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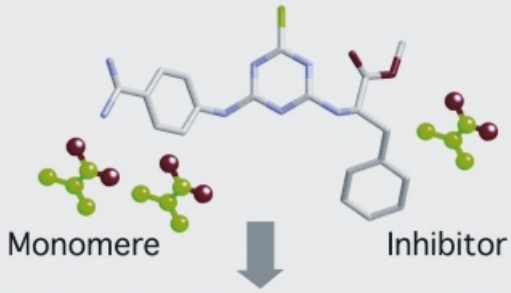

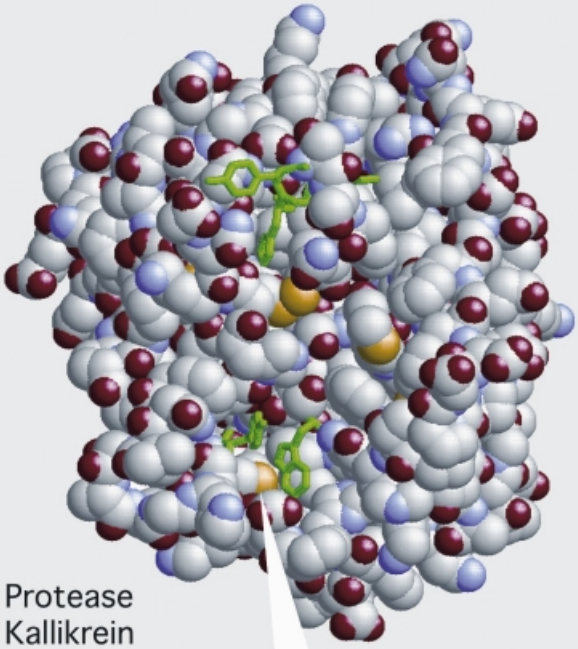

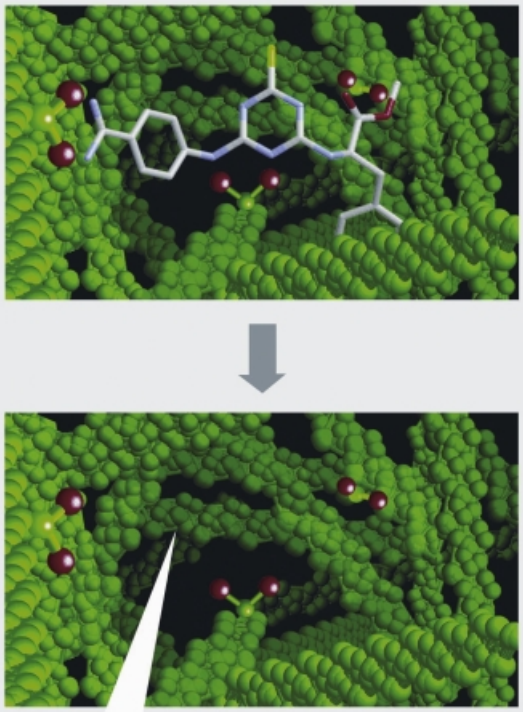

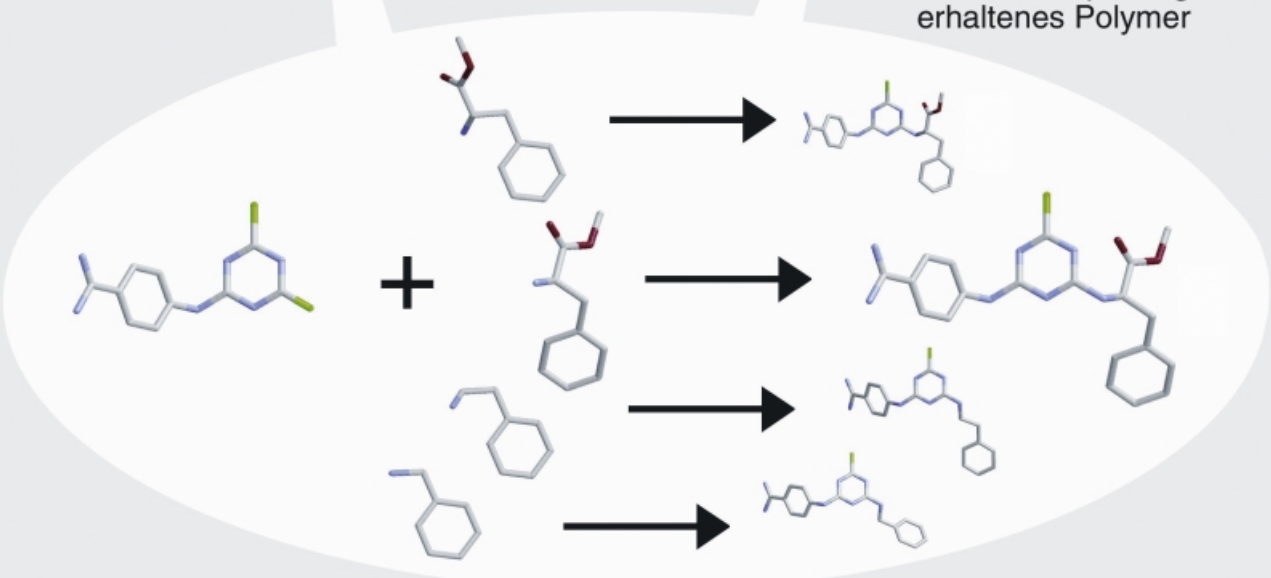

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Formation of a Class of Enzyme Inhibitors (Drugs), Including a Chiral Compound, by Using Imprinted Polymers or Biomolecules as Molecular-Scale Reaction Vessels**

Yihua Yu, Lei Ye,* Karsten Haupt, and Klaus Mosbach*

The design of novel materials with cavities that can behave as specific hosts to virtually any target molecules has become a topic of extreme scientific and technological interest in recent years. Within this broad area, the class of materials referred to as molecularly imprinted polymers (MIPs) has gained increasing interest.[1] Synthesis of MIPs is a process in which functional and cross-linking monomers are copolymerized in the presence of a target species that acts as a molecular template. The functional monomers initially form a complex with the template, and following polymerization, their functional groups are held in position by the highly cross-linked polymer structure. Subsequent removal of the template results in artificial receptors that are complementary in size, shape, and chemical functionality to the template. MIP materials are frequently used for solid-phase extraction, chromatography, and biomimetic sensor applications. One aspect that also deserves mention in the context of this article are studies carried out by several research groups towards the formation of enantiospecific imprints with potentially useful properties, for example, in the resolution of racemic drugs or for "enantiomeric polishing".[2]

For several years our research group has explored what we consider to be the next generation of molecular imprinting, which may improve synthesis and/or lead to new drugs and better affinity materials. Herein we describe two new approaches towards realizing this goal, in which we use kallikrein as a model system. Kallikrein was chosen because of its medicinal interest as well as its well-characterized structure and properties. In addition a number of inhibitors have been isolated or prepared against this proteinase enzyme.[3]

In the first approach we imprinted a known kallikrein inhibitor. After extracting the template inhibitor, we performed condensation reactions within the cavities of the MIP. The products of these condensations were evaluated for their ability to inhibit kallikrein. Thus, the products synthesized within the MIP cavities can be considered as "double imprints". We call this method the "anti-idiotypic" approach, as it relates to the known biological phenomenon of the formation of anti-antibodies to existing immunoglobulins.[4] In the second approach we target the enzyme itself by allowing it to direct the assembly of small building blocks within the biologically active site. New inhibitors for the enzyme are obtained by extraction following the condensation of these building blocks. We call this approach "direct molding". The theme that unites these two approaches is the use of cavities, either within a synthetic polymer (anti-idiotypic imprinting) or within the active site of an enzyme (direct molding), as molecular-scale reaction vessels (Figure 1).

