Generation and *in Situ* Evaluation of Libraries of Poly(acrylic acid) Presenting Sialosides as Side Chains as Polyvalent Inhibitors of Influenza-Mediated Hemagglutination

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Abstract: This paper describes a simple, microscale method for generating and evaluating libraries of derivatives of poly(acrylic acid) (pAA) that present mixtures of side chains that influence their biological activity. The method is based on the one-step conversion of poly(acrylic anhydride) (pAAn) to linear polymers presenting multiple units of R on side chains, pAA(R): the polymers are obtained by ultrasonication of a suspension of pAAn and aqueous RNH_2 contained in a 250-μL well of a microtiter plate. Using this method, derivatives of pAA having *N*-acetylneuraminic acid (NeuAc-L-NH_2) as a side chain, pAA(NeuAc-L), were generated and assayed for ability to inhibit hemagglutination (HAI) of chicken erythrocytes by influenza virus A (X-31); the constant (K_i^{HAI}) describing this inhibition is calculated on the basis of the concentration of NeuAc groups in solution, rather than the concentration of polymer molecules. Co-polymeric pAA(NeuAc-L_n; L_n = different linking groups) with a range of mole fractions of NeuAc-L-NH_2 ($\chi^{NeuAc-L} = 0.02-0.11$) exhibited HAI activities with K_i^{HAI} values between 27 and 0.30 μM. Using combinations of NeuAc-L-NH_2 and one of 26 different primary amines RNH_2, a variety of ter-polymeric pAA(NeuAc-L; R) ($\chi^{NeuAc-L} \sim 0.05$; $\chi^{R} \sim 0.06$) were also generated and assayed. Certain ter-polymers yielded values of K_i^{HAI} that were lower by a factor of $\sim 10^4$ than that of the parent co-polymeric pAA(NeuAc-L): the most active inhibitor was pAA(NeuAc-L; L-3-(2′-naphthyl)alanine)) ($K_i^{HAI} \approx 0.5$ nM). Typically, the incorporation of hydrophobic—especially aromatic—side chains enhanced activities. These polymers (pAA(NeuAc-L; R)) belong to a new class of polymeric, polyvalent sialosides that are potent inhibitors of the adsorption of influenza virus to erythrocytes. They were active with only low to moderate levels of incorporation of functional groups into the side chains: $\chi^{NeuAc-L} \sim 0.05$; $\chi^{R} \sim 0.06$.

Introduction

We describe a convenient microscale strategy that allows rapid generation and *in situ* biological evaluation of libraries of polymers and polymer sequences based on poly(acrylic acid) (pAA) that present a variety of functional groups as amide side chains.[1] The use of the word "library" here differs slightly from that common in combinatorial synthesis of small molecules: it refers to different sets of sequences and configurations along polymer backbones, rather than to different, uniquely defined chemical compounds. The method is based on the conversion of poly(acrylic anhydride) (pAAn) to derivatives of pAA by reaction with various amines RNH_2 in water. We prepare these derivatized polymers by ultrasonication of the reactants directly in the wells of a microtiter plate; we then assay them in the same plate with no further manipulation. We illustrate the application of this methodology by screening derivatives of pAA presenting multiple copies of *N*-acetylneuraminic acid (NeuAc or sialic acid)[2] as polyvalent inhibitors of the agglutination of erythrocytes induced by influenza virus A and by the discovery of new ter-polymers active in this assay at concentrations less of than 1 nM in sialic acid groups.

Influenza initiates infection by adsorption to the surface of mammalian cells through the multiple interactions of the viral protein hemagglutinin (HA) and clusters of *N*-acetylneuraminic acid (NeuAc; Figure 1) moieties expressed on the cellular surface.[3−5] HA is a major surface glycoprotein of the virus and exists as a homotrimeric complex (HA_3; MW $\sim$ 225 kDa): each subunit of the complex contains a binding site for NeuAc at its outermost portion.[6−8] The virus presents approximately 200−300 copies of HA_3 on the surface; this number constitutes $\sim$80−90% of the total protein on the surface. Influenza also presents another surface protein, neuraminidase (NA or sialidase), which is an enzyme capable of catalyzing the hydrolysis of the α-*O*-glycosidic linkage of NeuAc connected to a variety of aglycons on the cellular surface.[9,10] NA exists as a homotetrameric complex (NA_4; MW $\sim$ 240 kDa) on the surface of the virion ($\sim$20−40 copies of NA_4 per virion) and constitutes

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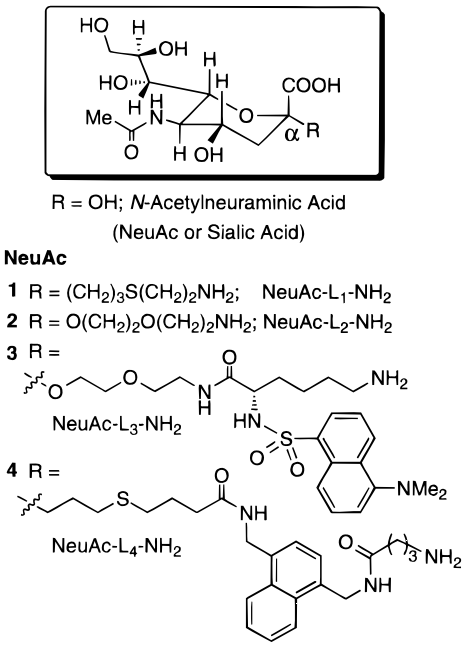

Figure 1. Structures of *N*-acetylneuraminic acid (NeuAc, or sialic acid), and synthetic α-*C*- and α-*O*-sialosides.

~10% of the total protein on the surface. This enzyme is usually considered to be unimportant in binding to target cells, but perhaps involved in allowing newly assembled virions to escape from the host cell and in helping the virus to escape from NeuAc-containing, protective proteins (such as α_2-macroglobulin[11])[11] in the mucosal secretions of the respiratory tract.

Monomeric sialosides are, in general, weak inhibitors of the adsorption of virus (as measured by the minimum concentration required to inhibit hemagglutination, $K_i^{HAI} \sim 10^{-3}$ M),[12,13] but multivalent sialosides that present multiple copies of sialosides tethered to liposomes,[14,15] dendrimers,[16] glycoproteins,[17−19] or polymers[17,20−24] can be highly effective inhibitors ($K_i^{HAI} \sim 10^{-5} - 10^{-11}$ M; expressed in terms of sialic acid groups). In particular, derivatives of poly(acrylamide) presenting NeuAc as side chains are now well established as potent inhibitors of hemagglutination.[20−24] These compounds are more effective than the *most* effective natural inhibitors (for example, equine α_2-macroglobulin) by factors up to ~10^3.[21] We have ascribed the high activities of these polyvalent sialosides to both a high-affinity binding of the polymers to multiple receptors on the virus and the ability of the adsorbed polymers to form gel layers that inhibit the approach of virus to the erythrocyte sterically (steric stabilization).[21−23] Practically, the effectiveness of polymers in inhibiting hemagglutination may be a function of multiple variables, including the structure and net charge of individual NeuAc moieties and non-sialoside side chains, the mole fraction of NeuAc-containing side chains in the polymer (χ^{NeuAc}), and the length and the flexibility of the polymer chains.[21−23]

Here, we describe a simple strategy that streamlines the generation of derivatives of pAA and the evaluation of biological activities of these polymers by carrying out both synthesis and assay in the wells of microtiter plates. The method allows convenient screening of libraries[25] of polymers and polymer sequences presenting multiple combinations of side chains at controlled mole fractions. Since microtiter plate assays are routine in biology and medical sciences,[26] this method may serve generally for screening and obtaining leads for a range of agglutination interactions and other processes that might be influenced by polyvalent inhibitors.[27]

Results and Discussion

Method of Generation of Libraries of Polymer. Figure 2 describes our strategy for rapid and efficient generation of libraries of potentially bioactive polymers. We synthesized poly(acrylic acid) having multiple R groups as side chains, pAA-(R), by sonicating a suspension (0.12 mg/μL) of poly(acrylic anhydride)[28,29] (pAAn) and an aqueous solution of an amine RNH_2 (0.1 M) contained in a 250-μL well of a microtiter plate (Figure 2). We characterized the resulting pAA(R) by examining ^{1}H-NMR (D_2O) spectra of lyophilized reaction mixtures as both a crude mixture and a purified form (following dialysis; MW cutoff of ~3.5 kDa). The ^{1}H-NMR signals of R from pAA(R) were distinguished readily from those of free, unreacted RNH_2 by their shape (the lines due to polymer-attached species are relatively broad) and by their chemical shift (the δ values of CH_2 or CH groups adjacent to the amide group are shifted downfield relative to those adjacent to the amine group). Molecular weights of purified pAA prepared using this method were estimated from gel permeation chromatography using polysaccharide standards: $M_w = 39.5$ kDa; polydispersity (M_w/M_n) = 1.91. We emphasize that our strategy is designed for rapid discovery of promising leads of bioactivity; once a polymer lead, pAA(R′), becomes known, one might synthesize the same pAA(R′) by use of other, more conventional methods of polymer synthesis. Further purification and fractionation may be necessary to yield an active polymer with a narrow molecular weight distribution.[30]

By comparing the integrated intensity of NMR signals of reacted RNH_2 (as RNH-CO− of the polymer) relative to that of unreacted free RNH_2, we estimated the yields (Figure 2) of incorporation of RNH_2. Figure 3 summarizes selected results with 4-aminobenzoic acid, 6-amino-1-hexanoic acid, and NeuAc-

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(30) The development of drugs based on non-biodegradable polymers is in its infancy, and the requirement for regulating clearance of such materials is not known.

