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Synthesis of Molecularly Imprinted Polymer and its Molecular Recognition Properties of *N*-Acetylneuraminic Acid

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Abstract: A molecular imprinting technique was applied in this work to detect *N*-acetylneuraminic acid (Neu5Ac) and its analogue structure. Two molecularly imprinted polymers (MIP) were prepared using Neu5Ac as the template molecule, as well as methacrylic acid (MAA) or 4-vinylpyridine (4-Vpy) and ethyleneglycol dimethacrylate (EGDMA) as the functional monomer and cross-linker, respectively. Free radical polymerization was carried out at 4°C under UV radiation or thermal (60 °C) polymerization. MIP thus obtained were ground into 11~25µm and 25-44µm. The binding results from Neu5Ac solution, mannose (Man) solution and *N*-acetyl-D-mannosamine (ManNAc) solution performed by Neu5Ac-MIP showed specific binding toward Neu5Ac rather than other analogue compounds on the host-guest system. The values of capacity for Neu5Ac-MIP were measured and experimental results were further used for simulation to obtain the binding isotherms. The principal advantage of this method is that Neu5Ac-MIP can recognize Neu5Ac and its analogue compounds.

Keywords: Molecularly imprinted polymer, *N*-Acetylneuraminic acid, Free radical polymerization, Specific binding, Host-guest system

Introduction

Sialic acid is ubiquitous in tissues and more than 25 different kinds of sialic acid have been reported in nature. Sialic acid is the generic name given to a family of acylated derivatives of a 9-carboxylated monosaccharide. Blix, Klenk and Gottshalk¹⁻³ established the nomenclature rules for sialic acid and did initial research in this field during the 1960s. From a chemical point of view sialic acid is a C₉-monosaccharide with several different functional groups, which must each to be considered when developing derivatization techniques.

The most commonly occurring sialic acids are *N*-acetylneuraminic acid (5-acetamido-3,5-dideoxy-D-glycero-D-galactononulosonic acid, Neu5Ac), *N*-glycolylneuraminic acid (Neu5Gc), deaminoneuraminic acid (KDN) and neuraminic acid. Other sialic acids arise from *o*-substitution of one or more of the hydroxyl groups of Neu5Ac with acetyl, methyl, lactoyl, sulfate groups or phosphate groups. Neu5Ac and Neu5Gc are the most abundant forms of sialic acids but neuraminic acid does not exist in nature. Neu5Ac concentration in serum has been reported in patients with different types of cancer while Neu5Gc has been found in human cancer tissues and sera of cancer patients⁴⁻⁶. On the other hand, KDN might also be used as a substitute for Neu5Ac, and can play an important role for selection antagonists and be used as an additive for health foods⁷. For these reasons, a variety of methods have been published for the analysis of sialic acids in glycoconjugates, including Thin-Layer chromatography⁸⁻⁹, gas chromatography¹⁰⁻¹², high-performance liquid chromatography¹³⁻¹⁴ and NMR spectroscopy¹⁵⁻¹⁶. Common limitations of these methods are the requirement for purified samples to avoid interference from other contaminants and their inability to differentiate the types of sialic acid.

In this report, we describe an optimized molecularly imprinted method for rapid and effective determination of Neu5Ac, Man and ManNAc (Figure 1). Compared to other methods, this method can be more convenient and simple.

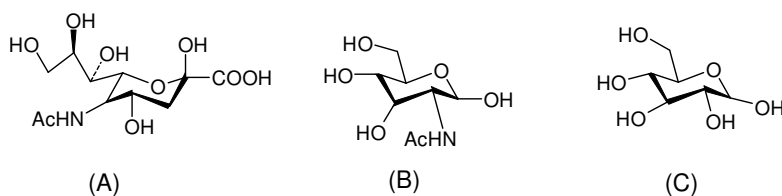


Figure 1. Structure of compounds used in this study. (A): *N*-Acetylneuraminic acid (Neu5Ac), (B): *N*-Acetyl-D-mannosamine (ManNAc) and (C): D-Mannose (Man).

