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Bioorganic & Medicinal Chemistry
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Bioorganic & Medicinal Chemistry 12 (2004) 1111–1120

Sialyllactose-binding modified DNA aptamer bearing additional functionality by SELEX

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Received 23 October 2003; accepted 3 December 2003

Abstract—We produced a novel cationic-charged modified DNA aptamer for sialyllactose that is a ubiquitous component of the cell surface responsible for the infection of several viruses by using the magnetic-particle-based SELEX method. After 13 rounds of selection we selected 22 clones as sialyllactose-binding DNA aptamers composed of several modified thymidines. The DNA aptamers could form a three-way junction structure that likely forms a binding site for siallyllactose. The three-way junction structure contains several modified thymidines bearing a positively-charged amino group at the C5 position, which could enhance the binding ability for silalyllactose which has a negatively-charged carboxyl group. The dissociation constant of the aptamer that showed the strongest sialyllactose-binding ability among the clones of the aptamers was 4.9 μM.

1. Introduction

Carbohydrates are important mediators in the specific recognition and adhesion between cells.[1] Sialyllactose (α-Neu5Ac-[2-3]- and-[2-6]-β-D-Gal-[1-4]-D-Glc) (Fig. 1) is a sugar head group of the GM3 ganglioside, sialic acid (*N*-acetylneuraminic acid) containing glycosphingolipid, which is a ubiquitous component of mammalian plasma-cell membranes.[1,2] This oligosaccharide appears to be an essential receptor component of many animal viruses from different families, such as influenza A and C viruses, Newcastle disease viruses, cardioviruses, and murine and primate polyoma viruses.[3] For such viruses, blocking of the sialyllactose of cell surfaces could lead to the loss of virion binding and inhibition of the initial step of viral infection. Thus, a compound that binds with sialyllactose specifically with high affinity will be useful as a tool for the studies of cell adhesion and viral infection, and as an anti-viral agent.

Repeated cycles of selection and enzymatic enrichment are a powerful method to produce RNA or single-stranded DNA molecules which bind to a specific target molecule. This methodology is called 'systemic evolution of ligands by exponential enrichment (SELEX)' or 'in vitro selection', and the RNA or ssDNA thus selected are termed aptamers.[4–8] A number of RNA or DNA aptamers that specifically recognize many kinds of target molecules, such as simple ions,[9] small molecules,[7] peptides,[10] single proteins,[8] organelles,[11] viruses,[12] and even entire cells[13] have been obtained by SELEX. However, an aptamer is rarely obtained from a modified DNA bearing an additional functional group,[14,15] because the modified DNA produced by PCR (polymerase chain reaction) is limited.

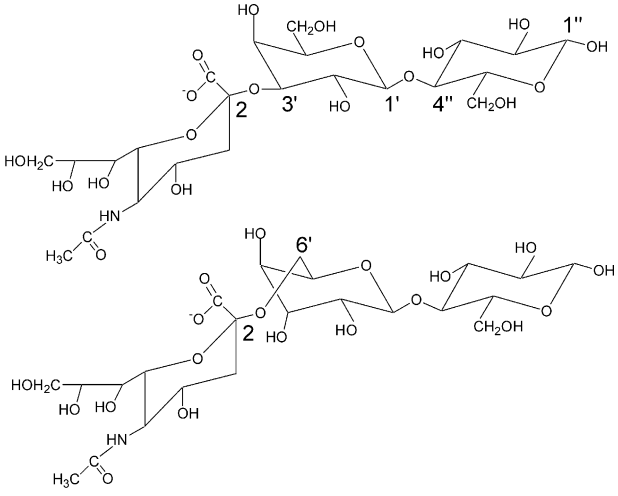

Figure 1. Structure of sialyllactose.