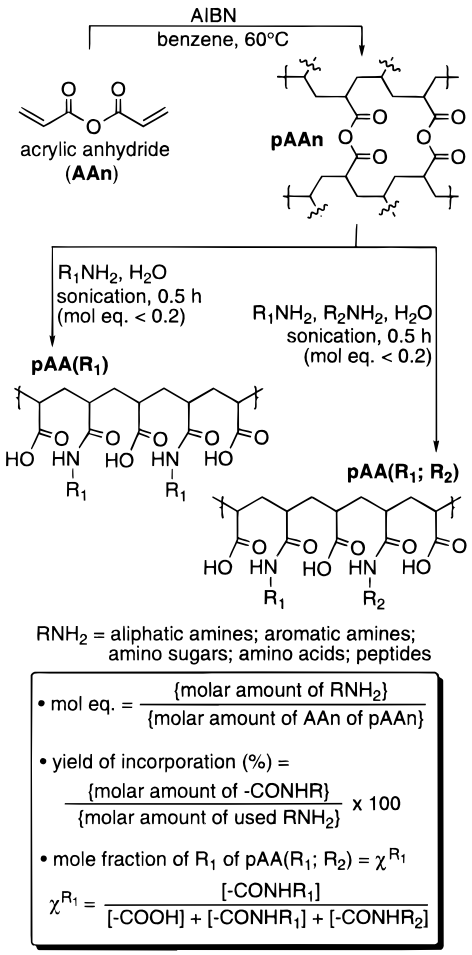

Figure 2. Quasi-solid-phase strategy for synthesis of derivatives of poly(acrylic acid), pAA. Co-polymer, poly(acrylic acid) presenting R_1 group as amide side chains, pAA(R_1), was generated in a well of a 96-microtiter plate by ultrasonicating a mixture of solid poly(acrylic anhydride) and an aqueous solution of a primary amine, R_1NH_2. Efficiency of the reaction was optimal when the ratio of molecular equivalents (mol equiv) of R_1NH_2 to anhydride groups of pAAn was <0.2, and the pH of the aqueous solution used was either ~7 (for aromatic amines) or ~12 (for aliphatic amines). Ter-polymers, pAA(R_1; R_2), were prepared similarly using an aqueous solution containing two amines: R_1NH_2 and R_2NH_2.

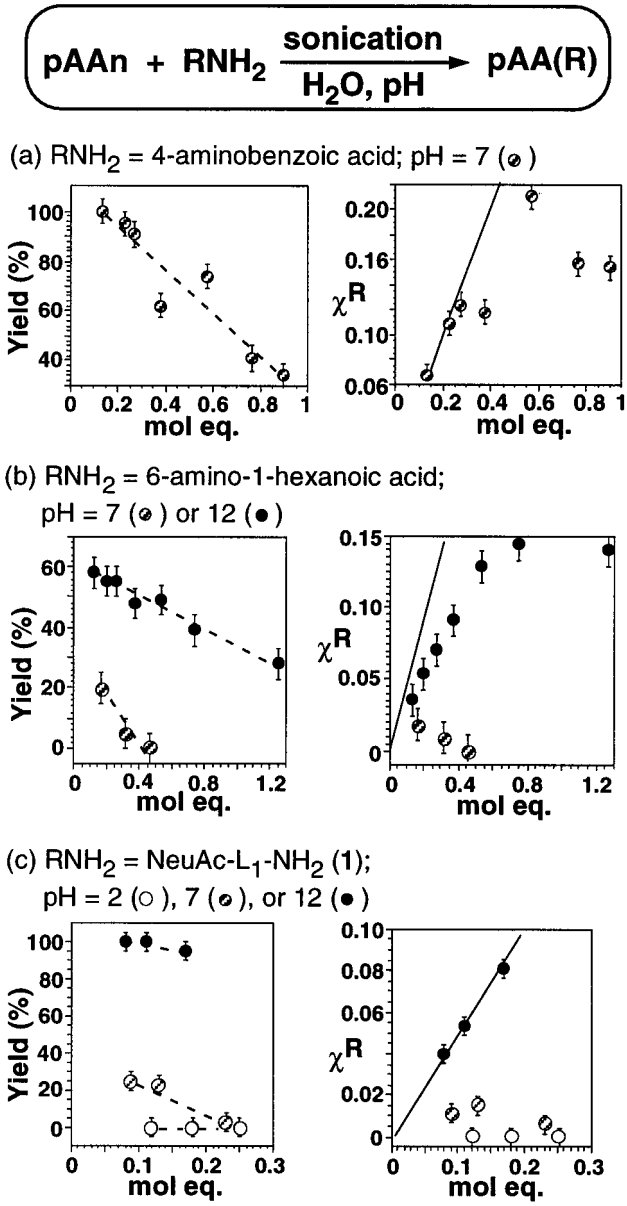

Figure 3. Dependence of the efficiency of the quasi-solid-phase reaction on mol equiv of reactants and pH of aqueous medium. The variables (yield, mol equiv, χ^R) are defined in Figure 2. (left) Plot of yields of incorporation (%) versus mol equiv of RNH_2 to anhydride groups of pAAn as a function of pH (2, 7, 12). (right) Plot of mole fraction of R (χ^R) of pAA(R) versus mol equiv of RNH_2 to anhydride groups of pAAn: this plot was derived from the previous plot (left), and the straight line is a theoretical reference line that represents quantitative reaction (where yield of incorporation is 100%). $\chi^R = 0.5$ (mol equiv of RNH_2 to anhydride groups of pAAn)(incorporation yield of RNH_2)/100.[36] (a) RNH_2 = 4-aminobenzoic acid. (b) RNH_2 = 6-amino-1-hexanoic acid. (c) RNH_2 = NeuAc-L_1-NH_2 (**1**).

L_1-NH_2 (**1**). Because amide formation and hydrolysis of anhydride groups were occurring competitively, the efficiency of the former was affected by the relative reactivity of each RNH_2 and was also sensitive to both the pH of the aqueous solutions of RNH_2 and the ratio of molecular equivalents of RNH_2 to anhydride groups of pAAn. The optimal pH for amide formation using the aqueous amine solutions was either ~7 (for aromatic amines) or ~12 (for aliphatic amines); these values are consistent with the values of pK_a for these groups. The efficiency of amide formation was greatest when the ratio of molecular equivalents (mol equiv) of RNH_2 to anhydride groups of pAAn was less than 0.2. The average value of the optimized yield of incorporation of RNH_2 groups into the polymer under these optimized conditions was ~90% (±5) from experiments using five different primary amines RNH_2 (4-aminobenzoic acid, 6-amino-1-hexanoic acid, *N*-methylhydroxylamine, L-arginine, and **1** (NeuAc-L_1-NH_2)).

It was straightforward to extend this method to generate libraries of ter-polymers, pAA(R_1; R_2), presenting both R_1 and R_2 groups (Figure 2). These materials were prepared simply by sonicating a three-component mixture of R_1NH_2, R_2NH_2, and pAAn as an aqueous suspension. Here, the reaction in each well produced a library of pAA(R_1; R_2) with R_1 and R_2 as amide side chains; we believe the sequence of R_1 and R_2 groups was approximately random. These polymers, pAA(R_1; R_2), are thus heterogeneous in terms of the sequence of side chains. Synthetic polymers prepared by other methods are probably also heterogeneous: direct polymerization (CH_2=$CHCO_2H$, CH_2=$CHCONHR_1$, CH_2=$CHCONHR_2$); modification of a preformed polymer (R_1NH_2, R_2NH_2, poly(*N*-(acryloyloxy)-succinimide) in DMF; then OH^-).