The latter studies have been influenced by our interest over the years in immobilization and affinity chromatography of biomolecules. These studies have involved, for example, the coupling of a preformed inhibitor–enzyme complex directly to supports through the active group of the inhibitor, and affinity precipitation of, for example, bis-NAD dehydrogenase complexes.[5] In addition, bio-imprinting within the active sites of enzymes leading to induced alteration of stereo- and substrate selectivities has been studied.[6] In these examples the active sites of enzymes have been utilized, either to direct the

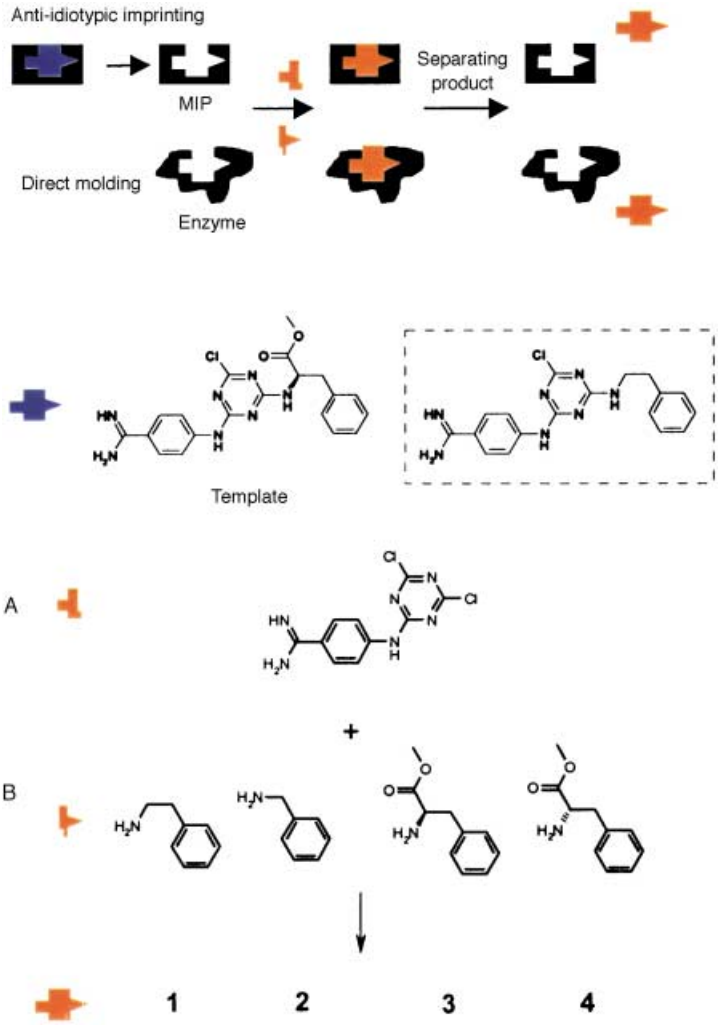

Figure 1. Schematic representation of the anti-idiotypic and direct molding approaches used, and subsequent synthetic reactions investigated in this study. The inserted structure in the middle (boxed) shows the template used in our previous study.

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assembly of properly oriented enzyme complexes or to provide binding pockets for non-native substrates or inhibitors.

We extended our earlier anti-idiotypic imprinting by using a chiral template, namely a L-phenylalanine methyl ester derivative (Figure 1).[7] This template permitted us to investigate enantiospecific synthesis inside the MIP binding cavities. (2-Trifluoromethyl)acrylic acid (TFMAA) and divinylbenzene (DVB) were used as the functional monomer and cross-linker, respectively. After preparing the MIP and removing the template, we allowed the nucleophilic reaction to take place between the positively charged triazine derivative and various hydrophobic amines inside the cavities to give either the same or related inhibitors, depending on the added amine. Condensation in the polymer cavities allowed formation of the larger product (**3**), which was not possible with an imprinted polymer prepared for the smaller template (**1**) in a previous study—presumably because of steric hindrance.[7]

Apart from the formation of the original template, the smaller compounds (**1** and **2**) could also be formed in the cavity (Figure 2). In contrast, the blank polymer (BP), which was prepared in the absence of the template, exhibited very little affect in facilitating the syntheses. This result can be explained by the fact that the BP is most unlikely to contain any specific binding site.