Keywords: Modified DNA; Aptamer; Sialyllactose; Thymidine analogue.
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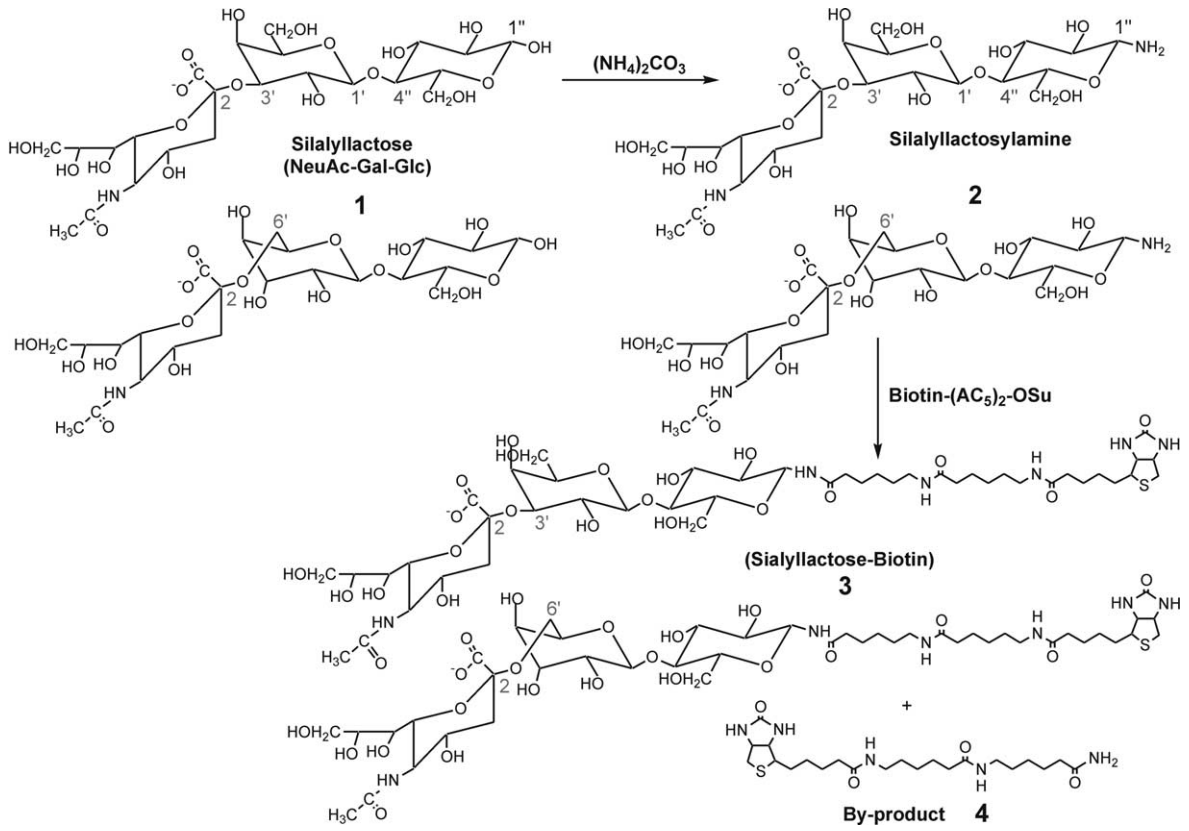
doi:10.1016/j.bmc.2003.12.009



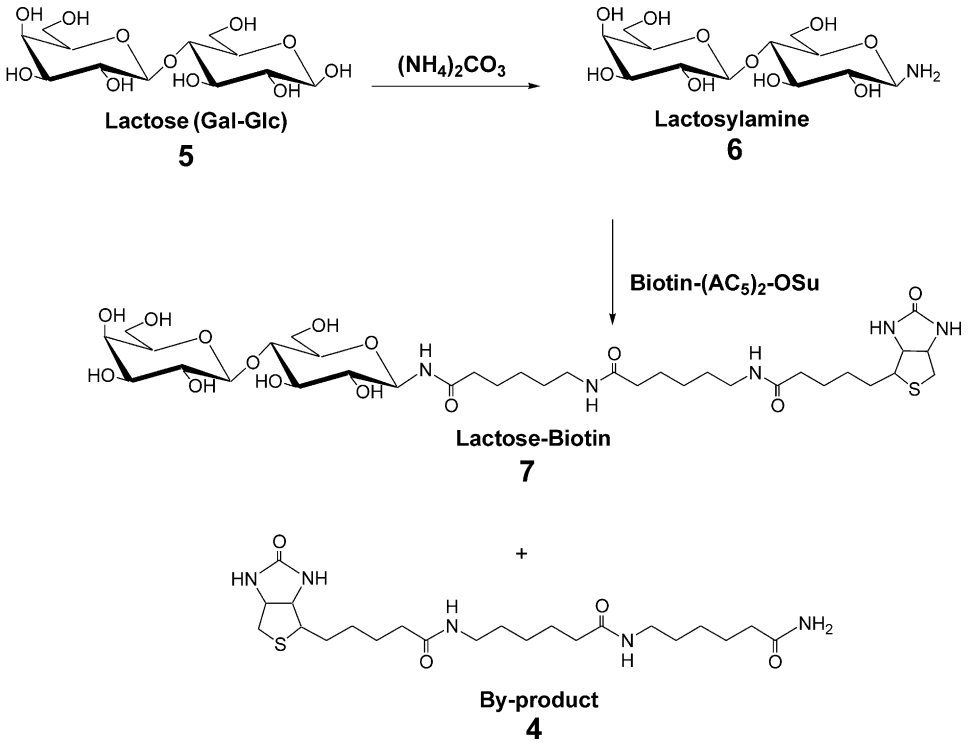
Scheme 1. Synthesis of sialyllactose-biotin conjugate.