Throughout this work, we choose poly(acrylic anhydride), pAAn,[28,29] as the precursor polymer because it was easily available, formed amides by reaction with primary amines in

aqueous solution under conditions of quasi-solid-phase reaction, and could be converted rapidly to derivatives of pAA in good yield. We also applied this strategy to other precursor polymers (reactive to amines) such as poly(methyl vinyl ether-*co*-maleic anhydride), pMEMAn, and poly(*N*-(acryloyloxy)succinimide),[21] pNAS. When applied to pMEMAn, the procedures yielded polymers with acceptable solubilities in water and allowed us to generate poly(methyl vinyl ether-*co*-maleic acid) presenting the side chains of R as amide groups, pMEMA(R). The same quasi-solid-phase reaction with pNAS, however, did not proceed well when tested using either of two primary amines (4-aminobenzoic acid and 6-amino-1-hexanoic acid); the sonication left pNAS as a mostly unreacted, white suspension. In addition, the reaction with pNAS had the disadvantage that it generated a side product, *N*-hydroxysuccinimide (NAS), in reaction wells; this reaction product may interfere with some bioassays. Our strategy is, therefore, most convenient when used with anhydride-based, precursor polymers, which produce no interfering side products.

Generation and in Situ Bioassay of Co-polymeric Sialosides. We applied this quasi-solid-phase synthetic method to derivatives of NeuAc having different linking groups L (**1**−**4**; NeuAc-L_n-NH_2)[31−33] to generate co-polymeric derivatives of pAA presenting NeuAc-L as a side chain (pAA(**1**)−pAA(**4**)). Following sonication, the crude solutions of polymers were evaluated immediately for hemagglutination inhibition (HAI) activities using an assay based on chicken erythrocytes and influenza virus A (X-31).[34,35] Table 1 gives the values of K_i^{HAI} (the minimum concentration of NeuAc-L groups from pAA(NeuAc-L) in solution that prevents hemagglutination) at various mol equiv of NeuAc-L-NH_2 to anhydride groups of pAAn (for definition of mol equiv, see Figure 2). Here, we assume that the mol equiv of NeuAc-L-NH_2 to anhydride groups of pAAn is related directly to the mole fraction of NeuAc-containing side chains in the polymer ($\chi^{NeuAc-L}$); $\chi^{NeuAc-L}$ can be deduced from an equation including mol equiv and yield of incorporation.[36] The HAI activities of these crude pAA(**1**) (mol equiv of **1** to anhydride groups of pAAn = 0.1−0.2) are comparable to that of purified poly(acrylamide) presenting **1** ($K_i^{HAI} \sim 0.3\ \mu$M at $\chi^{\mathbf{1}} \sim 0.05$[21]). Table 1 also shows three other derivatives of pAA (pAA(**2**) − pAA(**4**)) with HAI activities in the (sub)micromolar range. We used **3** and **4** for incorporation into side chains of the polymer because aromatic moieties in the middle of the linkage have been previously shown to enhance the binding affinity of monomeric NeuAc-L to the HA site.[37] The HAI activities of all monomeric sialic acids (**1**−**4**) were, however, low, $K_i^{HAI} \geq 5$ mM. We rationalize the relative activities of these polymers using the structures of the monomers. For instance, pAA(**3**) was active at a 4-fold lower concentration than pAA(**2**); this increased activity is probably due to the increased hydrophobicity of **3** relative to **2**.

Table 1. Hemagglutination Inhibition Activities of pAA(NeuAc-L) and Libraries of pAA(NeuAc-L; R)[a]

polymer	mol equiv[b] of RNH_2	mol equiv[b] of NeuAc-L-NH_2	K_i^{HAI} (μM)[c]
pAA(**1**)		0	15000[d]
		0.04 (**1**)	27
		0.06 (**1**)	13
		0.08 (**1**)	3.9
		0.10 (**1**)	3.4
		0.11 (**1**)	4.4
		0.12 (**1**)	1.1
		0.14 (**1**)	1.1
		0.17 (**1**)	0.50
		0.21 (**1**)	0.30
pAA(**2**)		0.11 (**2**)	0.80
pAA(**3**)		0.11 (**3**)	0.20
pAA(**4**)		0.11 (**4**)	3.1
pAA(**1**; R)	0.12 (RNH_2)	0.10 (**1**)	
RNH_2			
3-aminobenzoic acid			1.5
3-amino-5-hydroxybenzoic acid			3.1
4-aminobenzoic acid			3.1
4-amino-2-hydroxybenzoic acid			3.1
4-aminobenzenesulfonic acid			1.5
2-aminonicotinic acid			1.5
N-methylhydroxylamine			1.5
D-2-amino-2-deoxyglucose			2.2
D-2-amino-2-dexoymannose			0.055
1-amino-1-cyclopropanecarboxylic acid			1.1
1-amino-1-cyclopentanecarboxylic acid			0.20
1-amino-1-cyclohexanecarboxylic acid			0.028
aminocyclohexane			0.0043
L-arginine			1.5
L-glutamate			2.5
L-histidine			1.5
D-4-hydroxyproline			1.5
DL-leucine			0.30
L-phenylalanine			0.024
L-4′-nitrophenylalanine			0.048
L-phenylalanine methyl ester			0.024
1-amino-2-phenylethane			0.0021
L-3-(2′-naphthyl)alanine			0.00050
L-tryptophan			0.0043
L-Gly-L-Gly-L-Gly			3.1
L-Gly-L-Phe			1.5
pAA(**3**; R)	0.13 (RNH_2)	0.11 (**3**)	
RNH_2			
1-amino-2-phenylethane			0.0015
L-3-(2′-naphthyl)alanine			0.00070

[a] The hemagglutination inhibition (HAI) assay was performed as described previously using erythrocytes from 2-week-old chickens and influenza virua A (strain X-31).[21,34,35] [b] Mol equiv refers to the ratio of molecular equivalents of RNH_2 (or NeuAc-L-NH_2) used to anhydride groups of pAAn in a reaction well. [c] Each reported value of K_i^{HAI} represents an average value from at least five independent trials; the experimental uncertainty in each value is approximately ±50%. [d] Refers to the lowest concentration of carboxylic acid groups from pAA in solution that prevented influenza-mediated hemagglutination of erythrocytes: this low HAI activity of underivatized pAA is our control.

Bioassay of Libraries of Ter-polymeric Sialosides. We extended this method to generate libraries of ter-polymers, pAA-(NeuAc-L; R), presenting both NeuAc-L and one other R group, simply by sonicating a three-component mixture of NeuAc-L-NH_2, RNH_2, and pAAn. Application to more than one RNH_2 group should also be possible. Table 1 summarizes the values of K_i^{HAI} of pAA(NeuAc-L; R), obtained from combination of NeuAc-L-NH_2 (mol equiv to anhydride groups of pAAn = 0.10) and one of 26 different RNH_2 (mol equiv to anhydride groups

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(36) The mole fraction of R-containing side chains in pAA(R) (χ^R), which is defined below, can be deduced from an equation including mol equiv and yield of incorporation (%):

$$\chi^R = \frac{[-\mathrm{CONHR}]}{[-\mathrm{COOH}] + [-\mathrm{CONHR}]} = \frac{\{\text{no. of moles of } -\mathrm{CONHR}\}}{2\{\text{no. of moles of anhydride groups of pAAn}\}}$$

χ^R = 0.5 (mol equiv of RNH_2 to anhydride groups of pAAn) × (incorporation yield (%) of RNH_2)/100.
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of pAAn = 0.12). We chose a variety of commercially available primary amines with combinations of functional groups: hydrophobic, hydrophilic, charged (negatively; positively), aromatic, aliphatic, amino sugars, amino acids, and peptides. The wide selection of the second R group was intended to suggest a structure−activity relationship for the various ter-polymers; the incorporation of R group influences the activity of the ter-polymer by affecting multiple factors such as conformation, flexibility, and solubility of the polymer as well as by displaying independently intrinsic biospecific activity of the R group.