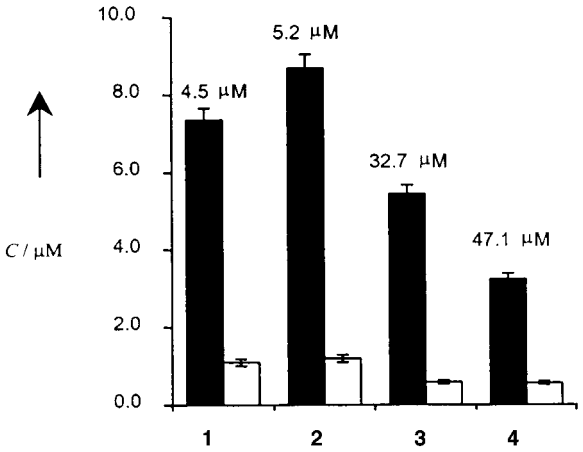

Figure 2. MIP-directed generation of inhibitors (anti-idiotypic imprinting). *C*: Concentration of the inhibitor extracted after its synthesis from the imprinted polymer (solid column) and the blank polymer (open column). The imprinted polymer was prepared using a molar ratio of template/TFMAA/DVB = 1/4/40. The inhibition constants for kallikrein are as indicated.

We observed that the MIP could produce more L enantiomer (67% more) than the D enantiomer, while the blank polymer yielded virtually equal amounts of the two enantiomers. Thus, we can conclude that the MIP cavities are themselves chiral, since they tend to direct the synthesis of products with the same stereochemistry as the initial template. This result is somewhat surprising in light of the relatively weak binding strength of the amine component that contains the asymmetric carbon atom. All products of the condensation reactions were tested for their ability to inhibit kallikrein. The lower yield of **3** and **4** are likely caused by some steric hindrance within the MIP cavities.

In the direct molding approach[8] kallikrein was chosen as a model enzyme to direct the formation of its own inhibitors. Previous studies along these lines also involved the enzyme trypsin.[9] It should be mentioned that related studies based on the self-assembly of small building blocks have been reported by other research groups utilizing either the dynamic combinatorial chemistry technique[10] or "click chemistry".[11]

To test the feasibility of using the active site of the enzyme directly to generate its inhibitors, kallikrein was first incubated with 2-(4-amidinophenylamino)-4,6-dichlorotriazine (building block A, Figure 1), followed by exposure to various amines (building blocks B, Figure 1).

As seen from Figure 3, several inhibitors could be synthesized in the presence of kallikrein. As a control, we used the strong protein inhibitor aprotinin to block the active site of

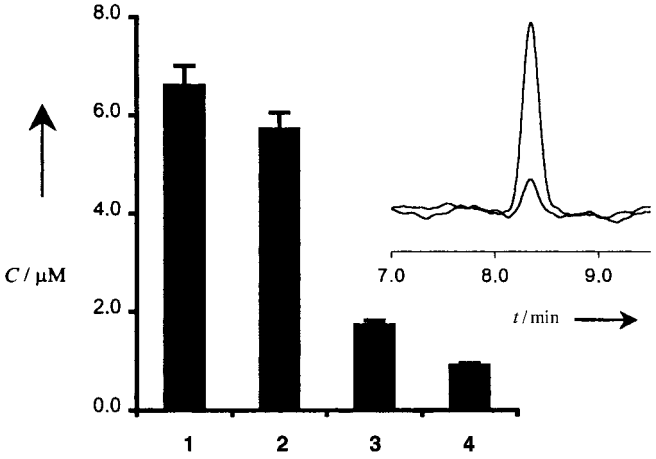

Figure 3. Enzyme-directed generation of inhibitors (direct molding). *C*: Concentration of the inhibitor obtained using kallikrein as a biotemplate. Insert: Chromatograms of **1** obtained using kallikrein as the biotemplate, in which the bottom trace represents the result of blocking the active site of the enzyme with one equivalent of aprotinin.

kallikrein, and we then proceeded to attempt the synthesis of **1**. HPLC chromatograms illustrating the formation of **1** in the presence and absence of aprotinin are also shown in Figure 3. As seen, the yield of **1** was dramatically reduced in the presence of aprotinin ($K_i = 1.0 \times 10^{-9}$ M). This result demonstrates the key role of the enzyme's active site in facilitating the synthesis of the inhibitor. This conclusion was further confirmed by substituting a nonrelated protein, bovine serum albumin (BSA), for kallikrein. The amount of **1** obtained was reduced by more than 90% when BSA was used.[12] It is interesting to note that kallikrein also showed some chiral selectivity in generating the inhibitors (**3** (L form) and **4** (D form)) in that twice the amount of **3** was formed compared to **4**. The K_i value of the D enantiomer is larger than that of the L enantiomer (47 and 33 μM, respectively), which supports the above finding of a higher yield of the L form and is consistent with a higher affinity of the L enantiomer with the enzyme compared to the D enantiomer.