Modified oligonucleotides with thymidine analogues bearing a functional group at the C5 position are useful for biological and biochemical studies.[16–20] Previously we reported that triphosphate of thymidine analogues bearing an amino linker at the C5 position could be accepted as a substrate for PCR when using KOD Dash DNA polymerase to form the corresponding DNA.[21] The resulting modified DNA could be used as a DNA aptamer by SELEX. Thus, we have decided to produce the modified DNA aptamer that binds to sialyllactose with high affinity. The modified DNA has a cationic protonated-amino group at the C5 position of the thymidine residue, which could enhance binding with sialyllactose that has an anionic carboxyl group. The resulting aptamers may block virion binding to cell surfaces. In this paper, we report the selection of the modified DNA aptamer that binds to sialyllactose using the sialyllactose-conjugated magnetic particles.

2. Results and discussion

Magnetic-particle-based SELEX was employed to select a sialyllactose-binding aptamer from a library of modified DNA bearing a cationic amino group at C5 position of the thymidine residue. For SELEX, sialyllactose was conjugated with biotin, as shown in Scheme 1, and immobilized on streptavidin-magnetic particles by a biotin-streptavidin interaction. First, sialyllactose (**1**) was converted to sialyllactosylamine (**2**) as described previously.[22] Then, **2** was used in a reaction with biotin-$(AC_5)_2$-OSu to obtain sialyllactose–biotin conjugates (**3**). Conjugate **3** was purified by preparative HPLC. The yield of **3** was 22.5%. The by-product (**4**), which could be formed from biotin-$(AC_5)_2$-Osu with a contaminated ammonium ion, was also purified by HPLC and used for negative selection. We also synthesized lactose-biotin conjugate (**7**) from lactose by the same procedure shown in Scheme 2 and used it for negative selection. The glyco-conjugates can be prepared in a mild and simple way from free oligosaccharides via glycosylamine. The anomeric hydroxyl group of a terminal sugar is converted to an amino group with the β-linkage. The resulting terminal amino group of the oligosaccharide undergoes chemo-selective acylation with an activated ester of the conjugating agent. This sugar-conjugation method is superior to the conventional method in which saccharides react directly with an activated ester or epoxy group of the conjugating agents at several hydroxyl sites. Next, conjugate **3** was immobilized on streptavidin-magnetic particles. The quantity of **3** immobilized onto the streptavidin particles was estimated by the quantification of the unbound **3** using a biotin-titration method.[23] 1.8 nmol of **3** was bound to 10 mg of the streptavidin magnetic particles. Similarly, we prepared **4**-streptavidin particles and **7**-streptavidin particles to use for negative selection.

We used the **3**-streptavidin particles for SELEX to produce a sialyllactose-binding aptamer from a library of a 102mer-modified DNA. The modified DNA library contained a central stretch of 60 random nucleotides and two 21mer-primer regions. The modified DNA was prepared by symmetric and subsequent asymmetric



Scheme 2. Synthesis of lactose-biotin conjugate.