Several pAA(**1**; R) (and pAA(**3**; R)) were 100 to ∼7000 more active than the parent co-polymeric pAA(**1**) (mol equiv of **1** (and **3**) to anhydride groups of pAAn = 0.11) in which there is no R group.[38] Although we measured the activities directly from crude pAA(NeuAc-L; R), several control experiments confirmed that these ter-polymers were indeed responsible for the high activities. Co-polymers that did not contain sialic acid groups, pAA(R), were inactive at ∼15 mM (except pAA(cyclohexyl) and pAA(L-phenylalanine methyl ester), which gave weak HAI activities K_i^{HAI}, ∼1.6 and ∼3.2 mM, respectively). The mixture of pAA(**1**) and RNH_2 gave the same result as that obtained with pAA(**1**) alone. Finally, the activities of pAA(**1**; R) (RNH_2 = L-3-(2′-naphthyl)alanine, L-tryptophan), either crude or purified (after dialysis; MW cutoff of ∼3.5 kDa), were the same within a factor of 2: the purified polymers were characterized by ^{1}H-NMR spectroscopy (D_2O).

Typically, the incorporation of derivatives of hydrophobic or aromatic amino acids enhanced the activities greatly. The results are related closely to the previous observation that the incorporation of benzylamine ($\chi^{benzyl} \sim 0.05$ or 0.1 as amide side chains) into poly(acrylamide) presenting **1** ($\chi^{\mathbf{1}} \sim 0.2$) increased HAI activities 2- to ∼4-fold.[21] In addition, the results suggest that there is some specificity in the ability of the R groups to increase the activity of pAA(**1**; R): (i) the incorporation of D-2-amino-2-deoxymannose as R group of pAA(**1**; R) gave an activity, 40-fold higher than that from D-2-amino-2-deoxyglucose; (ii) the HAI activities of pAA(**1**; R) (R = 1-amino-1-cycloalkanecarboxylic acid) are correlated to the ring size of cycloalkane groups (the value of K_i^{HAI} of pAA(**1**; 1-amino-1-cyclohexanecarboxylic acid) was less than that of pAA(**1**; 1-amino-1-cyclopentanecarboxylic acid) < that of pAA(**1**; 1-amino-1-cyclopropanecarboxylic acid). The present work thus demonstrates that specific structural features (and not hydrophobicity alone) may be responsible for some of the observed differences in values of K_i^{HAI}.

Mechanism of Inhibition of Viral Adhesion by Polymeric Polyvalent Sialosides. We previously showed that polyvalent presentation of NeuAc as a polymer greatly increased its ability to inhibit viral attachment to the surface of the erythrocyte (as measured by K_i^{HAI}) and that potent HAI activity might be explained by a combination of two proposed mechanisms: (i) polyvalent, affinity-enhanced binding of polymeric NeuAc to viral hemagglutinin (HA) and (ii) steric stabilization of viral surface by gel layer of adsorbed, water-swollen polymer (Figures 4 and 5).[21−23] Regarding the high activity of several of the new pAA(NeuAc; R) demonstrated here, we hypothesize that the non-sialoside groups (R) may be involved in (non)specific binding to hydrophobic sites on the surface of virus. One of the plausible sites for binding of the hydrophobic R group is nonfunctional, local hydrophobic clefts on the surface of the virus, and another is the viral M_2 protein. The M_2 protein is a membrane-bound proton-conducting channel,[39] and its surface density (∼20−60 copies per virion) is however small, ∼50- and 5-fold lower than those of HA and NA, respectively. Antiviral agents such as amantadine (1-aminoadamantane) and its structural analogue, rimantadine (Figure 4), interfere with replication of virus by binding to the M_2 site (by diffusing into the lumen of the channel and preventing H^+ flux across membrane).[40,41] We tested the above hypothesis (the possibility of involvement of hydrophobic surface sites of the virus in polyvalent attachment of pAA(NeuAc; R)) using poly(acrylamide) presenting derivatives of amantadine as an amide side chain, pA(Ad-**1**) to pA(Ad-**4**) ($\chi^{Ad} = 0.1$; Figure 4). The polymers were synthesized by reacting poly(*N*-(acryloyloxy)-succinimide) (pNAS) sequentially with 0.1 equiv of adamantane-**2** (and -**4**)-NH_2 to activated NHS ester of pNAS, and with excess ammonia. The values of K_i^{HAI} of purified pA(Ad-**2**) ($\chi = 0.1$) and pA(Ad-**4**) ($\chi = 0.1$) are ∼0.5 and ∼0.3 mM, respectively; here, K_i^{HAI} refers to the lowest concentration of adamantane groups from pA(Ad) in solution that prevented hemagglutination. The values of K_i^{HAI} of the polymers are lower at least ∼5−10-fold than those of corresponding monovalent derivatives of adamantane. We interpret the result by assuming that pA(Ad) attaches to the surface of virus by binding of adamantane ligand to hydrophobic sites (like M_2) and contributes to inhibiting the attachment of virus to erythrocyte, sterically (steric stabilization; Figure 5).

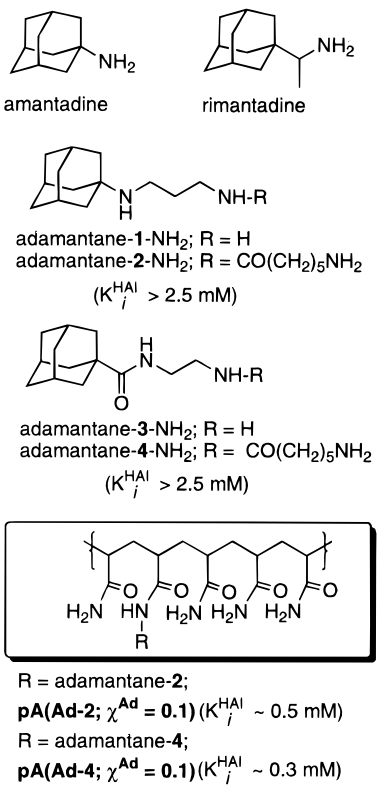

Figure 4. Structure of monovalent derivatives of adamantane and polymeric polyvalent adamantane. Amantadine (1-aminoadamantane) and its structural analogue, rimantadine, are used commercially as two of several agents effective against Influenza virus. These molecules bind to M_2 protein on the surface of Influenza virus and prevent H^+ flux through the proton-conducting channel formed by M_2.

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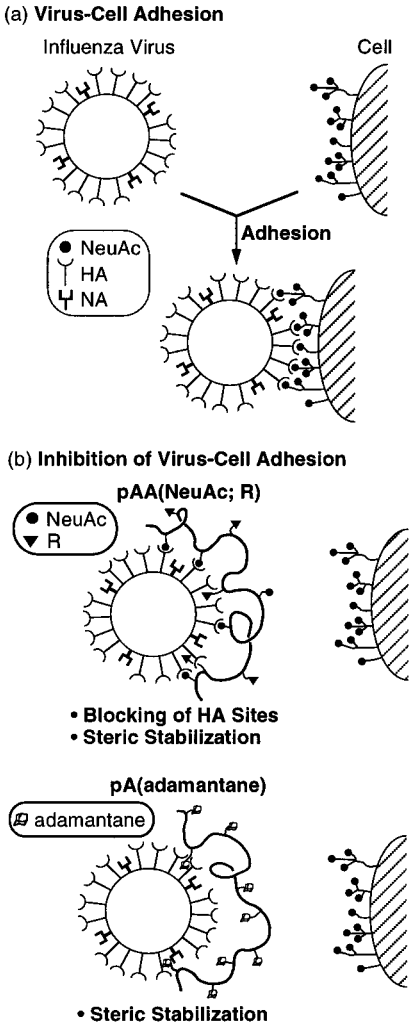

Figure 5. Scheme of influenza virus-cell association (a) and of the proposed mechanism of inhibition of the viral adhesion by polymeric polyvalent inhibitors (b). Influenza virus attaches tightly to the surface of cell using multivalent binding of its surface hemagglutinins to multiple copies of sialosides clustered on the surface of the target cell. Ter-polymers, pAA(NeuAc; R), are believed to inhibit the adsorption of influenza to the surface of erythrocyte by polyvalent binding of polymeric NeuAc to HA sites (affinity-enhanced, competitive binding) and by stabilizing the surface of virus with a layer of adsorbed, water-swollen polymer gel (steric stabilization). Certain polymers presenting non-sialoside groups as side chains, pA(adamantane), also inhibited agglutination of virus to cell, although the inhibitory effect was weak. These polymers might attach to the surface of virus by binding of non-sialoside ligands (adamantane) to non-HA sites of virus (such as M_2 site) or to adventitious hydrophobic sites, and inhibit the attachment of virus to cell sterically.