In this study, two different template/monomer systems are examined: (a) Neu5Ac/methacrylic acid (MAA); (b) Neu5Ac/ 4-vinylpyridine (4-VP). It was observed that different monomers interact very differently with the template molecules, depending on the chemical properties of the template molecule. During imprinting, the template creates a specifically imprinted cave in the polymer with a permanent memory to recognize the template. After removal of the template molecule, the polymer can be used as a selective binding medium for structurally related compounds. Selectivity of the MIP was evaluated by measuring its ability to resolve structural analogs in the adsorption process. The selectivity experiment demonstrates that the Neu5Ac-MIP is able to recognize the structural differences between the template and its analogs. Affinity and selectivity evaluation indicated that an imprinted polymer is a potential separation material having selectivity for Neu5Ac.

Experimental

Deionized water was passed through a Millipore system (Bedford, MA, USA). *N*-Acetylneuraminic acid (Neu5Ac), *N*-acetyl-D-mannosamine (ManNAc), and D-mannose (Man) were purchased from Sigma (St.Louis, MO, USA). Methacrylic acid (MAA, 99%) and ethylene glycol dimethacrylate (EGDMA, 98%) were obtained from Merck (Darmstadt, Germany). 2,2'-Azo-bisisobutyronitrile (AIBN) and *N,N*-dimethylformamide (DMF) were obtained from TCI (Tokyo, Japan). Methanol, ethanol, acetone, 4-vinylpyridine (4-VP), sodium dihydrogen phosphate and acetonitrile were of HPLC grade and obtained from TEDIA (Fairfield, OH, USA). Chloroform and acetic acid were from J.T.Baker (Phillipsburg, NJ, USA) and were of GC grade. All chemicals were of the HPLC or analytical grade. MAA, EGDMA and DMF were distilled to remove the inhibitors before polymerization.

Molecularly imprinted polymer preparation

Two polymers, Neu5Ac-MIP (PA and PB), and two blank polymers were prepared as shown in Figure 2. A typical preparation process for a molecularly imprinted polymer is as follows: 2mol% of Neu5Ac (template) and either 3mol% of MAA or 3 mol% of 4-VP (functional monomers) in 5 mL of DMF was added to 95mol% EGDMA (cross-linker). The 2, 2-azobisisobutyronitrile was used as an initiator. The mixture was transferred into a conical Erlenmeyer flask with a screw top. The conical Erlenmeyer flask was placed in an ultrasonic water bath until a clear solution was obtained. The solution was then degassed and purged with dry nitrogen for 5 min, the flask was sealed and placed under a UV-lamp (365 nm, 100 W) at 4 °C for 6 h or thermally (60 °C) polymerized. Following polymerization, the DMF was removed. The hard polymers were dried in a vacuum oven for 24 h at room temperature. Polymers were then ground using a laboratory mortar grinder. The 11-25, 25-44 μ m particle size fractions were collected. The non-MIP particles were also prepared according to the above procedures, except for the absence of Neu5Ac template during polymerization. The prepared MIP particles were washed with an excess amount of the 9:1(v/v) MeOH/AcOH. Then the particles were washed with water and finally dried in a vacuum.

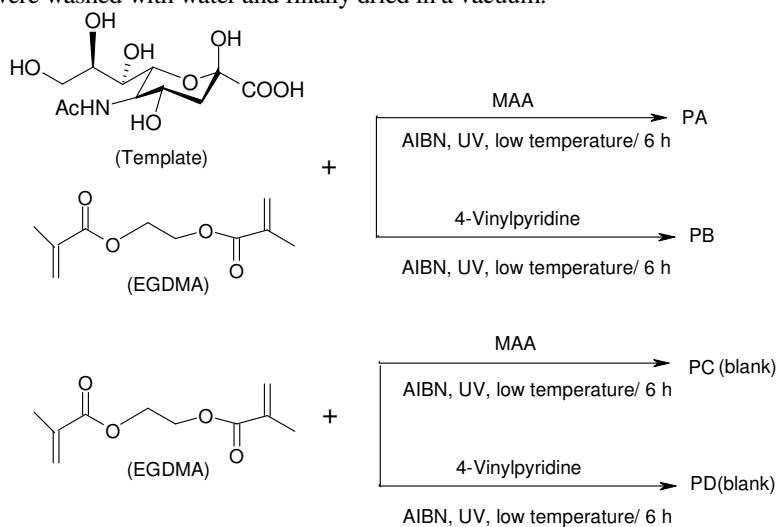


Figure 2. Schematic representation of the synthesis of MIPs for Neu5Ac as template and MAA, 4-VP as functional monomer.