PCR using a thymidine derivative bearing a terminal amino group at the C5 position (Fig. 2) and three other natural nucleotides with KOD Dash DNA polymerase, by the modification of the published procedure.[21] All thymidine residues were replaced with the modified thymidine in the resulting DNA, except for the primer 1 region. In the initial pool we used ~1.5 nmol modified ssDNA. Tris–HCl buffer (50 mM, pH 7.6) containing 250 mM NaCl and 5 mM $MgCl_2$ was used as a binding/selection buffer during SELEX. In the first 3 rounds a very small amount of loaded ssDNA was bound to the target. A significant increase in binding to sialyllactose was observed in the subsequent rounds. In round 5, negative selection was carried out with compound **4**, and in rounds 7, 8, 10, 12 and 13 with compound **7**, before positive selection with **3** to remove the ssDNA that binds to **4** and **7** on the magnetic particles. After 13 rounds of selection, 0.4 nmol of 1 nmol of input ssDNA was bound to 0.8 nmol of sialyllactose on the magnetic particles. The use of magnetic particles for affinity separation of aptamers from a DNA library has several advantages such as easy manipulation and small sample volumes. However, this SELEX method requires heating to elute aptamers from the target-conjugated magnetic particles surfaces and careful separation of the magnetic particles by a magnetic particle separator.

Twenty-two clones were selected and sequenced after 13 rounds of selection to define a common sequence motif responsible for binding (Table 1). After searching for a sequence-match among the 22 clones, we grouped them into 6 groups. Clones of each group show a similarity in sequence except for group 1. Two clones were grouped into group 1 and they show no similarity in the randomized region sequence. The sequences of the clones grouped into 4 and 5 were almost the same except for a few bases in both sides of the randomized (60 nt) region, and 12 clones were grouped into these two groups, which were 55% of the isolated aptamers. Thirteen among the 22 clones contained exactly 60 nucleotides in the randomized region whereas 5 clones contained one excess base (61 nt). Four clones contained 36–39 nucleotides in the randomized region instead of the initial 60 nt and were grouped into group 6. This increase or decrease in number may be due to addition or deletion during the selection-PCR cycles. Randomized regions of almost all of the clones are comparatively C-rich, except clones SL-1, SL-17 and SL-18, which are G-rich.

Estimation of the secondary structure of several isolated clones from Group 1 to 5 (SL-1, SL-2, SL-3, SL-4, SL-5, SL-8 and SL-11), was done by the single-stranded nucleic acid folding program, GENETYX-MAC. As shown in Figure 3, all clones estimated are composed of a three-way junction except for clone SL-2, which has a stem-loop structure. The primer region of the clones also participates in the formation of the three-way junction. One to three modified Ts are present at the junction region and several modified Ts at the stem regions. The three-way junction structure containing the

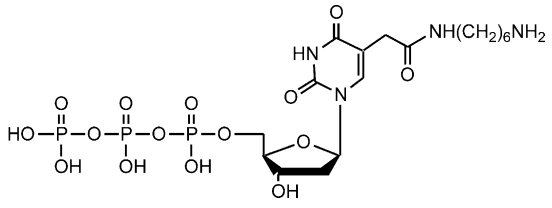


Figure 2. Structure of modified TTP ($pppT^{HM}$).