In summary, we have described two novel techniques that may be useful for the discovery of new inhibitors and drugs. In

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the anti-idiotypic approach the MIP behaves as an enzyme or receptor binding site mimic and allows the screening of building blocks for the production of new compounds that mimic the biological activity of the original bioactive template. Three distinct advantages of this approach are: 1) there is virtually no need for detailed structural information or isolation of the real biological target; 2) it does not involve costly synthesis of a large amount of products; and 3) other functional monomers such as, for example, silanes might be used, the latter with possibly higher in vivo stability. It should also be emphasized that the MIPs prepared can be used repeatedly. In general, one can regard a MIP cavity as a single molecular reactor that guides, through its complementary size, shape, and chemical functionality, the condensation of small building blocks into a structure similar or identical to that of the initial template. With the "direct molding" approach, one would not have to rely on known bioactive molecules. However, the number of utilizable reactions is limited, because the side-chain functionalities of many amino acid residues of an enzyme/receptor may react with the building blocks. Clearly other additional applications, such as for the preparation of specific affinity ligands for separation, can be envisaged for both approaches.

Experimental Section

3: 2-(4-Amidinophenylamino)-4,6-dichlorotriazine (473 mg, 1.5 mmol) was dissolved in DMF (15 mL) and a solution of L-phenylalanine methyl ester (269 mg, 1.5 mmol) in DMF (7.5 mL) added. The mixture was stirred at 20–30 °C for 48 h, during which time the reaction was monitored by HPLC. After the reaction was completed, the solvent was completely removed by rotary evaporation (50 °C). The residue was then dissolved in chloroform/methanol (3/1, v/v), and purified by column chromatography on silica gel. Yield: 76 %. ^{1}H NMR (400 MHz, [D$_6$]DMSO): $\delta = 10.56$ (s, 1H; NH), 9.25 (s, 1H; NH), 8.95 (br s, 2H; NH), 8.02 (m, 1H), 7.80 (m, 3H), 7.4–7.15 (m, 5H; Ph), 4.78 (br d, 1H; NH), 3.71 (m, 4H; OCH_3, CH), 3.25 (dd, 1H, J = 13.5, 4.9 Hz; CH_A), 3.09 ppm (m, 1H; CH_B). Optical activity ($[\alpha]_{589}^{20}$) in methanol: +4.6.

Preparation of imprinted polymer: **3** (0.1 mmol), (2-trifluoromethyl)acrylic acid (TFMAA, 0.4 mmol), divinylbenzene (4.0 mmol), and recrystallized 2,2′-azobis(2-isobutyronitrile) (10 mg) were dissolved in anhydrous acetonitrile (0.5 mL) in a borosilicate glass tube equipped with a screw cap. The template could be dissolved in acetonitrile after addition of TFMAA, which indicated favorable molecular association between the template and TFMAA. The solution was purged with dry nitrogen for 5 min and sealed immediately. Polymerization was induced thermally at 60 °C and continued for 16 h. The resulting polymer monolith was ground manually and suspended in acetone. The slurry was forced through a 25-μm test sieve (Retsch, Germany). A second grinding was performed, if necessary, until all the particles passed through the test sieve. The particles with diameters of 10–25 μm were collected by repeated sedimentation in acetone. To remove the template, the MIP particles were extensively washed with methanol/acetic acid (9/1, v/v) until no UV adsorption at 300 nm was detected in the supernatant. The polymer particles were washed with acetone for several times and dried in a desiccator under vacuum. A nonimprinted blank polymer was prepared in the same way as that used for preparing the MIP, except that no template was used during the whole process.