HPLC analysis for sialic acid quantification

HPLC analysis was used to determine binding capacity of the Neu5Ac-MIPs after the adsorption experiments. The instrument was a JASCO PU-980 pump system with SHODEX-RI detector. An Aminex HPX-87H Ion Exclusion Column, 300 mm x 7.8 mm (Bio-Rad), was employed for the analysis. The mobile phase was 0.005 mol L⁻¹ H₂SO₄ with a flow rate of 1.0 mL min⁻¹. The concentration of standard solutions was 20 mmol L⁻¹~1.0 mmol L⁻¹ substrate (Neu5Ac, Man, ManNAc). A calibration graph was found between concentration of substrate and the absorbance. In each adsorption experiment, unless otherwise specified an accurately weighed 1g portion of the polymer particles was transferred into a 50 mL centrifuge tube, 25 mL of vary concentration standard solution was added and the tube rotary for 12 h at 4 °C. This solution was centrifuged at 2000 rpm for 10 min at 4 °C. The supernatant was transferred into 50 mL volumetric flask. The absorbance of the solution was measured by HPLC with RI detector. The concentration of substrate was estimated based on the standard curve.

Results and discussion

Characterization of the Neu5Ac-imprinted polymer

Neu5Ac was chosen as the template, polymerization occurred non-covalently among the functional monomer in the presence of DMF. The polymer materials were ground into powder and the fraction had a particle size ranging from 11 to 25 and 25 to 44µm. The template was extracted from the polymer using MeOH/AcOH (9:1; v/v) solution. For comparison, non-covalent imprinted polymers were prepared using either MAA or 4-VP as the functional monomer. Figure 2 show that polymer PC contains EGDMA and MAA, while PD contains EGDMA and 4-VP; these polymers have no templates and serve as the blank for the non-covalent imprinted polymers. The addition of MAA to PA provides insight into the formation of templates, and functional monomer assemblies. PB is a non-covalent MIP in which 4-VP was used in place of MAA.

The solvent plays an important role in formation of the porous structure of MIP. It is also clear that the polarity of solvents used in the imprinting analyses affects the specificity of the polymers. In this work, DMF was chosen as the solvent. Non-polar porogens such as toluene, chloroform or polar porogen such as acetonitrile did not sufficiently dissolve Neu5Ac at low temperature. DMF is a non-protonic solvent that may not make the hydrogen bonding formation in the polymerization system. Thus, using DMF as the polymerization solvent is advantageous for the present imprinting, rebinding to the template. In this study, DMF is successfully used as solvent for preparing polymer-introduced recognition sites for Neu5Ac.

The results of this study show that better selectivity is obtained at the lower temperature (4 °C) polymerization versus the identical polymers thermally (60 °C) polymerized. To polymerize at lower temperatures, it is necessary to use photochemical reaction (UV radiation). Figure 3 shows the binding results of MIP under different temperature polymerized.

Absorptivities and binding assays: evidence for imprinting effect

The proposed method was applied to the selectivity of Neu5Ac and other similar structure compounds. Most imprinted polymers were synthesized using EGDMA as cross-linker and MAA or 4-VP as functional monomers by light or heat initiated polymerization¹⁷⁻¹⁹. The Neu5Ac molecule has carboxyl and five hydroxyl groups. These groups can form a hydrogen bond with functional monomers after removing the template, thereby leaving a

three-dimensional molecular imprint. The recognition properties of MIP are due both to the arrangement of the functional groups of the monomer units around the print molecules and to the MIP's shape or size. In order to remove the template molecules, it is necessary to break the hydrogen bond between template molecule and carboxyl or pyridine groups of the polymer by washing with an excess amount of the 9:1(v/v) MeOH/AcOH.