In proposing steric stabilization of influenza virus as the mechanism of action of pA(adamantane), we are suggesting attaching a polymer to the surface of virus by binding of non-NeuAc ligands to non-HA sites on the viral surface; these sites would normally be unimportant in adhesion of the virus to the cell. The substantial HAI activity of pA(adamantane), along with those of other co-polymers, pAA(cyclohexyl) and pAA(L-phenylalanine methyl ester), suggests that the polymer might inhibit virus-cell adhesion (HA-NeuAc mediated), sterically by attaching to non-HA sites on the surface of the virus. Furthermore, it implies that the potent activities of certain ter-polymeric sialosides may be due to binding of a second non-sialoside group to the viral surface (in addition to binding of NeuAc to HA) and thus to increased binding affinity of these ter-polymeric sialosides to the virus.

In summary, certain non-sialoside groups enhanced the activities of pAA(NeuAc-L; R) in hemagglutination inhibition by factors up to $\sim 10^4$, even though they showed no HAI activity by themselves. We suggest that the large increases in the potency of these ter-polymers relative to pAA(NeuAc) are partly due to binding of non-sialoside groups to non-HA sites and thus to enhancing the affinity of polymer for the viral surface. The results of assay with several co-polymeric poly(acrylamide) and poly(acrylic acid) presenting non-sialoside side chains suggest that steric stabilization may play a role in inhibiting adhesion of pathogen to cell and may be useful as a concept to designing new types of pharmaceuticals. The best of these pAA(NeuAc-L; R) belongs to a new class of hemagglutination inhibitors with unusually high activities at relatively modest mole fractions of NeuAc-L ($\chi \sim 0.05$) and R ($\chi \sim 0.06$): the value in mole fraction of NeuAc-L or R is equivalent to $\sim 30-40$ side chains of NeuAc-L or R per polymer molecule. This finding emphasizes the importance of combinations of side chains in modulating the activities of these polyvalent inhibitors.

Conclusion

In short, this procedure is a rapid, economical method for synthesizing and screening polyvalent, polymeric inhibitors for bioactivity. Particularly when minor structural modifications of side chains and/or polymer backbone can induce unexpectedly large changes in activities,[27] the present method may improve the efficiency of screening candidate compounds. We believe that this strategy should be broadly applicable to studies of polymeric modulators involved in a range of biological processes including pathogen-cell interaction,[42,43] tumor metastasis and immunomodulation,[44−46] and cell migration and adhesion.[46−49]

Experimental Section

General Procedure. Reagents were, unless otherwise specified, used as received. Microtiter plate (96 conically-shaped wells) equipped with cover plate was purchased from ICN, Flow. Ultrasonication was performed on ultrasonic bath-type cleaner (Fisher). Dialysis membranes (cellulose; MW cutoff of ~3.5, or 12−14 kDa) were purchased from Spectrum. Hemagglutination inhibition (HAI) assays were performed as described,[34,35] using Influenza virus (strain X-31) and chick red blood cells (RBCs, or erythrocytes). A suspension of erythrocytes from 2-week-old chicks was purchased from Spafas Co. Thin layer chromatography (TLC) was performed on silica gel precoated glass plates (E. Merck, Darmstadt). Flash column chromatography was performed on silica gel 60_{F254} (230−400 mesh, E. Merck).

Synthesis of Poly(acrylic anhydride).[28,29] A solution (120 mL) of anhydrous benzene containing acrylic anhydride[29] (11.8 g, 93.56 mmol) and AIBN (azobis(isobutyronitrile), 500 mg) was degassed for 5 min *in vacuo* and saturated with N_2 gas for 30 min. The solution was heated slowly to reflux under a stream of N_2 gas and allowed to reflux for 8 h. White precipitate was collected on a Büchner funnel, washed quickly with anhydrous benzene (~150 mL) and anhydrous THF (~100 mL), and immediately dried *in vacuo*. The polymerization reaction yielded 11.5 g of poly(acrylic anhydride), pAAn, as colorless solid. The molecular weight distribution of pAAn was determined after hydroly-

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sis: (i) pAAn was hydrolyzed to poly(acrylic acid), pAA, by ultrasonicating a mixture of pAAn and 0.1 M NaOH for 0.5 h; (ii) crude pAA was purified by dialysis (MW cutoff of ~3.5 kDa) against 0.1 M NH_4Cl and deionized water; (iii) molecular weights of purified pAA were estimated by gel permeation chromatography (GPC) using polysaccharide standards: M_n = 20.7 kDa, M_w = 39.5 kDa, polydispersity = M_w/M_n = 1.91. ^{1}H-NMR (300.1 MHz, purified pAA in D_2O): δ (ppm) 2.7−2.4 (br s), 2.4−2.1 (br s), 2.0−1.7 (br s), 1.7−1.5 (br s), 1.5−1.2 (br s).

Typical Procedure for Generation of pAA(R) Using Quasi-Solid-Phase Reaction (Figures 2 and 3). Solutions of co-polymers pAA-(R) were prepared by reacting of RNH_2 (4-aminobenzoic acid, 6-amino-1-hexanoic acid, or NeuAc-L_1-NH_2[30]) with poly(acrylic anhydride) (pAAn) using different numbers of molar equivalents of RNH_2 to anhydride groups of pAAn (mol equiv = {number of moles of NeuAc-L-NH_2}/{number of moles of anhydride groups of pAAn}) and using aqueous solutions of amines adjusted to different pHs (2, 7, or 12). The polymer for which the mol equiv is 0, is a homo-polymeric pAA obtained from sonication (hydrolysis) of pAAn alone in phosphate-buffered saline (PBS) solution (137 mM NaCl, 2.7 mM KCl, 7.7 mM Na_2HPO_4, 1.5 mM KH_2PO_4, 0.05% NaN_3), pH 12. Co-polymeric pAA-(R; RNH_2 = 4-aminobenzoic acid) for which the mol equiv is >0 was generated in microtiter plates with 96 conically-bottomed wells as follows: (i) placing an amount of pAAn powder (2.8, 3.3, 4.4, 6.7, 9.3, 11, 19 mg) into a well; (ii) soaking the powder with 100 μL of aqueous 4-aminobenzoic acid (0.2 M in PBS buffer, pH 7); (iii) immediately sealing the plate (taping four sides of a plate with Parafilm before placing a cover plate tightly), and then ultrasonicating (Fisher ultrasonic bath-type cleaner) the mixture for 0.5 h: the sonication also increased the temperature of water in the bath (and accordingly, the reactants), slowly up to ~50 °C. Each of the crude polymer solutions was neutralized with 1.0 M NaOH, lyophilized to dryness, and characterized by ^{1}H-NMR spectroscopy. The yield of incorporation was estimated by comparing the intensity of integrated signal of reacted RNH_2 (as RNH-CO− of the polymer) relative to that of free unreacted RNH_2. Crude pAA(R) (RNH_2 = 4-aminobenzoic acid; mol equiv of RNH_2 to anhydride groups of pAAn = 0.38; pH ~7.0): yield of incorporation = 62%. ^{1}H-NMR (300.1 MHz, D_2O): δ (ppm) 7.8−7.5 (br), 7.7−7.6 (d, J = 7.5 Hz; 2H from unreacted RNH_2), 7.5−6.9 (br), 6.7−6.6 (d, J = 7.5 Hz; 2H from unreacted RNH_2), 2.7−2.0 (br m), 1.9−1.3 (br m). Crude pAA(R) (RNH_2 = 6-amino-1-hexanoic acid; mol equiv of RNH_2 to anhydride groups of pAAn = 0.26; pH ~12): yield of incorporation = 55%. ^{1}H-NMR (300.1 MHz, D_2O): δ (ppm) 3.2−2.9 (br s), 2.9−2.8 (t, J = 7.0 Hz; 2H from unreacted RNH_2), 2.5−2.0 (br m), 1.8−1.1 (br m). Crude polymer solutions were purified by dialysis (MW cutoff of ~12−14 kDa; against water): ^{1}H-NMR spectra of pure pAA(R) showed no signals for free RNH_2. The effect of the pH on the extent of incorporation was studied by performing the reaction using aqueous solutions of primary amines with pH 2, 7, or 12.