MIP-directed generation of inhibitors (anti-idiotypic imprinting): Imprinted polymer (20 mg) or blank polymer (20 mg) was incubated with 40 μg of 2-(4-amidinophenylamino)-4,6-dichlorotriazine (building block A) in acetonitrile (1.0 mL) at 20 °C for 4 h. Polymers with bound 2-(4-amidinophenylamino)-4,6-dichlorotriazine (building block A) were separated by centrifugation (NANOSEP centrifugal concentrator, Gelman Laboratory, Michigan, USA, molecular weight cut-off 300 K) at 14000 rpm for 5 min. The polymers were resuspended in acetonitrile (1.0 mL), followed by adding the individual amine reactant (2-phenylethylamine: 152 μg, benzylamine: 134 μg, L-phenylalanine methyl ester: 224 μg, D-phenylalanine methyl ester: 224 μg). The mixture was gently stirred at 20 °C for 16 h. Centrifugal filtration was performed using the NANOSEP concentrator. To extract the product, the polymer particles were resuspended in 10 % acetic acid in MeOH (1.0 mL) and mixed at 20 °C for 2 h. After centrifugation (14000 rpm, 10 min), the concentration of the product in the supernatant (0.8 mL) was measured by HPLC analysis.

Enzyme-directed generation of inhibitors (direct molding): Kallikrein solution (0.4 mL at 1.0 mg mL^{-1} in tris(hydroxymethyl)aminomethane-hydrochloric acid (Tris-HCl) buffer, 50 mM, pH 7.0) was mixed with 2-(4-amidinophenylamino)-4,6-dichlorostriazine (0.6 mL at 1.0 mg mL^{-1} in Tris-HCl buffer, 50 mM, pH 7.0) in a 1.5-mL eppendorf tube and incubated at 20 °C for 4 h. Centrifugation was performed using a NANOSEP centrifugal concentrator (molecular weight cut-off 3 K) to remove excess of building block A. The kallikrein with bound building block A was transferred into Na_2CO_3/$NaHCO_3$ buffer (1.0 mL, 50 mM, pH 10.0), to which excess of amine (building block B, 10 mg mL^{-1}) was added separately (phenylethylamine: 30 μL, benzylamine: 34 μL, L-phenylalanine methyl ester: 44 μL, D-phenylalanine methyl ester 44 μL). After incubation at 20 °C for 48 h, a NANOSEP centrifugal concentrator (molecular weight cut-off 3 K) was used to remove the excess of free amine, and the kallikrein–inhibitor complex was transferred into 6.0 M guanidine hydrochloride (1.0 mL) and incubated at 20 °C for 2 h to disassociate the inhibitor from kallikrein. After centrifugation (NANOSEP, molecular weight cut-off 3 K), the filtrate was analyzed by HPLC for product quantification. When aprotinin was used to block the active site of kallikrein, it was added to the enzyme solution and incubated for 2 h prior to the addition of the building blocks.

HPLC analysis: Reverse-phase HPLC analysis was carried out by using a LaChrom L-7100 solvent delivery system, a L-7455 diode array detector, and a software package D-7000 HPLC System Manager (Merck KgaA, Darmstadt, Germany). A chromolith performance column (RP-18 e) from Merck (Darmstadt, Germany) was used with a gradient elution (0–10 min, 20–50 % acetonitrile in water, both containing 0.1 % trifluoroacetic acid) at a flow rate of 1.0 mL min^{-1}. For each analysis 20 μL of sample was injected.

Enzyme assays: The inhibitory activities of the compounds against porcine pancreatic kallikrein were determined from their effect on the enzyme-catalyzed hydrolysis of the chromogenic substrate D-Val-Leu-Arg-4-nitroanilide. Each assay (total volume of 1 mL) contained: *N*-tris(hydroxymethyl)methylglycine (tricine)/NaOH buffer (100 mM, pH 8.2, 940 μL); D-Val-Leu-Arg-4-nitroanilide (20–60 nmol in 20 μL of water); the inhibitor (2.5–20 nmol in a mixture of 10 μL of DMSO and 10 μL of water); and porcine pancreatic kallikrein (4 μg in 20 μL of water). The change in absorbance at 410 nm was monitored at 20 °C to determine the initial rates (ρ) of hydrolysis. Assays were carried out at a minimum of five inhibitor concentrations ($[I]$) and three substrate concentrations. The inhibition constant (K_i) was determined from the Dixon plot ($1/\rho$ versus $[I]$), where K_i was taken as the absolute value of $[I]$ that corresponded to the intersection point of a series of curves at different substrate concentrations.[13]

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Facile Assembly of Cell Surface Oligosaccharide Mimics by Copolymerization of Carbohydrate Modules**

