent effect on the properties of the polymers prepared by either method: both the molecular weight distribution and the K_i^{HAI} measured for the polymers containing SA and BT were indistinguishable from those prepared without BT groups.



The procedure we used in our ELISA binding assay is outlined in Scheme 3. First, fetuin (a glycoprotein containing SA groups in oligosaccharide side chains) was adsorbed noncovalently on the hydrophobic surface of polystyrene 96-well plates. Second, viral particles were immobilized on the plates by treatment of the fetuin layer with a suspension of the virus.

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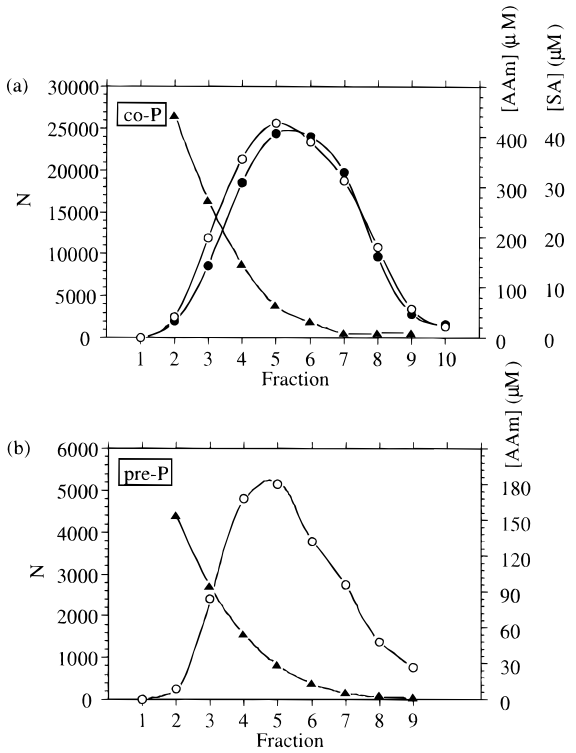


Figure 3. Characterization of narrow molecular weight fractions of (a) pA(co-P: Am, SA) and (b) pA(pre-P: Am, SA). The polymer chain length (▲), given as the number of monomeric units per chain (*N*), and the concentration of polymer (○), given as the total concentration of monomeric units [AAm], is provided for each fraction. For the pAA-(co-P; Am, SA) fractions, the total concentration of polymer-bound SA groups [SA] is also given (●).

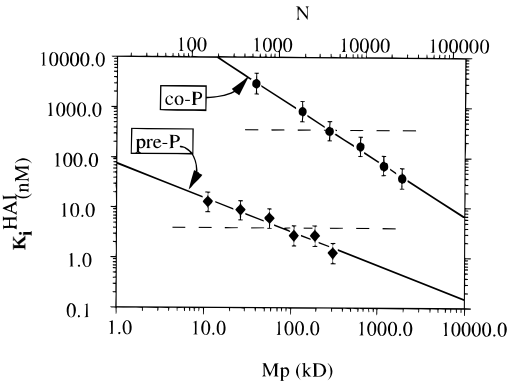
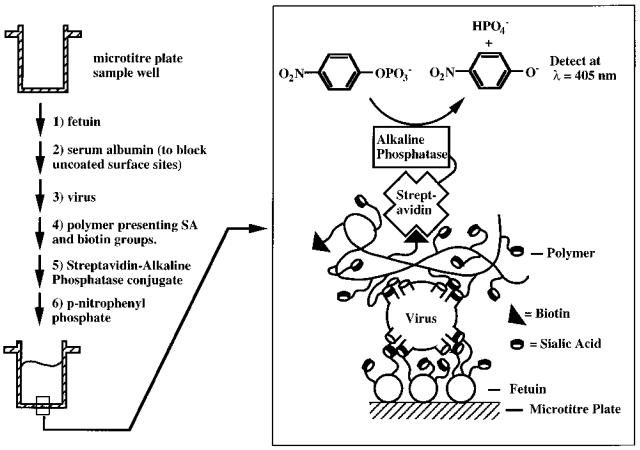


Figure 4. Dependence of hemagglutination inhibition on molecular weight and polymer chain length (*N*) for narrow molecular weight fractions of pA(co-P: Am, SA) (●) and pA(pre-P: Am, SA) (◆). K_i^{HAI} is the minimum concentration of polymer (given as the concentration in solution of polymer-linked sialic acid groups) required to prevent hemagglutination of chicken red blood cells. The molecular weight (M_p) of the polymer fractions was determined from the retention time of the GFC peak for polymer that had been hydrolyzed in acid to polyacrylic acid. The chain length (*N*) is defined as the number of monomeric units per polymer chain. The dashed lines show the values for the unfractionated polymers.

Third, the immobilized virus was treated with a solution of the biotin labeled polymer, and incubated for 30 minutes (we have shown previously that incubation for 30 min is sufficient for polymer-virus mixture to reach equilibrium[33a]). Fourth, bound

(33) (a) Mammen, M.; Dahmann, G.; Whitesides, G. M. *J. Med. Chem.* **1995**, *38*, 4179−4190. (b) At the highest concentrations of polymers tested, the ELISA signal decreases with increasing concentration. This behavior could be caused by inaccessibility of biotin groups to the streptavidin−enzyme complex at high surface coverage of polymer, or by an increase in

Scheme 3. ELISA-Type Assay for Measuring the Binding of a Biotin-Labeled Polymer-Bearing Sialic Acid Groups to the Influenza Virus[a]



[a] The cartoon shows schematically the important binding events occurring during the assay.

polymer was determined by using a streptavidin−alkaline phosphatase conjugate. Fifth, the rate of enzyme-catalyzed hydrolysis of *p*-nitrophenyl phosphate was measured colorimetrically with a kinetic microplate reader. Figure 5 shows the binding curves as a function of the concentration in solution of polymer-bound SA moieties. Although the interaction of these polymers with the virus is probably complex mechanistically, the data can be adequately fit over most of the concentration range by assuming they follow a Langmuir isotherm—a one-step reaction with a single binding constant (eq 1).[33b]

$$v = v_{sat}\left(\frac{[SA]}{K^{ELISA} + [SA]}\right)$$

Where [SA] = the concentration in solution of polymer-bound SA groups during polymer binding; v = the rate of *p*-nitrophenyl phosphate hydrolysis due to bound streptavidin−enzyme; v_{sat} = the rate at hydrolysis that results from saturation of the viral surface with polymer; K^{ELISA} = the effective dissociation constant, calculated on a per SA basis (i.e. on the basis of the total concentration of polymer-linked SA residues in solution).