Typical Procedure for Generation of Libraries of pAA(NeuAc) and pAA(NeuAc; R). Solutions of co-polymers pAA(NeuAc-L) were prepared by reaction of NeuAc-L-NH_2 (**1**, **2**, **3**, or **4**) with pAAn using different numbers of mol equiv of NeuAc-L-NH_2 to anhydride groups of pAAn (Figure 2). Co-polymeric pAA(NeuAc-L) for which the mol equiv is >0 was generated in microtiter plates with 96 conically-bottomed wells as follows: (i) placing 6 mg of pAAn into a well; (ii) soaking the powder with a variable amount (19−100 μL) of 0.1 M NeuAc-L-NH_2 in PBS buffer, pH 12; (iii) immediately sealing the plate, and then ultrasonicating the mixture for 0.5 h. Each solution of co-polymers (pH ~ 3) generated in a well was neutralized to pH ~ 7 by adding 60 μL of 1.0 M NaOH and adjusted to 100 or 200 μL (total volume) with PBS, pH 7.2, before the HAI assay. pAA(**1**) (mol equiv of **1** to anhydride groups of pAAn = 0.08; pH ~ 12). ^{1}H-NMR (300.1 MHz, D_2O): δ (ppm) 3.8−3.5 (m), 3.5−3.3 (m), 3.3−3.1 (br), 2.6−2.3 (br m), 2.3−1.8 (br m), 1.7−1.0 (br m). The above protocol was extended similarly to the preparation of ter-polymers pAA(NeuAc-L; R); here, a three-component mixture (6 mg of pAAn, 50 μL of 0.1 M NeuAc-L-NH_2 (**1** or **3**) and 30 μL of 0.2 M RNH_2) was sonicated. Purified (dialyzed) pAA(**1**; R) (RNH_2 = L-3-(2′-naphthyl)alanine; mol equiv of **1** to anhydride groups of pAAn = 0.10; mol equiv of RNH_2 to anhydride groups of pAAn = 0.12; pH ~ 12): ^{1}H-NMR (300.1 MHz, D_2O): δ (ppm) 7.6−7.4 (br m), 7.4−7.1 (br m), 3.7−3.5 (br m), 3.6−3.4 (br m), 3.3−3.1 (br), 3.1−2.8 (br m), 2.6−1.9 (br m), 1.8−1.0 (br m).

Hemagglutination Inhibition (HAI) Assay.[21,34,35] Influenza virus (strain X-31) was kept as a suspension in a super stock solution (~15 mg of protein/mL of PBS, pH 7.2) at 4 °C. A solution of erythrocytes from 2-week-old chicks (which was provided as a suspension (~5% v/v) in Elsevers solution) was washed with PBS, pH 7.2 (4 times), as described[21] and then resuspended in PBS (~0.5% v/v). The HAI assay of polymer was performed at room temperature (~20 °C), using 2-fold serially diluted solutions of polymeric NeuAc: (i) each well (50 μL) containing certain amount of a polymer was mixed with 50 μL of a suspension of X-31 virus (~0.025 μg protein/mL PBS); (ii) after 30 min of incubation at ~20 °C, 100 μL of a suspension of chicken erythrocytes (~0.5%) was added to each well, followed by gentle agitation and incubation for 1 h at room temperature (rt). The end point of the HAI assay is the last well in which an agglutinated pellet is observed. The value of HAI activity, K_i^{HAI} is defined as the lowest concentration of NeuAc of polymer in solution (at this end point) that inhibited the agglutination of erythrocytes induced by influenza virus. The reported value of K_i^{HAI} represents an average of at least five independent measurements.

Synthesis of Monovalent and Polymeric Polyvalent Adamantanes (Figure 4). Adamantane-**1**-NH_2 was synthesized from 1-aminoadamantane using two steps of reaction: (i) Michael addition to acrylonitrile (EtOH, 24 h, reflux, 55%) and (ii) reduction of nitrile to amino group ($LiAlH_4$, ether, 5 h, 0 °C, 74%). ^{1}H-NMR (300.1 MHz, CD_3OD): δ (ppm) 2.68 (t, J = 7.1 Hz, 2H), 2.61 (t, J = 7.1 Hz, 2H), 2.06 (br s, 3H), 1.75−1.69 (br d, 12H), 1.63−1.59 (quin, J = 7.1 Hz, 2H). After a coupling reaction of adamantane-**1**-NH_2 and 6-NHCbz-1-hexanoic acid *N*-hydroxysuccinimide (NHS) ester (MeOH, rt, 24 h, 32%) and hydrogenolytic deprotection of *N*-Cbz group (H_2 (1 atm), 10% Pd/C, MeOH, rt, 24 h, ~90%), the product (adamantane-**2**-NH_2) was obtained as a pale yellow oil. R_f = 0.45 in *i*-$PrNH_2$/MeOH/CH_2Cl_2 (1:5:15). ^{1}H-NMR (250.1 MHz, CD_3OD/$CDCl_3$): δ (ppm) 3.16 (s, 4H), 2.63−2.57 (t, J = 7.1 Hz, 2H), 2.10−2.04 (t, J = 7.3 Hz, 2H), 1.91 (br s, 3H), 1.69−1.68 (br s, 6H), 1.59 (br s, 6H), 1.53−1.41 (m, 4H), 1.25−1.22 (quin, J = 6.72 Hz, 2H). FAB-MS (NBA): *m/z* 344 $[M + Na]^+$. Adamantane-**3**-NH_2 was synthesized by a coupling reaction of adamantane-1-carboxylic acid and 1,2-diaminoethane (DCC, NHS, CH_2Cl_2; then the diamine, MeOH, 84%). ^{1}H-NMR (300.1 MHz, $CDCl_3$): δ (ppm) 3.23 (m, 2H), 2.75 (t, J = 7.0 Hz, 2H), 1.97 (br s, 3H), 1.78 (m, 6H), 1.65 (m, 6H). After a coupling reaction of adamantane-**3**-NH_2 and 6-NHCbz-1-hexanoic acid NHS ester (DMF, 24 h, rt, 68%) and deprotection of *N*-Cbz group (H_2 (1 atm), 10% Pd/C, MeOH, rt, 24 h, ~90%), the product (adamantane-**4**-NH_2) was obtained as a white solid. R_f = 0.62 in *i*-$PrNH_2$/MeOH/CH_2Cl_2 (1:2:14). ^{1}H-NMR (300.1 MHz, CD_3OD/$CDCl_3$): δ (ppm) 3.24−3.19 (t, J = 6.7 Hz, 2H), 2.74−2.62 (m, 4H), 2.20−2.15 (t, J = 7.4 Hz, 2H), 2.08 (br s, 3H), 1.68−1.58 (m, 14 H), 1.48−1.44 (quin, J = 7.3 Hz, 2H), 1.37−1.30 (quin, J =7.3 Hz, 2H). ^{13}C-NMR (100.6 MHz, CD_3OD): δ (ppm) 181.08, 176.39, 51.37, 41.73, 41.26, 39.96, 37.57, 36.79, 30.43, 29.73, 27.22, 26.49. FAB-MS (NBA): *m/z* 358 $[M + Na]^+$. HRMS: calcd for $C_{19}H_{33}N_3O_2Na$ 358.2468, found 358.2470. To a solution of DMF (2 mL) containing poly(*N*-(acryloyloxy)succinimide) or pNAS (50 mg, equivalent to 0.3 mmol of NAS) was added adamantane-**4**-NH_2 (10.1 mg, 29 μmol) dissolved in DMF (1 mL), followed by addition of Et_3N (0.1 mL). After stirring of the mixture (3 d, rt; then 6 h, 50 °C), aqueous NH_3 (10% w/w; 1 mL) was added to the mixture, followed by stirring for additional 6 h at rt. At the conclusion of reaction, the mixture was transferred into a dialysis bag (cellulose membrane, MW cutoff of ~12−14 kDa) and was dialyzed at rt over 3 days: water (2 × 4 L), 5% (w/w) NH_4Cl (4 L), and water (2 × 4 L). After dialysis, the content of the bag was frozen, and lyophilized to afford 25 mg of pA(Ad-**4**) as a fluffy white solid (*ca.* yield = 83%). ^{1}H-NMR (300.1 MHz, D_2O): δ (ppm) 3.13−3.12 (br s), 2.25−1.95 (br m), 1.83 (br s), 1.7−1.01 (br m), 1.6 (s). pA(Ad-**2**) was prepared similarly from adamantane-**2**-NH_2. ^{1}H-NMR (300.1 MHz, D_2O): δ (ppm) 3.14 (br s), 2.98 (br m), 2.84 (br m), 2.4−1.9 (br m), 1.7 (s), 1.6−1.0 (br m).