Kenji Sasaki, Yoshihiro Nishida,* Tomoaki Tsurumi, Hirotaka Uzawa, Hirosato Kondo, and Kazukiyo Kobayashi*

Artificial glycoconjugate polymers (glycopolymers) and other multivalent carbohydrate ligands constitute a new class of biomimetic supramolecules.[1] They have shown many biological applications in, for example, cultivation, tumor diagnosis and detection, and the trapping of viruses and bacterial toxins.[2] Their utility is ascribed mainly to their strong and species-specific interactions with the receptor proteins as a result of multivalent binding and/or carbohydrate cluster effects. Although most of the glycopolymers so far prepared and applied were made up of simple mono- and disaccharides, more intense interest has been directed to glycopolymers carrying cell-surface oligosaccharides such as sialyl Lewis^X (sLe^X)[3] and globosyl oligosaccharides[4] that have potentially greater biological significance. From a practical viewpoint, however, the synthesis and application of these glycoconjugates are seriously restricted by the difficulty in preparing target oligosaccharides in sufficient amounts prior to their incorporation into the multivalent models.

In the course of our synthetic study of glycopolymers carrying the mimics of cell-surface oligosaccharides,[5] we found recently that an acrylamide copolymer carrying α-L-fucopyranoside and 3-sulfo-β-D-galactopyranoside as a side chain shows strong activity in blocking L-selectin/sLe^X tetrasaccharide adhesion.[6] As judged from the observation that none of the acrylamide copolymers carrying only one of the two glycosides showed a notable activity, the observed activity was ascribed to the cooperative binding of the α-L-fucoside and 3-sulfo-β-D-galactoside to L-selectin. This finding indicated the potential utility of the module approach and prompted us to generalize it as a "carbohydrate module method".

For the facile understanding of our "carbohydrate module method", let us assume a model consisting of the binding of a branching pentasaccharide with the receptor protein (Figure 1). In this model the two glycoside residues (A1 and A2) at the nonreducing terminal provide key binding interactions with the receptor protein at the *r*(A1) and *r*(A2) binding sites. Conventional mimic syntheses may target a branching trisaccharide carrying A1, A2, and B residues, thus developing the multivalent model (polymer-(A1 + A2 + B)) in Figure 1b. Our approach targets a copolymer-(A1/A2) species carrying the key interactive sugars (Figure 1c) to circumvent the difficulty in preparing the branching saccharide. The copolymer-(A1/A2) species is assumed to have a certain probability of occupying both of the binding sites and, thus, is expected to show a higher binding activity than the polymers carrying only one of the key sugars (poly-(A1) and poly-(A2)). This situation means that copolymerization of two key interactive sugars (A1 and A2 in the present case) provides a facile way to mimic the biologically active structures of oligosaccharides. The glycopolymers thus derived may show potent biological activity in blocking the binding of the oligosaccharide to receptor proteins.

The present approach involves the following three steps: segmentation of a targeted oligosaccharide into smaller sugars, synthesis of the corresponding glycosylated monomers (defined as "carbohydrate modules" in this study), and the reassembly of oligosaccharide mimics by copolymerization of the modules. Previously, we employed this process for sLe^X tetrasaccharide **1a**, which was segmented into modules of allyl α-L-fucoside and *p*-acrylamidophenyl 3-sulfo-β-D-galactopyranoside to afford the copolymer (**2**) with potent activity in blocking L-selectin/sLe^X binding.[6] In the present study, we took the module approach to 6-sulfo-sialyl Lewis^X (6-sulfo-sLe^X) tetrasaccharide **1b** which has a 6-sulfo-GlcNAc at the reducing residue. The biological significance of **1b** is evident from recent studies[7] in which it was substantiated that the 6-sulfo analogue **1b** is a better ligand than sLe^X (**1a**) for L-selectin. We aimed to not only prepare multivalent 6-sulfo-Le^X mimics but also to investigate the possible role of the 6-sulfo-GlcNAc residue in binding to L-selectin.

The oligosaccharide sequence was divided into Lewis^X (Le^X), sLe^X, and 6-sulfo-GlcNAc to investigate key epitopes for L-selectin. To construct the library of Le^X, sLe^X, and 6-

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