The use of eq 1 to fit the ELISA binding isotherms requires the assumption that the concentration of polymer in solution is not significantly affected by the binding of polymer to immobilized virus. As long as this assumption holds, the effective dissociation constant that we measure, K^{ELISA}, should be independent of the concentration of virus immobilized on the surface of the 96-well plate. If this surface concentration of virus is high enough to bind and remove a significant fraction of the polymer from solution, the calculated value of K^{ELISA} will be larger (weaker binding) than the actual value and will increase with an increase in the surface concentration of virus. To test the validity of the assumption, we measured the binding isotherms of unfractionated pA(co-P: Am, SA, BT) and pA(pre-P: Am, SA, BT) on fetuin-coated plates that had been treated with different concentrations of influenza virus. Figure

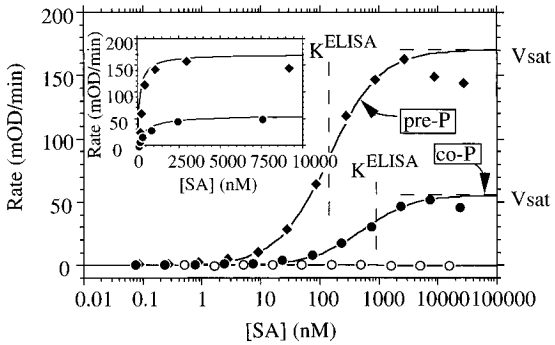


Figure 5. ELISA sandwich assay measuring the binding of unfractionated pA(co-P: Am, SA, BT) (●) and pA(pre-P: Am, SA, BT) (◆) to the immobilized influenza virus as a function of the concentration of polymer-linked SA groups in solution during adsorption of the polymer. The virus was immobilized on a fetuin-coated surface from a suspension containing 50 μg of viral protein/mL. Adsorbed polymer was quantified by binding a streptavidin−alkaline phosphate conjugate and measured as the rate of enzymatic hydrolysis of *p*-nitrophenyl phosphate (given as the rate of change of absorbance at 405 nm). PA(co-P: Am, BT), a polymer synthesized under the same copolymerization conditions as pA(*co*-P: Am, SA, BT) except for the omission of the SA-labeled acrylamide monomer, was tested as a negative control to verify the need for SA groups for binding (○). The concentrations of the pA(co-P: Am, BT) solutions are given as the concentration of polymer-linked SA groups present that would be present in equivalent dilutions of pA(co-P: Am, SA, BT). The curves superimposed over the data show nonlinear fits of the data to Langmuir isotherms as described in the text (the fitted constants, K^{ELISA} and v_{sat}, are shown with dashed lines). At the higher polymer concentrations there was a reproducible decrease in rate with increasing [SA]; these data were not included in the nonlinear fits.

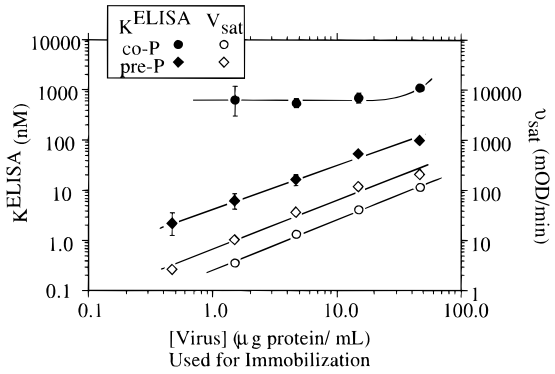


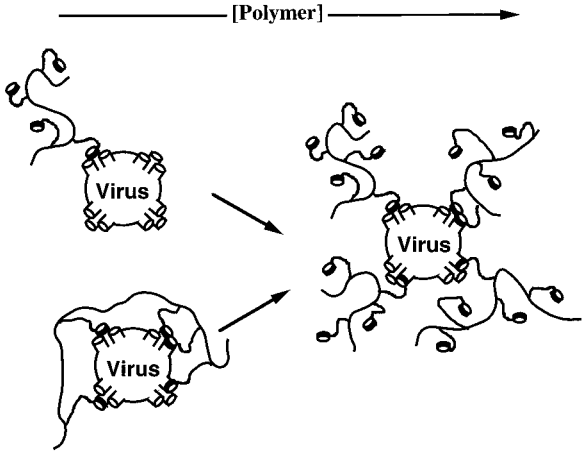
Figure 6. Dependence of binding constants, calculated for the binding of SA-containing polymers to influenza virus by the ELISA assay, on the amount of virus immobilized on the ELISA plates. The surface concentration of immobilized virus was varied by treating fetuin-coated ELISA plates with suspensions of virus containing different concentrations of virus. For each concentration of virus used, the binding constants K^{ELISA} and v_{sat} were determined for both pA(co-P: Am, SA, BT) and pA(pre-P: Am, SA, BT) from binding isotherms as described in the text and Figure 5.

6 shows the results of this study. The correlation of v_{sat} with the concentration of virus shows that the number of viral particles that adsorbed to the plates was dependent on the concentration in solution of virus during the immobilization step. Except for the highest surface concentration of virus, the K^{ELISA} values determined for pA(co-P: Am, SA, BT) were independent of the concentration of immobilized virus: for this polymer, the effective binding constant K^{ELISA} = 541 ± 112 nM. In contrast, the values of K^{ELISA} determined for pA(pre-P: Am, SA, BT) were strongly dependent on the concentration of immobilized virus over the entire range of surface concentrations that were tested. Thus, using this assay, we cannot accurately

the number of biotin groups bound per tetravalent streptavidin at high concentrations of biotin groups on the surface. Another possibility is that high concentrations of the SA-containing polymers are able to competitively desorb immobilized virus from the fetuin-coated surface. Data from polymer concentrations exhibiting this behavior were not included in the curve fits.

(34) The differences in inhibition constants are not due to the different SA derivative and linker chains used in the pre-P and co-P polymers. Polymers with the same linker and SA derivative as the pre-P polymer but prepared by copolymerization behave similarly to the co-P polymer used in this study (see ref 17).

Scheme 4. The Increase in the Mass of Adsorbed Polymer with Polymer Concentration May Represent an Increase in Occupancy of SA Binding Sites on the Virus (Top Arrow), a Decrease in the Number of Attachment Sites per Polymer Chain without a Change in Occupancy of SA Binding Sites (Bottom Arrow), or a Combination of These Two Mechanisms



measure the effective binding constant, but we can place an upper limit of $K^{ELISA} < 3$ nM. We were unable to decrease the concentration of virus on the surface of the virus further because the signal to noise of the assay became too low.

The effective dissociation constants (K^{ELISA}) that were measured indicate that the polymers bound from 10^3 to $>10^6$ tighter, on a per SA group basis, than monomeric SA and its simple analogs. This result is evidence for a strong enhancement in the affinity of these polyvalent inhibitors through binding of multiple binding elements. Lees et al. showed that ligands attached to a polymer backbone may be less accessible sterically to receptors than free ligands in solution.[18] The enhancement of binding due to polyvalency effect, therefore, may be even greater than indicated. We note explicitly that the ELISA assay gives no indication of the degree of occupancy of SA binding sites on the virus. The mass of adsorbed polymer adsorbed on virus may increase with polymer concentration for at least two reasons. First, additional polymer chains may bind to unoccupied sites on the virus. Second, it is possible to adsorb more polymer chains per viral particle without changing the occupancy of the SA binding sites by making fewer attachments per polymer chain. These two possibilities are illustrated in Scheme 4. At very low concentrations of polymer on the surface, the first mechanism will dominate. At higher concentrations on the surface, unfavorable interactions between polymer chains may preclude the occupation of all SA binding sites—under these conditions, the adsorption of additional polymer chains is likely to follow a combination of both mechanisms.