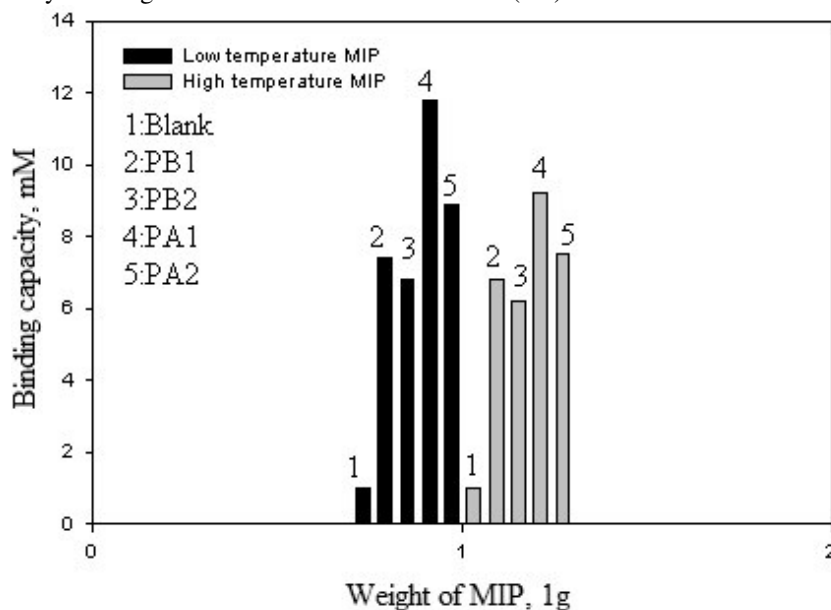


Figure 3. Different binding results of Neu5Ac from the MIPs under high temperature (60 °C) and low temperature (4 °C) polymerized.

The adsorptivities of Neu5Ac and other compounds from four MIP are shown in Table 1. Comparison of the data demonstrates the difference in their selectivities. High adsorptivities for Neu5Ac were obtained in the 94.54% and 92.51% for PA1 and PA2 polymers, 91.94% and 91.91% for PB1 and PB2 polymers. PA and PB could almost completely absorb the print molecule Neu5Ac. By contrast, low adsorptivities were obtained with the mannose at 41.17% and 37.57% for PA1 and PA2 polymers, at 66.72% and 49.77% for PB1 and PB2 polymers. The adsorptivities with ManNAc were 56.71% and 47.75% for PA1 and PA2, 53.77% and 39.59% for PB1 and PB2 polymers. Neu5Ac has 4-carbon alkylchain and its carboxyl or hydroxyl group could form a strong hydrogen-bonding complex with MAA or a remaining pyridine group. This binding may coordinate two or more functional groups of the polymer in a favorable position. ManNAc and mannose seemed to have difficulty diffusing into the molecularly imprinted sites; and would encounter interference due to steric hindrance and weak, non-specific hydrophobic interactions with the MIP. Therefore binding strength between the resulting MIP site and the analyse compounds is weaker than binding with the well-positioned MIP functional groups. This causes the relative low adsorptivities of Man and ManNAc from the MIP (PA and PB). The adsorptivities of blank polymers (PC and PD) were clearly lower than that of PA and PB, showing that the adsorptivities of blank polymer were physical adsorption (non-selective). PC and PD lacked template molecules during polymerization, so they were not imprinting polymers. Overall, it can be seen that all the MIP prepared in this research showed significant selectivity to the print molecule alone.

Table 1. Absorptivities^b (%) of Neu5Ac, Man and ManNAc from PA1, PA2, PB1, PB2 and non-imprinted polymers.

	Neu5Ac, % (blank)	Man, % (blank)	ManNAc, % (blank)
Sialic acid/MAA(PA) ^a			
11~25 μ m(PA1)	94.54 (13.67)	41.17 (12.34)	56.71 (13.59)
25~44 μ m(PA2)	92.51 (12.59)	37.57 (11.39)	47.75 (11.47)
Sialic acid/4-VP(PB)			
11~25 μ m(PB1)	91.94 (17.70)	66.72 (16.21)	53.77 (15.43)
25~44 μ m(PB2)	91.91 (15.64)	49.77 (16.49)	39.59 (14.64)

^a In each experiment, 1g of polymer particle was used. Polymer polymerized at low temperature(4°C).