Synthesis of Derivatives of *N*-Acetylneuraminic Acid (Figure 6). The α-*C*- and α-*O*-sialosides containing an amine-terminated linker

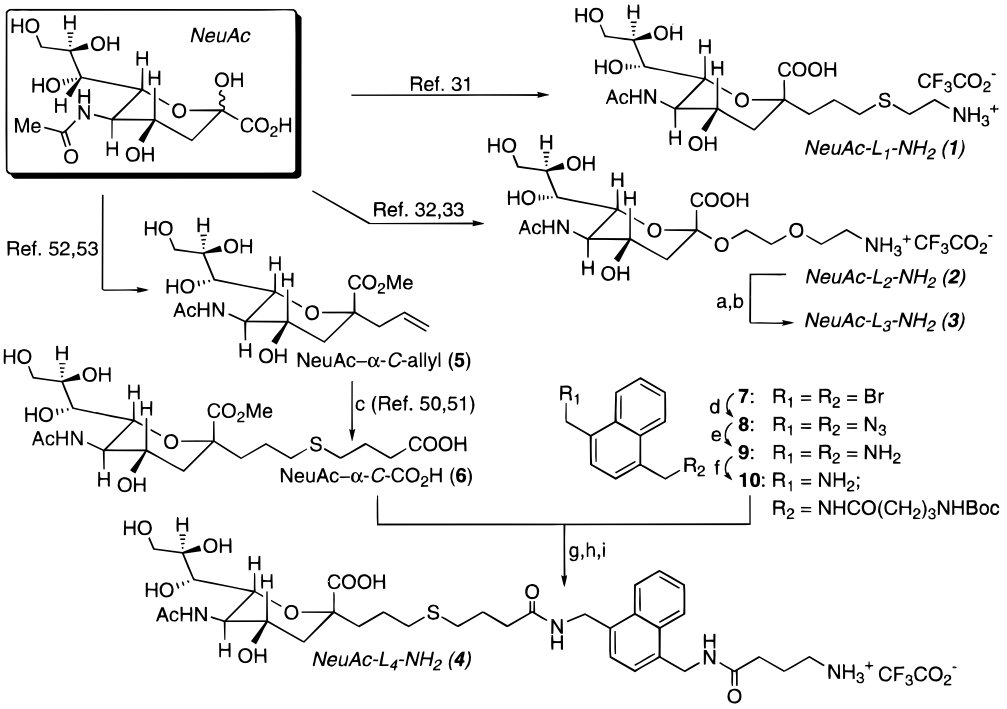

Figure 6. Summary scheme for synthesis of α-*C*- and α-*O*-sialosides. (a) N^{ϵ}-Cbz-N^{α}-dansyllysine, $COIm_2$, Et_3N, DMF, rt, 93%. (b) H_2 (1 atm), 10% Pd/C, MeOH, rt, ∼100%. (c) *hν* (254 nm), 4-mercapto-1-butanoic acid, 4,4′-azo-bis(isocyanovaleric acid), H_2O, 10 h, 62−74%. (d) NaN_3, DMF, rt, ∼100%. (e) H_2 (1 atm), 10% Pd/C, MeOH, rt, 60%. (f) 4-NHBoc-butyric acid *N*-hydroxysuccinimide ester, DMF, 80 °C, 2 d, 35%. (g) $COIm_2$, DMF, 80 °C, 2 d, 42%. (h) LiOH·H_2O, H_2O, rt, 24 h, ∼100%. (i) TFA, CH_2Cl_2, 0 °C, 1 h, ∼100%.

group, NeuAc-L_1-NH_2 (**1**)[31] and NeuAc-L_2-NH_2 (**2**),[32,33] were synthesized according to literature methods.

NeuAc-L3-NH_2 (3). A mixture of 1,1′-carbonyldiimidazole (53 mg, 0.33 mmol) and N^{ϵ}-Cbz-N^{α}-dansyllysine (160 mg, 0.31 mmol) in DMF (2 mL) was stirred for 3 h at rt, followed by addition of NeuAc-L_2-NH_2 (**2** as TFA salt; 120 mg, 0.24 mmol) and Et_3N (0.15 mL, 1.08 mmol). After stirring (24 h, rt), the mixture was concentrated *in vacuo* to yield a pale yellow oily residue. This residue was dissolved in MeOH (15 mL) containing 5 g of ion exchange resin (Dowex-50W, H^+-form). After shaking for 5 min, the resin-containing solution was filtered through filter paper and was concentrated prior to flash column chromatography (50 g of silica gel; 10% MeOH/CH_2Cl_2 to 5% HCO_2H/30% MeOH/CH_2Cl_2). The coupling product was obtained as a pale yellow oil (195 mg, 93%) from fractions with R_f of 0.5 (5% HCO_2H/30% MeOH/CH_2Cl_2). ^{1}H-NMR (500.1 MHz, CD_3OD): δ (ppm) 8.53−8.50 (m, 1H), 8.38−8.36 (br d, J = 8.6 Hz, 1H), 8.21−8.19 (br d, J = 8.6 Hz, 1H), 7.57−7.52 (m, 2H), 7.32−7.24 (m, 5H; $-COOCH_2C_6\mathbf{H_5}$), 7.22−7.18 (m, 1H), 5.03 (s, 2H; $-COOC\mathbf{H_2}C_6H_5$), 3.88−3.80 (m, 3H), 3.75−3.48 (m, 9H), 3.27−3.26 (m, 2H), 3.08−3.07 (m, 2H), 2.84 (s, 6H; $N(\mathbf{CH_3})_2$), 2.71−2.67 (m, 3H), 2.0 (s, 3H; $C\mathbf{H_3}CONH$), 1.63−1.58 (t, J = 12.0 Hz, 1H; $\mathbf{H}_{3\alpha}$), 1.49−1.48 (br s, 2H), 1.1−1.0 (m, 4H); FAB-MS (glycerol): *m/z* 914 $[M + Na]^+$. A suspension of the above *N*-Cbz protected, derivative of sialic acid (180 mg, 0.20 mmol) and 10% Pd/C (100 mg) in MeOH (20 mL) was stirred under an atmosphere of H_2 (1 atm) for 1 d at rt. After removal of the palladium catalyst by filtering through a pad of Celite, the filtrate was evaporated *in vacuo* to yield a crude product (NeuAc-L_3-NH_2 (**3**)) as a pale yellow oil. We estimated the yield of hydrogenolysis to be approximately quantitative on the basis of a ^{1}H-NMR spectrum that indicates the complete removal of the *N*-Cbz group. ^{1}H-NMR (399.9 MHz, CD_3OD): δ (ppm) 8.56−8.54 (t, J = 8.69 Hz, 1H), 8.38−8.35 (dd, J = 3.27, 8.69 Hz, 1H), 8.22−8.20 (d, J = 8.7 Hz, 1H), 7.62−7.52 (m, 2H), 7.27−7.23 (m, 1H), 3.91−3.77 (m, 4H), 3.76−3.61 (m, 4H), 3.58−3.56 (m, 4H), 3.48−3.45 (t, J = 4.75 Hz, 2H), 3.01−2.98 (m, 2H), 2.86 (s, 6H; $N(\mathbf{CH_3})_2$), 2.72−2.62 (m, 3H), 2.0 (s, 3H; $C\mathbf{H_3}CONH$), 1.64−1.56 (m, 3H), 1.43−1.40 (m, 2H), 1.18 (m, 2H). ^{13}C-NMR (100.6 MHz, CD_3OD): δ (ppm) 175.47, 175.10, 173.72, 129.34, 124.42, 120.56, 119.59, 119.18, 116.68, 116.45, 113.77, 101.72, 74.34, 73.03, 71.12, 70.17, 69.34, 64.56, 63.93, 58.65, 57.77, 54.15, 42.23, 40.34, 40.27, 33.65, 33.34, 27.86, 23.24, 22.90, 22.72. FAB-MS (glycerol): *m/z* 758 $[M + H]^+$, 780 $[M + Na]^+$.