The values of K^{ELISA} that were measured were similar to the corresponding values of K_i^{HAI}: inhibition of hemagglutination requires concentrations of polymer on the viral surface that are approximately half of the values at saturation. The 100-fold difference in K_i^{HAI} for polymers prepared by copolymerization compared to polymers prepared by modification of a preformed polymer is completely accounted for by differences in the effective dissociation constants of the polymers, as opposed to differences in the abilities of the polymers, once bound to the virus, to prevent the virus from binding the red blood cell sterically. The reason for the large differences in dissociation constants measured for polymers prepared by the two methods is not clear but may reflect differences in the distribution of SA groups along the polymer backbone.[34] We believe, for instance, that the co-P class of polymers may not have a random distribution of SA groups along the polymer backbone because of differences in the relative rates of copolymerization of the substituted and unsubstituted monomers.[19] The uneven distribution of SA groups over the length of a polymer chain limits the surface area of the virus (and therefore the number of SA binding sites) that can interact with the polymer, and thereby decreases the effective binding constant of the polymer. In contrast, the preparation of the pre-P polymers is more likely to give a random distribution of SA groups because the distribution is not influenced by the relative rates with which each functional group is incorporated into the polymer. The distribution of SA groups in the pre-P polymers is controlled by differences in the reactivities of the activated side chains along the length of the polymer; these differences are probably small because neutral polyacrylates in good solvents tend to behave as random coils.[35]

No binding occurred, by the ELISA assay, if the polymers did not present both SA and BT groups; this observation demonstrated that no non-specific interactions were occurring. The specificity of the binding interactions was confirmed by transmission electron microscopy (TEM). A carbon film on a copper grid was treated with fetuin, virus, and pA(pre-P: Am, SA, BT) by the same procedure used to coat the ELISA plates. The adsorbed polymer was then labeled with a streptavidin—colloidal gold conjugate. Figure 7 is a TEM micrograph that shows extensive labeling of the adsorbed polymer with the colloidal gold tag. The colloidal gold is predominantly confined to the surface of the viral particles; the polymer is therefore also localized on the viral surface.

Narrow molecular weight fractions of the biotin-labeled polymers were prepared exactly as described for the unlabeled polymers, and the values of K_i^{HAI} showed the same dependence on chain length. Figure 8a shows that for pA(co-P: Am, SA, BT), K^{ELISA} was inversely dependent on chain length (slope of the log−log plot $= -0.8$), while v_{sat} did not vary with chain length; fractions containing longer polymer chains (and, therefore, more SA groups per chain) had higher affinities, but the total mass of polymer bound under conditions of saturation was independent of chain length. Figure 8b shows that K_i^{HAI} and K^{ELISA} had similar dependencies on chain length; the dependence of inhibitory potency on chain length, therefore, is due to the higher affinity of the longer chains. A similar analysis was not possible for the pre-P inhibitor because the binding constants were too tight to measure accurately by the ELISA assay (all the fractions have a $K^{ELISA} < 15$ nM; data not shown).

One can develop a simple model for relating binding constants to chain length by assuming that each additional SA on the longer chains is capable of participating in an additional interaction with HA on the viral surface, and that each additional SA−HA interaction contributes roughly equally to the free energy of binding. It follows from the exponential relationship of equilibrium constants to free energy that the binding constant should be an exponential function of the chain length. The roughly linear (i.e. less than exponential) relationship that we observed implies that, in the range of molecular weights we analyzed, there must be a decreasing energetic contribution from the binding of each additional SA group. The decrease in the effectiveness of additional SA groups can be explained in two ways: (i) a decrease in the accessibility of SA groups due to the larger conformational entropy of the longer chains; and (ii) geometric constraints that prevent some SA groups from reaching unoccupied SA-binding sites.

(35) For a listing of the solution properties of various polyacrylic acid derivatives in good solvents see: Kurata, M.; Stockmayer, W. H. *Adv. Polym. Sci.* **1963**, *3*, 196−312.

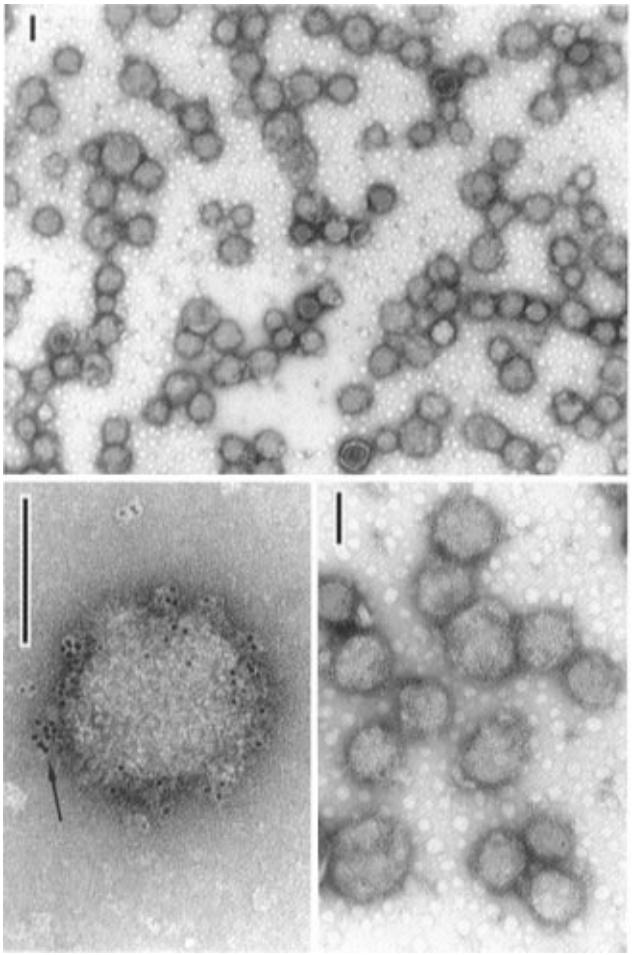


Figure 7. Transmission electron microscope (TEM) pictures showing the binding of pA(pre-P: Am, SA, BT) to virus under the conditions used for the ELISA sandwich assay. Polymer bound to the virus was labeled with streptavidin adsorbed on 5 nm diameter colloidal gold. The adsorbed complexes were stained with a uranyl acetate negative stain before TEM analysis. The picture shows a dense distribution of collioidal gold label (seen as dark black dots) on and around the virus particles. The arrow in the micrograph with the highest magnification shows one group of colloidal gold labels. The scale bars indicate 100 nm.