^b Absorptivities (%) calculated according to the equation:(C_i-C_f)/C_i × 100% , where C_i is the initial sialic acid concentration (mmole L⁻¹), C_f is the final sialic acid concentration (mmole L⁻¹).

Effects of the amount of MIP on the adsorption

The adsorption profiles of Neu5Ac, ManNAc and Man with respect to different weights of MIP in respective solution with a concentration of 22 mmol L⁻¹ are showed in Table 2. The experimental data was evaluated by the batch adsorption method. It is found that the amount of MIP taken greatly affected the binding capacity. ManNAc and Man were very little bound to either PA or PB polymers, much less than Neu5Ac. The data (Table 2) confirms that Neu5Ac retention on the MIP is significant. The most selective and strongest bonds could be possibly formed with two or more pairs of hydrogen bonds. Due to the important role of these hydrogen bonds in the binding of the 5-carbon ring, the adsorption is expected to be quite strictly oriented.

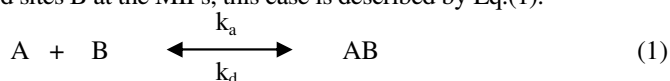
Table 2. Binding ability to Neu5Ac, ManNAc and Man with different weight MIP^a (PA1, PA2, PB1 and PB2)

Weight of the pellets, g	Neu5Ac, mmol L ⁻¹				ManNAc, mmol L ⁻¹				Man, mmol L ⁻¹			
	PA1	PA2	PB1	PB2	PA1	PA2	PB1	PB2	PA1	PA2	PB1	PB2
0.05	11.5	13.9	11.6	12.0	1.6	3.8	1.9	1.0	5.1	2.6	2.8	3.2
0.2	12.1	14.5	14.7	13.1	5.3	6.6	5.5	2.6	6.8	5.0	5.6	4.5
0.4	16.2	16.4	15.8	13.4	5.5	7.1	7.2	6.2	6.4	5.3	6.2	7.1
0.8	17.8	17.2	18.6	15.5	3.4	6.3	8.2	6.8	8.8	7.3	8.3	7.1
1.0	20.1	19.7	19.5	19.5	9.7	8.2	9.2	6.8	7.4	6.8	11.8	8.9

^aMIP were polymerized at 4 °C low temperature

Determination of binding parameters of the imprinted polymers

The Langmuir adsorption isotherm equation is one of the most widely used models to describe the equilibrium behaviors of adsorbate uptake. When an adsorbate (A) adsorbs to an adsorbent or a solid phase which contains specific binding sites (B). Let's look at the simplest case, *i.e.* A is binding to molecularly imprinted sites B at the MIPs, this case is described by Eq.(1).



For Eq.(1), the equilibrium constant (K) can be expressed as:

$$K = \frac{k_d}{k_a} = \frac{[AB]}{[A][B]} \quad (2)$$

The number of the moles of A bound to per mole of B (q) can be expressed as²⁰:

$$q = \frac{[AB]}{[B] + [AB]} \quad (3)$$

By combining Eq.(2) and (3) gives the traditional Langmuir isotherm model(Eq.4) for single-solute adsorption.

$$\frac{1}{Q} = \frac{1 + K[A]}{KQ_{max}[A]} \quad (4)$$

in which Q is adsorption capacity, and Q_{max} is the maximum adsorption capacity. A represents the concentration of adsorbate and $1/K$ is the dissociation constant at binding site. It should be emphasized that $[A]$ in Eq. (4) must be expressed as the molar concentration of adsorbate at equilibrium. Eq. (4) was used to fit the experimental data and change in standard free energy of adsorption (ΔG^0) was then determined by:

$$\Delta G^0 = -RT \ln K \quad (5)$$

The test found a relationship between $1/Q$ and $1/[A]$, namely, Langmuir plot showed in Figure 4. The plot obtained for the being of Neu5Ac to its polymer imprint consists of four distinct straight lines with slope which gave dissociation constant. The maximum adsorption capacity (Q_{max}), equilibrium constant (K) and standard free energy (ΔG^0) are summarized in Table 3. From the slope and intercept of the plot, the equilibrium constant K and the apparent maximum number Q_{max} of the higher affinity binding sites can be calculated to be $4.828 \times 10^2 \sim 4.623 \times 10^2 \text{ L mol}^{-1}$ and $57.89 \sim 48.49 (\mu\text{mol g}^{-1})$ for PA1, PA2, PB1 and PB2, respectively. In the same way, K and Q_{max} of the lower affinity bonding sites were calculated to be $2.364 \times 10^2 \sim 2.419 \times 10^2 \text{ L mol}^{-1}$ and $21.03 \sim 22.14 (\mu\text{mol g}^{-1})$ for PC and PD, respectively. Table 3 shows the values of ΔG^0 calculated from K by using Eq. (5), indicating that Neu5Ac interacts most strongly with MAA and most weakly with blank polymers. Therefore, the MIP synthesized with MAA or 4-VP is expected to give the highest selectivity to Nec5Ac. As described, above we can estimate the selectivity of MIPs by recovery measurements of an analyte and other structurally related analogues.

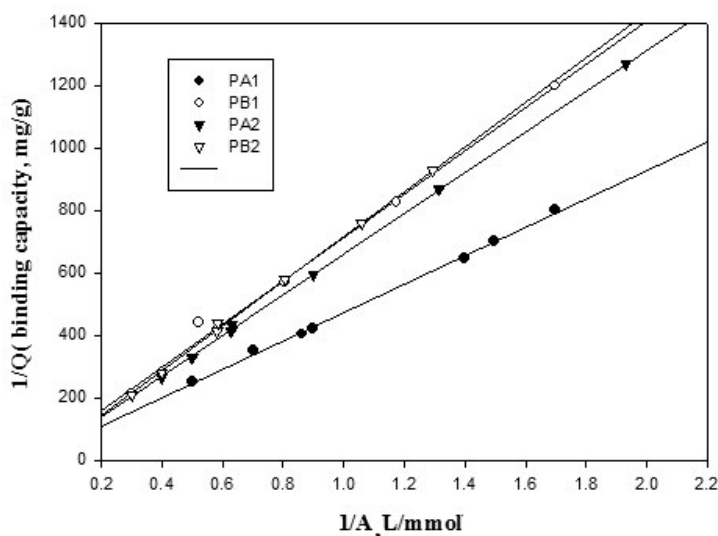


Figure 4. Langmuir plots to estimate the binding parameters of Neu5Ac-MIPs

Table 3. Fitted parameters equilibrium constant K , the maximum adsorption capacity (Q_{max}), and standard free energy (ΔG^0) by using the Eq.(4) for Neu5Ac on the imprinted polymers (PA1, PA2, PB1 and PB2) and on the non-imprinted polymers (PC and PD).

Affinity system	$K \times 10^2, \text{L mol}^{-1}$	$Q_{max}, \mu\text{mol g}^{-1}$	$\Delta G^0, \text{kJmol}^{-1}$
PA1	4.828	57.89	-14.23
PA2	4.792	54.23	-14.19
PB1	4.623	49.12	-14.11
PB2	4.634	48.49	-14.11
PC(blank)	2.364	21.03	-12.57
PD(blank)	2.149	22.14	-12.35

Conclusions

In this paper, two imprinted polymers, $p(\text{EGDMA-co-MAA})$ and $p(\text{EGDMA-co-4-VP})$ were synthesized by non-covalent method using Neu5Ac as template and their molecular recognition properties were studied. Both MIP were applied as selective sorbents, which proves that recognition ability can be ascribed to the imprinting process. The influence of recognition conditions on the receptivity and selectivity of the MIP demonstrated that hydrogen bonding between substrates and the binding sites played an important role.

The Langmuir model was used to calculate the experimental data of Neu5Ac, Man and ManNAc adsorption to these polymers. The imprinting effect and selectivity of the MIPs were confirmed and it was seen that $p(\text{EGDMA-co-MAA})$ was deemed to be the most promising polymer for further study it showed the greatest imprinting effect and the highest recoveries for all compounds. These studies add evidence to the research about the use Neu5Ac as a template in field of the molecularly imprinted technology, and support the application of biopharmaceutical analysis.

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