NeuAc-α-C-CO_2H (6). Compound **6** was prepared using the radical-initiated addition reaction of a thiol to an olefinic group of α-*C*-allyl sialoside (**5**), according to literature methods.[31,50,51] A suspension of α-*C*-allyl-sialic acid methyl ester[52,53] (0.6 g, 1.73 mmol), 4-mercapto-1-butanoic acid[54] (0.8 g, 6.67 mmol), and 4,4′-azobis(4-cyanovaleric acid) (0.4 g, 1.43 mmol) in water (10 mL) was degassed for 10 min *in vacuo* prior to being saturated with N_2 (by bubbling N_2 gas through the solution for 1 h). A reaction flask containing the mixture was placed in a photochemical reactor (Rayonet) and was irradiated at 254 nm for 10 h. Evaporation of the irradiated mixture afforded a pale yellow oil, which was purified with flash silica gel chromatography (10% MeOH/CH_2Cl_2 to 5% HCO_2H/30% MeOH/CH_2Cl_2). The adduct was obtained as an oil from fractions with R_f = 0.6 (5% HCO_2H/30% MeOH/CH_2Cl_2). The combined oily material was dissolved in MeOH (5 mL), and it was poured slowly into ether (50 mL), which led to instant precipitation of the product as a white solid (0.5−0.6 g, 62−74%). ^{1}H-NMR (500.1 MHz, CD_3OD): δ (ppm) 3.84−3.76 (m, 3H), 3.78 (s, 3H; $COOC\mathbf{H_3}$), 3.70−3.48 (m, 4H), 2.60−2.55 (dd, J = 4.62, 13.16 Hz, 1H; $\mathbf{H}_{3\beta}$), 2.53−2.47 (m, 4H; $C\mathbf{H_2}SC\mathbf{H_2}$), 2.36−2.35 (t, J = 7.24 Hz, 2H; $C\mathbf{H_2}COOH$), 2.04 (s, 3H; $C\mathbf{H_3}CONH$), 1.87−1.80 (m, 4H; $C\mathbf{H_2}CH_2SCH_2C\mathbf{H_2}$), 1.73−1.70 (m, 1H), 1.60−1.55 (t, J = 11.89 Hz, 1H; $\mathbf{H}_{3\alpha}$), 1.51−1.41 (m, 1H); FAB-MS (glycerol; negative ion mode): *m/z* 466 $[M - H]^-$; HRMS: calcd for $C_{19}H_{32}N_1O_{10}S_1$ 466.1745, found 466.1747.

Compound 10. 1,4-Bis(bromomethyl)naphthalene (**7**)[55] was converted to 1,4-bis(azidomethyl)naphthalene (NaN_3, DMF, rt);[56] the azido group was then reduced to an amino group using catalytic hydrogenation (H_2 (1 atm), 10% Pd/C, MeOH). 1,4-Bis(azidomethyl)naphthalene (**8**). ^{1}H-NMR (400 MHz, $CDCl_3$): δ (ppm) 8.06 (m, 2H), 7.63 (m, 2H),

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7.45 (s, 2H), 4.77 (s, 4H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) 132.32, 131.73, 126.97, 126.51, 124.33, 52.94. CI-MS (NH_3): *m/z* 273 $(M + NH_3)^+$. 1,4-Bis(aminomethyl)naphthalene (**9**). ^{1}H-NMR (300 MHz, $CDCl_3$): δ (ppm) 8.10 (m, 2H), 7.54 (m, 2H), 7.43 (s, 2H), 4.31 (s, 4H). ^{13}C-NMR (100 MHz, $CDCl_3$): δ (ppm) 138.31, 131.60, 126.06, 124.31, 124.08, 44.13. CI-MS (NH_3): *m/z* 187 $(M + H)^+$. A solution of DMF (150 mL) containing 1,4-bis(aminomethyl)naphthalene (1.4 g, 7.53 mmol) and 4-NHBoc-1-butyric acid NHS ester (2.17 g, 7.53 mmol) was stirred at 80 °C for 2 d under a stream of N_2. Evaporation of DMF afforded an oily residue which was purified with flash silica (200 g) chromatography (10% MeOH/CH_2Cl_2, 5% *i*-$PrNH_2$/10% MeOH/CH_2Cl_2). A monoadduct, 1-[(4′-NHBoc-1′-oxo-1′-aminobutyl)methyl]-4-(aminomethyl)naphthalene (**10**) (R_f = 0.58 in 5% *i*-$PrNH_2$/10% MeOH/CH_2Cl_2), was obtained in 35% yield (0.98 g). ^{1}H-NMR (300.1 MHz, $CD_3OD/CDCl_3$): δ (ppm) 7.98−7.95 (m, 2H), 7.51−7.49 (m, 2H), 7.33−7.32 (s, 2H), 4.81−4.77 (two s, 4H), 3.01−2.98 (t, J = 6.84 Hz, 2H), 2.18−2.14 (t, J = 6.84 Hz, 2H), 1.74−1.70 (q, 2H, J = 6.84 Hz), 1.34 (s, 9H). FAB-MS. *m/z* 393 $(M + Na)^+$. The reaction also yielded a bis-adduct, 1,4-bis[(4′-NHBoc-1′-oxo-1′-aminobutyl)methyl]naphthalene (1.47 g).

NeuAc-L$_4$-NH$_2$ (4). A solution of DMF (3 mL) containing NeuAc-α-*C*-CO_2H (**6**) (292 mg, 0.625 mmol) and 1,1′-carbonyldiimidazole (105 mg, 0.654 mmol) was stirred for 2 h at rt, prior to addition of **10** (300 mg, 0.810 mmol). The final mixture was stirred for 2 d at 80 °C under a stream of N_2. Evaporation of DMF afforded a pale yellow residue, which was then dissolved in MeOH (10 mL) containing 5 g of ion exchange resin (Dowex-50W; H^+ form). After the suspension was stirred for 5 min, resins were removed by filtration through filter paper. The concentrated residue of the filtrate was purified by flash column chromatography (silica gel; 5% MeOH/CH_2Cl_2 to 20% MeOH/CH_2Cl_2). The coupling product (218 mg, 42%) was obtained as a light yellow oil (R_f = 0.71 in 20% MeOH/CH_2Cl_2). ^{1}H-NMR (500.1 MHz, CD_3OD): δ (ppm) 8.07 (m, 2H), 7.55−7.53 (m, 2H), 7.41−7.39 (m, 2H), 4.90−4.85 (s, 2H), 4.82 (s, 2H), 3.95−3.71 (m, 3H), 3.70−3.47 (m, 4H), 3.73 (s, 3H; COOC**H$_3$**), 3.05−3.03 (t, J = 6.82 Hz, 2H; C**H$_2$**-NHBoc), 2.55−2.45 (m, 7H), 2.25−2.23 (t, J = 6.82 Hz, 2H; $ArCH_2$-NHCOC**H$_2$**), 2.02 (s, 3H; C**H$_3$**CONH), 1.85−1.65 (m, 7H), 1.62−1.58 (t, **H$_{3\alpha}$**, J = 12.84 Hz), 1.40 (m, 10H). FAB-MS (glycerol): *m/z* 721 $(M + H\text{-Boc})^+$. To a solution of MeOH (5 mL) containing the above product (120 mg, 0.146 mmol) was added $LiOH\cdot H_2O$ (20 mg, 0.477 mmol) in H_2O (5 mL). After being stirred (24 h, rt), the mixture was adjusted to pH ∼ 3 by adding ion exchange resin (Dowex-50W, H^+ form). After filtration of resins, the filtrate was concentrated *in vacuo* to afford an oily residue: this material was pure enough to proceed to next step without further purification. ^{1}H-NMR (500.1 MHz, CD_3OD): δ (ppm) 8.06 (m, 2H), 7.57−7.54 (m, 2H), 7.42−7.41 (m, 2H), 4.85−4.81 (two s, 4H), 3.85−3.70 (m, 3H), 3.68−3.50 (m, 4H), 3.05−3.02 (t, J = 6.7 Hz, 2H; C**H$_2$**NHBoc), 2.62−2.59 (m, **H$_{3\beta}$**), 2.55−2.48 (m, 4H; C**H$_2$**SC**H$_2$**), 2.40−2.38 (t, J = 7.23 Hz, 2H; C**H$_2$**CONH), 2.26−2.22 (t, J = 7.16 Hz, 2H; NHCOC**H$_2$**), 2.04 (s, 3H); C**H$_3$**CONH), 1.86−1.82 (m, 5H), 1.76−1.74 (t, J = 7.16 Hz, 2H), 1.59−1.58 (t, **H$_{3\alpha}$**, J = 11.0 Hz), 1.40 (m, 10H). FAB-MS (glycerol; negative ion mode): *m/z* 805 $[M - H]^-$. HRMS: calcd for $C_{39}H_{57}N_4O_{12}S_1$ 805.3690, found 805.3694, calcd for $C_{39}H_{58}N_4O_{12}S_1Na_1$ 829.3666, found 829.3670. To an *N*-Boc protected **4** (50 mg, 0.062 mmol) suspended in CH_2Cl_2 (1 mL) at 0 °C was added a mixture of CF_3CO_2H (1 mL) and CH_2Cl_2 (1 mL). The mixture was stirred for 1 h at 0 °C, and it (a pale red solution) was poured slowly into cold ether (50 mL), which led to formation of a white precipitate. It was collected by centrifugation and washed with ether (50 mL). The yield on deprotection of *N*-Boc was estimated to be quantitative on the basis of ^{1}H-NMR (CD_3OD) spectrum of the product (NeuAc-L$_4$-NH_2 (**4**) as TFA salt). FAB-MS (glycerol; negative ion mode): *m/z* 705 $[M - H]^-$.

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