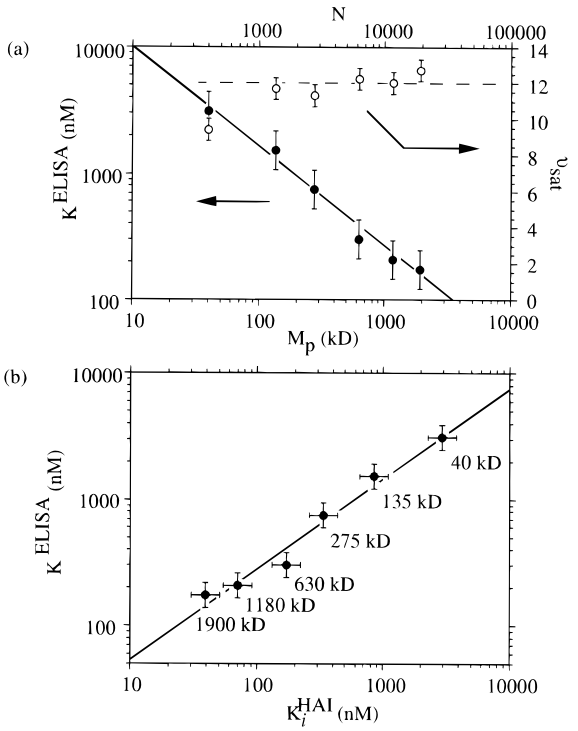


Figure 8. ELISA sandwich assay measuring the binding of narrow molecular weight fractions of pA(co-P: Am, SA, BT) to immobilized virus. (a) The constants K^{ELISA} and υ_{sat} (obtained by fitting binding isotherms to the Langmuir model described in the text) are plotted as a function of molecular weight and chain length, *N*. (b) K^{ELISA} and K_i^{HAI} are plotted for each polymer fraction. The molecular weight of each fraction is listed next to the corresponding symbol on the plot.

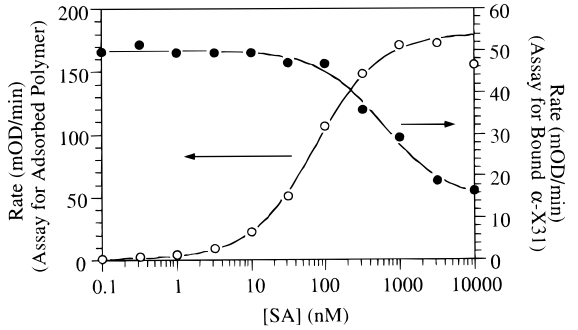


Figure 9. Inhibition of the binding of a polyclonal antibody (●) to influenza virus by adsorbed pA(pre-P: Am, SA, BT) (○) as a function of the concentration of polymer-linked SA groups in solution during adsorption of the polymer. The virus was immobilized on a fetuin-coated surface from a suspension containing 50 μg of viral protein/mL. Polymer was adsorbed on the immobilized virus and quantified by binding a streptavidin−alkaline phosphatase conjugate and measuring the enzymatic activity as described in Figure 5. To measure the binding of the polyclonal antibody, α-X31, the immobilized virus−polymer complex was treated first with α-X31. Bound antibody was quantified by binding a goat anti-rabbit Ig antibody linked to alkaline phosphatase and measuring the bound alkaline phosphatase activity.

Steric Stabilization of the Viral Surface by Adsorbed Polymer: Inhibition of Antibody Binding by Adsorbed Polymer. To determine whether adsorption of the polymeric inhibitors causes steric stabilization of the viral surface, we examined the effect of pA(pre-P: Am, SA, BT) on the binding of a rabbit polyclonal antibody generated by immunizing a rabbit with X-31 virus (α-X31). The polyclonal antibody should include individual antibodies that bind to a variety of epitopes over the entire surface of the virus. A significant inhibition of the binding of α-X31 to the viral surface by the polymeric inhibitor would indicate that the adsorbed polymer not only blocks the SA binding site but also creates an excluded volume around the virus that is large enough to interfere with binding events removed from the SA binding site.

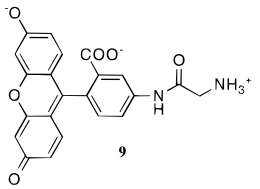
The format of the assay we used to measure the binding of α-X31 to influenza virus was similar to the ELISA assay we used for measuring the binding of the polymeric inhibitors. First, we treated viral particles immobilized on fetuin-coated microtiter plates with varying concentrations of a polymeric inhibitor. In contrast to the assay for the binding of polymer (in which bound polymer was quantified using a streptavidin−alkaline phosphate conjugate), we instead treated the immobilized virus−polymer complex sequentially with α-X31 followed by an alkaline phosphatase-labeled goat anti-rabbit Ig polyclonal antibody (to quantify the amount of α-X31 that bound).

Figure 9 compares the relative mass of polymer adsorbed on the virus (as determined by the polymer-binding assay) from solutions containing different concentrations of pA(pre-P: Am, SA, BT) with the relative mass of α-X31 that bound in the presence of the adsorbed polymer (as determined by the antibody-binding assay). The presence of adsorbed polymer decreased the binding of α-X31 by as much as 64%. This inhibition of α-X31 binding was not solely due to the blocking

of antibodies directed at the SA binding site; the presence of the SA derivative **2** ($K_d = 3\ \mu$M) at a concentration of 300 μM had no discernible effect on the binding of α-X31 (data not shown). These results shows that pA(pre-P: Am, SA, BT) is capable of steric stabilization of the viral surface. A threshold concentration of polymer on the surface of the virus was necessary for steric stabilization to occur; no inhibition was observed until the concentration of polymer on the surface was approximately half of the value at saturation. The similarity of the threshold values of adsorbed polymer that were necessary for both steric stabilization against antibody binding and inhibition of hemagglutination (also approximately half of saturation) suggests that steric stabilization may have a role in the inhibition of hemagglutination.

We note that even in the presence of saturating concentrations of polymer, the binding of α-X31 was not completely inhibited. Although we have not investigated further this residual binding, we propose two possible explanations: (i) incomplete coverage of the virus by the polymer or (ii) penetration of the antibodies through the polymer layer. In both cases, larger structures, such as red blood cells, would be expected to be more strongly influenced by the steric stabilization than would the relatively small antibodies.

Measuring the Stochiometry of the Binding of the Polymeric Inhibitors to the Surface of Influenza Virus: Binding Assays Using Fluorescein Labeled Polymers. To understand better the requirements for both polyvalent binding of the polymeric inhibitors to influenza virus and stabilization of the viral surface, we needed a technique for measuring the amount of polymer adsorbed onto the surface of the virus. A disadvantage of the ELISA assay for measuring the binding of polymeric inhibitors to the virus is that the assay determines only the *relative* mass of bound polymer and, therefore, cannot be used to calculate the stochiometry of binding. We developed an alternative method for determining the *absolute* mass of polymer bound to the virus. A fluorescein-labeled SA-containing polymer was bound to virus under conditions predicted to lead to the maximum amount of polymer bound to the viral surface (as determined by ELISA assay using a comparable biotin-labeled polymer). Free and bound polymer were separated by centrifugation of the virus. The amount of bound polymer was determined by comparing the fluorescence of the supernatant to a control solution that was not treated with virus. The polymer used in these experiments—pA(pre-P: Am, SA, F), where F stands for fluorescein—was prepared by the same procedure as pA(pre-P: Am, SA, BT) except the biotin-containing amine **8** was replaced with the fluorescein-containing amine **9** at a concentration calculated to give a polymer containing a proportion of monomer units linked to fluorescein, $\chi_F = 0.005$. Figure 10 shows that the fraction of a solution of pA(pre-P; Am, SA, F) (present at a total concentration of polymer-linked SA groups, [SA] = 1 μM) that adsorbed on a suspension of virus was linear with the concentration of virus up to 16 μg of viral protein/mL.



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The slope of this line (50 μmol of polymer-linked SA groups/g of viral protein) is the ratio of bound polymer to virus. The average molecular weight of the combined viral proteins

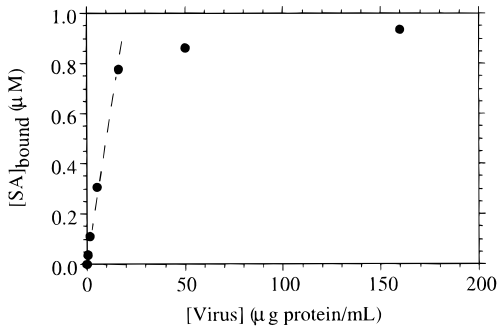

Figure 10. Titration of a solution of the fluorescein-labeled polymer pA(pre-P: Am, SA, F) with virus in suspension. The concentration of polymer-linked SA groups in solution is [SA] = 1.0 μM. The polymer solutions were incubated with the virus for 30 min at 4 °C. The bound polymer was then removed by centrifugation of the virus. The percentage of polymer bound to virus was determined by comparison of the fluorescence of the supernatant to a control run in the absence of virus. The concentration of polymer chains adsorbed on virus particles is given in terms of the concentration of polymer-linked SA groups.

in one influenza virus has been determined (using a variety of experimental techniques) to be roughly 2×10^8 g of protein/mol of viral particles.[36−38] Using this molecular weight, the ratio of bound polymer to virus is given by the following expression:

$$[\text{polymer}]_{\text{bound}}/[\text{virus}] = ([\text{SA}]_{\text{bound}}\ (\text{M})/[\text{virus}]\ (\text{g}))\text{MW}_{\text{virus}}$$
$$\approx (50 \times 10^{-6}\ \text{M/g})(2 \times 10^8\ \text{g/mol})$$
$$\approx 10\,000\ \text{polymer-linked SA groups per viral particle}$$

The mole fraction of SA-substituted monomer units in the polymer backbone is $\chi_{SA} = 0.17$. The average chain length (N_{av}) of polymers derived from pNAS is equal to the average molecular weight determined by GFC for acid-hydrolyzed pNAS (Table 1) divided by the molecular weight of acrylic acid: N_{av} = 70000 D/71 D ≈ 1000 monomer units per polymer chain. Using these constants, the ratio of bound polymer to virus can be rearranged to give the number of bound polymer chains per viral particle:

$$[\text{polymer}]_{\text{bound}}/[\text{virus}] =$$
$$(10\,000\ \text{SA groups/virion})/(\chi_{\text{SA}}N_{\text{av}})$$
$$\approx 60\ \text{polymer chains per viral particle}$$

The average radius of the viral particles is $R_v = 60$ nm.[37] The average molecular weight of the polymer with the SA groups attached is 140 kD. Using these values, a surface concentration of polymer on the virus can be calculated:

$$[\text{polymer}]_{\text{bound}}/\text{area}_{\text{virus}} =$$
$$\{60\ \text{chains} \times 140\ \text{kD}/(6 \times 10^{23})\}/(4\pi R_v^2) \approx 0.3\ \text{ng/mm}^2$$

These values are all semiquantitative but they provide a useful picture of the interaction of the polymeric inhibitors with the virus.

Discussion

From the stochiometry of binding, we can determine the conditions required for inhibition of hemagglutination. Assum-

(36) Cusack, S.; Ruigrok, R. W. H.; Krygsman, P. C. J.; Mellema, J. E. *J. Mol. Biol.* **1985**, *186*, 565−582.
(37) Ruigrok, R. W. H.; Andree, P. J.; Hooft Van Huysduynen, R. A. M.; Mellema, J. E. *J. Gen. Virol.* **1984**, *65*, 799−802.
(38) Reimer, C. B.; Baker, R. S.; Newlin, J. E.; Havens, M. C. *Science* **1966**, *152*, 1379−1381.

ing that the inhibition of hemagglutination required a surface concentration of polymer at least equal to half of the maximum value ($K_i^{HAI} \approx K^{ELISA}$), the minimum surface concentration on the virus of pA(pre-P: Am, SA), $\chi_{SA} = 0.17$, required to prevent hemagglutination was approximately 5000 polymer-linked SA groups per viral particle or 0.15 ng/mm^2. This value is approximately the same concentration of polymer on the surface that was required to cause significant inhibition of the binding of a polyclonal antibody against the virus. The number of polymer chains per viral particle that were required to inhibit hemagglutination is remarkably small: For a polymer with a chain length of 1000 monomer units (molecular weight = 140 kD), only 30 polymer chains per viral particle were required for inhibition. Since the weight of adsorbed polymer required for inhibition was roughly independent of chain length, polymers with molecular weights >1000 kD needed to bind <3 polymer chains per viral particle to inhibit hemagglutination.

The measured stochiometry can be used to determine the sensitivity limits of the HAI assay. The typical concentration of virus used in the HAI assay is 50−100 μg of viral protein/L. The required concentration of bound SA required to inhibit this suspension (1−2 nM) sets a lower limit of 1−2 nM on the values of K_i^{HAI} that can be measured using this assay. The measured values of K_i^{HAI} for the pre-P class of polymeric inhibitors approach this lower limit; the real values may be lower.

The fact that adsorbed polymers inhibited the binding of a polyclonal antibody to the viral surface is evidence that the polymers were capable of steric stabilization of the viral particles. Adsorbed or grafted polyacrylamide has been shown, in other systems, to stabilize a variety of colloids in water.[39] The required surface concentration of polyacrylamide required for stabilization, however, has not been carefully measured in a manner that allows for comparison between systems. Some data are available for other polymers. The stabilization of AgI sol in water by adsorbed poly(vinyl alcohol) requires a minimum surface concentration of polymer of 0.4 ng/mm^2.[40] More typically, stabilized colloids are prepared under conditions that result in relatively high concentrations of polymer on the surface (> 1 ng/mm^2).[41] The surface concentrations we observed for our inhibitors (∼ 0.3 ng/mm^2) were, therefore, slightly low compared to these other systems, but close enough to previously observed values, albeit in systems using different polymers, to be consistent with the hypothesis that steric stabilization of the viral surface was important in the phenomenon we were examining (especially considering the semiquantitative nature of our calculations of stochiometry).[42] The relatively low amounts of polymer that adsorbed on the viral surface may explain why we observed only partial inhibition of the binding of antibodies against influenza virus.

The surface of each viral particle contains on average 500 HA trimers (i.e. 1500 SA-binding sites).[37,43] At a surface concentration of 5000 polymer-linked SA groups per viral particle, there are sufficient SA groups available to block every SA-binding site. If all SA binding sites are, indeed, occupied, then steric stabilization of the virus by the polymer may be irrelevant. Despite the large excess of polymer-linked SA groups on the surface, however, the percentage of SA-binding sites that are occupied is probably low due to geometric constraints: the average distance between adjacent SA groups on the polymer chain (∼30 Å for the pre-P polymers) is too small to bridge the HA binding pockets (the intramolecular distance between SA binding sites on an HA trimer is 46 Å, the range is distances between HA trimers on the fluid viral surface is 50−115 Å),[18] and the binding of some SA groups may be prevented by inter- or intra-chain interactions. A direct measure of the occupancy of SA-binding sites would clarify further the importance of steric stabilization; there is, however, no good assay presently available for measuring this value.

Conclusions

SA-containing polyacrylamides are significantly better than monovalent SA at preventing the agglutination of red blood cells by influenza virus. Using an ELISA assay for the binding of polymer to virus, we showed that the differences in inhibition constants (K_i^{HAI}) between monomeric and polymeric inhibitors, measured on the basis of total SA groups in solution, correlated with differences in the binding affinities of the inhibitors for the viral surface. Similarly, we showed that differences in the affinity of binding also explained the differences in inhibitory efficacy of polymeric inhibitors prepared by two different methods (pre-P vs co-P) as well as the differences in inhibitory efficacy of polymer fractions differing in molecular weight. These results confirm the importance of enhanced binding through polyvalent interactions in the efficacy of the polymeric inhibitors. We expect that the synthesis of polymers bearing other weak-binding ligands will provide a general route to high-affinity polyvalent ligands for a variety of receptors on the surfaces of viruses or cells.

We note that large enhancements in binding and inhibitory efficacy were observed even for fractions of polymers with relatively low molecular weights. For polymers prepared by the pre-P method, a fraction containing just 150 monomer units (corresponding to ∼30 SA groups) both bound and inhibited at concentrations of polymer-bound SA groups <15 nM (>5 orders of magnitude better than the monomer). The fact that a relatively small number of SA groups is adequate to provide such large enhancements in binding is important in suggesting the design of new tight-binding inhibitors. In principle, it may be possible to construct polyvalent inhibitors with better controlled structure than the polyacrylamide inhibitors—perhaps based on dendrimers or modified cyclodextrins—with similar or better binding affinities. The detailed geometrical requirements imposed on the SA groups to achieve large binding enhancements are not, however, currently understood.

While enhanced affinity through polyvalent binding is clearly important in the high potency of the polymeric inhibitors for inhibiting hemagglutination by influenza virus, we as yet do not have a complete picture of the mechanism of inhibition. The fact that the polymers inhibit the binding of antibodies to the surface of the virus is strong indirect evidence that steric stabilization plays a role in the inhibition of virus−cell interactions. Other recent studies have provided additional indirect evidence for steric stabilization: (i) structure−activity relationships determined for polymeric inhibitors with modified side chains are consistent with a mechanism involving steric stabilization,[19] and (ii) high-affinity monomeric inhibitors of neuraminidase (NA), a hydrolytic enzyme present on the surface of influenza virus, enhance the efficacy of SA-containing

(39) Evans, R.; Davison, J. B.; Napper, D. H. *Polym. Lett.* **1972**, *10*, 449−453. Furusawa, K.; Tezuka, Y.; Watanabe, N. *J. Colloid Interface Sci.* **1980**, *73*, 21−26. Nowicki, W.; Nowicka, G. *Materials Sci. Forum* **1988**, *25*−26, 497−500. Tsubokawa, N.; Maruyama, K.; Sone, Y.; Shimomura, M. *Polym. J.* **1989**, *21*, 475−481.

(40) Fleer, G. J.; Koopal, L. K.; Lyklema, J. *Kolloid-Z. Z. Polym.* **1972**, *250*, 689−702.

(41) Klein, J.; Luckham, P. *Nature* **1982**, *300*, 429−431.

(42) Theoretical calculations predict that steric stabilization may be possible at surface concentrations of polymer as low as 0.2 ng/mL, but comparisons to experimental systems are not straightforward. See: Hesselink, F. Th.; Vrij, A.; Overbeek, J. Th. G. *J. Phys. Chem.* ***1971***, 75, *2094.*

(43) Skehel, J. J.; Schild, G. C. *Virology* **1971**, *44*, 396−408.

polymers in inhibiting hemagglutination.[44] This second observation, which is especially pronounced in polymers containing high proportions of SA, suggests that SA groups on the polymers bind the NA active site as well as the HA-binding pocket. The enhancement of inhibition by the polymers in the presence of NA inhibitors is probably due to expansion of the adsorbed polymer layer after the competitive release of SA groups from the NA active sites.[44] Proof for steric stabilization as an important factor would require showing that polymeric inhibitors can prevent hemagglutination without occupying a large percentage of the SA-binding sites on the protein. We are currently investigating this issue by testing the inhibitory efficacy of polymeric inhibitors targeted specifically at NA, a protein that does not play a role in virus−cell attachment.

Experimental Section

Materials. The SA-linked acrylamide monomer **4** was prepared according to Lees et al.[18] The amine **6** and pNAS were prepared according to Mammen et al.[19] The amine-containing derivative of fluorescein **9** was purchased from Molecular Probes, chicken red blood cells from 2 week old chicks were purchased from Spafas, and fetuin (from fetal calf serum) was purchased from Sigma. The alkaline phophatase-linked streptavidin and goat anti-rabbit Ig conjugates used in the ELISA sandwich assay were purchased as solutions from Fisher Scientific. Streptavidin adsorbed on colloidal gold (particle diameter = 5 nm) was purchased from Sigma. Influenza virus X-31 and the rabbit polyclonal antibody against X-31 (α-X31) were obtained from Prof. J. J. Skehel (National Institute for Medical Research, London). Phosphate buffered saline (PBS) is 137 mM NaCl, 3 mM KCl, 8 mM Na_2HPO_4, and 2 mM KH_2PO_4, adjusted to pH 7.2. BSA-PBS is 1.0 mg/mL bovine serum albumin in PBS. Polymer-linked BT groups and *O*-glycosides of SA were assayed by colorimetric assays.[30,32] Hemagglutination inhibition (HAI) assays were conducted as previously described.[18,20] Flash chromatography was performed using silica gel 60 (230−400 mesh, E. Merck). Melting points are reported without correction.

***N*-Boc-*N′*-biotinyl-3,6-dioxaoctane-1,8-diamine (10).** Tris(ethylene glycol)-1,8-diamine (6.6 g, 45 mmol) and 12 N HCl (4.6 mL, 55 mmol) were added to 60 mL of 1:1 water/dioxane and cooled to 0 °C. While the solution was stirred, 2-(*tert*-butoxycarbonyloxyimino)-2-phenylacetonitrile (BOC-ON, 3.7 g, 15 mmol) was added and the solution was allowed to warm to room temperature over 90 min. The solution was concentrated to an oil, suspended in 30 mL of water, and acidified to pH 4.0 with 6 N HCl. The aqueous solution was washed 3 times with 30-mL portions of ethyl acetate, adjusted to pH 11.0 with 10 N NaOH, and then extracted 5 times with ethyl acetate. The combined ethyl acetate fractions were dried over magnesium sulfate, then evaporated under reduced pressure to give 2.0 g (55%) of the crude mono-Boc protected diamine.

The crude mono-Boc protected diamine (1.0 g, 4.1 mmol) and 4.1 mL of 1 N HCl were combined in a mixture of 40 mL of 250 mM $NaHCO_3$ and 40 mL of dimethoxyethane. The *N*-hydroxysuccinimide ester of biotin[45] (1.32 g, 3.9 mmol) was added as a solid and the solution was stirred overnight at room temperature. Water (100 mL) was added to the solution and the product extracted six times into 100-mL portions of ethyl acetate. The combined extracts were washed with a saturated solution of NaCl, dried over magnesium sulfate, and concentrated to a white solid. Purification by flash chromatography using 9:1 methylene chloride/methanol as the eluent gave 1.3 g (71%) of the product as a white solid, yield 71%, mp 101.5−102.5 °C. ^{1}H-NMR δ (DMSO-d_6): 7.83 (t, 1H), 6.77 (t, 1H), 6.42 (s, 1H), 6.36 (s, 1H), 4.29 (dd, 1H), 4.12 (t, 1H), 3.48 (s, 4H), 3.36 (q, 4H), 3.16 (q, 2H), 3.08 (m, 3H), 2.80 (dd, 1H), 2.55 (d, 1H), 2.05 (t, 2H), 1.59 (m, 1H), 1.46 (m, 3H), 1.36 (s, 9H), 1.28 (m, 2H). HRMS-FAB $[M + H]^+$ calcd for $C_{21}H_{38}N_4O_6S$ 475.259, found 475.257.

***N*-Biotinyl-3,6-dioxaoctane-1,9-diamine (8).** The Boc-protected amine **10** was dissolved in 10 mL of trifluoracetic acid (TFA) per gram of protected amine and stirred for 20 min at room temperature. The TFA was evaporated under reduced pressure to give an oil. The oil was dissolved in water and reconcentrated to remove residual TFA leaving the product as the TFA salt. The oil was used without further purification. ^{1}H-NMR δ (D_2O): 4.31 (dd, 1H), 4.11 (dd, 1H), 3.45 (t, 2H), 3.39 (s, 4H), 3.32 (t, 2H), 3.09 (t, 1H), 3.02 (m, 1H), 2.90 (t, 2H), 2.70 (dd, 1H), 2.47 (d, 1H), 1.97 (t, 2H), 1.3−1.5 (m, 4H), 1.11 (m, 2H).

***N*-Acryloyl-*N′*-biotinyl-3,6-dioxaoctane-1,9-diamine (7).** The amine **8**, prepared as described above from 0.71 g (1.5 mmol) of the BOC-protected amine **10**, was dissolved in 50 mL of 250 mM $NaHCO_3$. *N*-Acryloylsuccinimide[46] was added dropwise with stirring in 10 mL of dimethoxyethane. The solution was stirred for 3 h at 4 °C in the dark. Unreacted starting materials and buffer salts were removed by adding 40 g of Amberlite mixed bed ion-exchange resin. The supernatant was lyophilized to give a crude product, which was then applied to a silica column and eluted with 4:1 methylene chloride/methanol to give 0.34 g of the product as a white solid, yield 52%, mp 123−125 °C. ^{1}H-NMR δ (DMSO-d_6): 8.20 (t, 1H), 7.85 (t, 1H), 6.43 (s, 1H), 6.37 (s, 1H), 6.23 (dd, 1H), 6.06 (d, 1H), 5.55 (d, 1H), 4.28 (dd, 1H), 4.11 (m, 1H), 3.49 (s, 4H), 3.43 (t, 2H), 3.37 (t, 2H), 3.27 (q, 2H), 3.16 (q, 2H), 3.07 (m, 1H), 2.80 (dd, 1H), 2.56 (d, 1H), 2.05 (t, 2H), 1.59 (m, 1H), 1.47 (m, 3H), 1.28 (m, 2H). HRMS-FAB $[M + H]^+$ calcd for $C_{19}H_{32}N_4O_5S$ 429.217, found 429.215.

Synthesis of SA-Containing Polymers by Copolymerization. The copolymer pA(co-P: Am, SA) was prepared as described previously.[18] Stock solutions of acrylamide (2.2 M), SA-linked acrylamide **4** (0.22 M), and azobis(cyanovaleric acid) (100 mM adjusted to pH 7.2 with 10 N NaOH) in deoxygenated water were combined to give a solution containing 1 M acrylamide, 0.1 M **4**, and 10 mM azobis(cyanovaleric acid). Irradiation with a long-wave UV lamp (Spectronics Corp.) gave a polymer assumed to contain a 10:1 ratio of unsubstituted acrylamide units to sialic acid linked units ($\chi_{SA} = 0.09$). The copolymer pA(co-P: Am, SA, BT) was prepared by the same method except that the polymerization solution also contained 10 mM biotin-linked acrylamide **7**, and resulted in a polymer assumed to contain a ratio of 100:10:1 of unsubstituted acrylamide units, sialic acid-linked acrylamide units, and biotin-linked acrylamide units, respectively ($\chi_{SA} = 0.09$, $\chi_{BT} = 0.01$).

Synthesis of SA-Containing Polymers by Modification of a Prepolymerized Chain.[19] A solution of SA-containing amine **6** (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 room temperature for 20 h, heated at 65 °C for 6 h, then stirred at room temperature for an additional 48 h. This procedure yielded a stock solution of preactivated polymer with $\chi_{SA} \approx 0.20$ (the stock solution contained 2.0 μmol SA/mL and 8.0 μmol NHS/mL).

(i) The polymer pA(pre-P: Am, SA) was prepared by adding the stock solution (600 μL, 4.8 μmol NHS) dropwise to NH_4OH (concentrated aqueous, 1.5 mL), then stirring at room temperature for 12 h and dialyzing against PBS.

(ii) The polymers pA(pre-P: Am, SA, BT) ($\chi_{BT} = 0.01$) and pA(pre-P: AM, SA, F) ($\chi_F = 0.005$) were prepared by combining the stock solution (115 μL, 1 μmol NHS) with the biotin-containing amine, **8** (0.01 μmol in 25 μL of DMF), or the fluorescein-containing amine, **9** (0.005 μmol in 25 μL of DMF) and heating for 6 h at 65 °C. The solutions were added dropwise to NH_4OH (concentrated aqueous, 1.5 mL), stirred at room temperature for 12 h, and dialyzed against PBS.

Gel-Filtration Chromatography. For determination of the molecular weight distribution of acrylamide polymers by GFC, the polymer was first hydrolyzed in acid to acrylic acid. This procedure results in the hydrolysis of >95% of the amide groups in polyacrylamide.[27] A

(44) (a) Choi, S.-K.; Mammen, M.; Whitesides, G. M. In press (b) All polymers made using *C*-glycosides gave similar values of K_i^{HAI} to those made using *O*-glycosides when assayed at 4 °C. At room temperature, however (19 °C), we see evidence that the hydrolysis of the *O*-glycosidic linkages becomes more significant: for *O*-glycosides, the values K_i^{HAI} measured at 19 °C were 10 times higher (worse) than that measured at 4 °C. We conclude that while the hydrolytic activity of NA may be significant at room temperature, it is not significant at 4 °C.

(45) Becker, J. M.; Wilchek, M.; Katchalski, E. *Proc. Natl. Acad. Sci. U. S. A.* **1971**, *68*, 2604−2607.

(46) Pollack, A.; Blumenfeld, H.; Wax, M.; Baughn, R. L.; Whitesides, G. M. *J. Am. Chem. Soc.* **1980**, *102*, 6324−6336.

solution was prepared containing the polymer at a concentration of 1 mg/mL in 300 μL of 10% (w/v) thioglycolic acid/6 N HCl (in the absence of thioglycolic acid, the hydrolysis of sugar-containing polymers resulted in the formation of an insoluble tar). The solution was sealed in a glass tube under nitrogen and heated to 105 °C for 24 h. Initially a precipitate forms due to formation of cyclic imides. All the material, however, redissolves as the reaction goes to completion. The hydrolysis mixture was quenched by the addition of 300 μL of 5 N NaOH, 200 μL of 1 M Na_2HPO_4, and 700 μL of water. The resulting solution was dialyzed extensively against 10 mM sodium phosphate, 150 mM sodium sulfate, pH 7.2.

GFC analysis was conducted using a 7.8 × 300 mm column containing a linear gradient of different pore size polyacrylamide beads (Ultrahydrogel Linear, Waters Chromatography). The flow rate of eluent (10 mM sodium phosphate, 150 mM sodium sulfate, pH 7.2) was 0.5 mL/min. The elution of polymer samples (20 μL sample injections) was monitored by the UV adsorption at 210 nm. The column was calibrated using the peak retention times (Mp $\sim$ $(M_nM_w)^{0.5}$) of polyacrylamide molecular weight standards (Polysciences).[28] Molecular weight distributions were calculated from the GFC traces.[28]

The preparation of narrow molecular fractions of the substituted polymers was carried out by GFC using similar conditions except that smaller columns with narrower distributions of pore sizes (GPC4000 or GPC300, Alltech Chromatography) were used to maximize throughput and minimize dilution. Unfractionated polymers (20 μL, ~2 mg/mL) were injected and 10 × 200 μL fractions were collected during elution of the polymer. For each sample, the GFC separation was repeated several times to obtain reasonable amounts of fractionated polymer.

ELISA Sandwich Assay for Measuring the Relative Binding of Biotin-Labeled, SA-Containing Polymers to Influenza Virus. The wells of a 96-well ELISA plate (Pro-Bind, Falcon) were coated with fetuin by treatment with 50 μL of a solution containing 100 μg/mL of fetuin in PBS for 2 h at 4 °C. The wells were washed three times with 200-μL portions of BSA-PBS. The fetuin-coated wells were then consecutively treated with 100 μL of a suspension of virus in BSA-PBS for 1 h, 100 μL of the polymer sample in BSA-PBS for 30 min, and 100 μL of a solution containing the streptavidin−alkaline phosphatase conjugate (prepared by a 1:1000 dilution of the stock solution in BSA-PBS) for 30 min. Each well was washed 3 times with BSA-PBS between each binding reaction and 3 times with PBS after binding of the streptavidin−alkaline phosphatase conjugate. Alkaline phosphatase substrate (100 μL of a solution containing 1 mg/mL of *p*-nitrophenyl phosphate, 5 mM $MgCl_2$, 1.6 M diethanolamine, adjusted to pH 9.8 with 6N HCl) was added to each well and the rate of hydrolysis of *p*-nitrophenyl phosphate measured using a kinetic microplate reader (Biorad) with a 405-nm filter.

Transmission Electron Microscopy (TEM). A 50 Å thick carbon film supported on a copper grid was coated with fetuin by placing a drop containing a solution of fetuin in PBS on the surface of the film for 2 h. The surface was washed with 2 drops of BSA-PBS then treated sequentially with a drop of each of the following solutions: influenza virus (50 μg of protein/mL in BSA-PBS, 1 h), pA(pre-P: Am, SA, BT) ([SA] = 1 μM in BSA-PBS, 30 min), and streptavidin−colloidal gold ([streptavidin] = 50 μg/mL, 30 min). The surface was washed with 2 drops of BSA-PBS after each binding reaction and with 2 drops of water prior to staining. The adsorbed complex was negatively stained with uranyl acetate: a drop of an aqueous solution of uranyl acetate (25 mM) was placed on the surface of the substrate; most of the drop was removed by absorption onto a piece of filter paper; the remaining thin film of liquid was allowed to evaporate. The sample was analyzed on a Philips EM420 TEM using an accelerating voltage of 120 kV.

ELISA Sandwich Assay for Measuring the Inhibition by SA-Containing Polymers of the Binding of Antibodies to Influenza Virus. Solutions containing pA(pre-P: Am, SA, BT) were adsorbed onto viral particles immobilized on 96-well microtiter plates as described in the procedure for the ELISA assay for the binding of the polymeric inhibitors to influenza virus. Without removing the solution of the polymeric inhibitor, serum from a rabbit immunized against the X-31 virus (100 μL of a 1:10000 dilution in BSA-PBS) was added and allowed to bind for 10 min. The wells were washed with 3 × 200 μL portions of BSA-PBS and then treated for 30 min with a goat anti-rabbit Ig antibody linked to alkaline phosphatase (100 μL of a 1:2000 dilution of the stock solution in BSA-PBS). The wells were washed with PBS and the alkaline phosphatase measured as described for the previous ELISA assay.

Fluorescence Assay for Measuring the Binding of Fluorescein-Labeled, SA-Containing Polymers to Influenza Virus. Solutions containing pAA(co-P; Am, SA, F) and virus in PBS were incubated 30 min at 4 °C. Adsorbed and free polymer were then seperated by centrifugation of the virus at 100000 × g (Airfuge Centrifuge, Beckman). For each polymer concentration tested, a control was also run in the absence of virus. The concentration of free polymer was determined by comparison of the fluorescence intensity (Ex = 489 nm, Em = 515 nm) of the supernatant of the test sample with that of the control.

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