Table 1. Sequences in the randomized region of selected DNA aptamers

Clone no		Random sequence region	%A	%T	%C	%G
Group 1						
(1)	SL-1	GAGCGTGCGCTTGCCTGCGTGTAGAGTCCGTCAACTCGCTCAGTGGGTGGGTGGGTGGTG	10	25	21.7	43.3
(1)	SL-2	GCGGGGCCCGTGTGCGTTGGTCGAATTCGTGTACACTTCTCGCCTGTCTACCAGCCAGCC	11.7	25	33.3	30
Group 2						
(1)	SL-3	TCGCTCGCGTGATAGAA**TTGAACTCACCACCTCTTGGGTAGTGTCCCGCCCGCCGAGT**CC	16.7	23.3	35	25
(1)	SL-4	TCGCTCGCGTGCTCGTC**TTGAACTCACCACCTCTTGGGTAGTGTCCCGCCCGCCGAGT**GG	10	25	36.7	28.3
Group 3						
(1)	SL-5	**GCTGCGGTAGGTGAACGGTAACGGCAAACTTCGCATGCTTGCTCGCCTGCTCGCCCCTC**C	15	21.7	35	28.3
(1)	SL-6	**GCTGCGGTAGGTGAACGGTAACGGCAAACTTCGCATGCTTGCTCGCCTGCTCGCCCCTC**GC 61nt	14.8	21.3	34.4	29.5
Group 4						
(1)	SL-7	**CCCGGGGCCAGCCACTTC**C**GTCAGTGAATTCCTGCTCGTATATCTACTCGCCCGCCTGCC**	13	23.3	41.7	21.3
(3)	SL-8	**CCCGGGGCCAGCCACTTCTGTCAGTGAATTCCTGCTCGTATATCTACTCGCCCGCCTGCC**	13	25	40	21.7
(1)	SL-9	**CCCCGGGGCCAGCCACTTCTGTCAGTGAATTCCTGCTCGTATATCTACTCGCCCGCCTGCC** 61nt	13	24.6	41	21.3
Group 5						
(2)	SL-10	**AGCGGGGCCAGCCACTTCTGTCAGTGAATTCCTGCTCGTATATCTACTCGCCCGCCTGCC**	15	25	36.7	23.3
(2)	SL-11	**AGCGGGGCCAGCCACTTCTGTCAGTGAATTCCTGCTCGTATATCTACTCGCCCGCCTGCG**	15	25	35	25
(1)	SL-12	**AGCGGGGCCAGCCACTTCTGTCAGTGAATTCCTGCTCGTATATCTACTCGCCCGCCTGGGG** 61nt	14.8	24.6	32.8	27.9
(1)	SL-13	**AGCGGGGCCAGCCACTTCTGTCAGTGAATTCCTGCTCGTATATCTACTCGCCCGCCTGGGC** 61nt	15	24.6	34.4	26.2
(1)	SL-14	**AGCGGGGCCAGCCACTTCTGTCAGTGAATTCCTGCTCGTATATCTACTCGCCCGCCTGCGG** 61nt	14.8	24.6	34.4	26.2
Group 6						
(1)	SL-15	CGCGCCCCCTCAGCCCT**GTACGAATTCACGAGGTTGCC** 38nt	15.8	18.4	42.1	23.7
(1)	SL-16	CCGGGCCGCCCCCCT**GTACGAATTCACGAGGTTGCC** 36nt	13.9	16.7	41.7	27.8
(1)	SL-17	CAGGTCGGGGGGGGCCCC**GTACGAATTCACGAGGTTGCC** 39nt	15.4	15.4	28.2	41
(1)	SL-18	GAGGTCGGGGGGGGCCCT**GTACGAATTCACGAGGTTGGG** 39nt	15.4	17.9	17.9	48.7

22 clones were classified into 6 groups based on the matching of sequence. On the left side, the number of clones with identical sequences are shown in parentheses, and on the right, the frequency of occurrence for each bases is shown. Similar sequence among the clones in each groups are shown in bold. Sequence similarity between group 4 and 5 are shown by background highlighting.

modified T likely forms a binding site for sialyllactose. The modified T has a positively-charged amino group at the C5 position, that could enhance the binding ability for silalyllactose which has a negatively-charged carboxyl group.

Six clones from different groups (SL-1, SL-2, SL-3, SL-5, SL-8 and SL-11), were subjected to a binding assay to compare their binding ability. The binding assay was carried out by incubation of the isolated DNA aptamer with the sialyllactose-magnetic particles, and separation and quantification of the unbound DNA. The binding ability, which was expressed as a percentage of the bound DNA to the input DNA, is listed in Table 2. Clones, SL-8 from group 4 and SL-11 from group 5, which have essentially the same sequence, had a stronger binding ability compared to the other clones. SL-11, which has the strongest binding ability among the isolated clones, showed 93% binding ability. We also prepared and determined the binding ability of SL-11-Natural, which contains the exact sequence of SL-11 but does not have any modified T. The SL-11 showed stronger affinity to sialylylactose than the unmodified SL-11-Natural. This result suggests that a positively-charged amino group at the C5 position of modified T in SL-11 could enhance its binding ability for silalyllactose which has a negatively-charged carboxyl group. We estimated the dissociation constant of SL-11 to sialyllactose to be 4.9 μM by the equilibirium filtration method.[24]

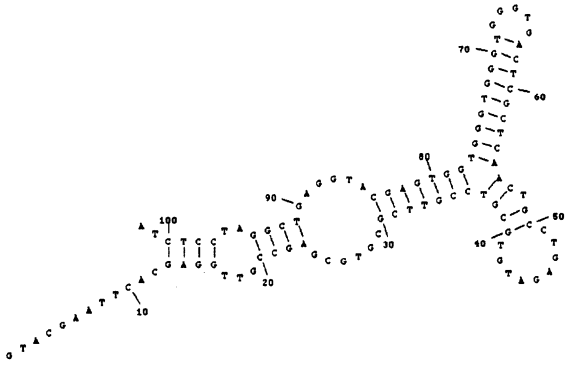
Table 2. Relative binding ability of modified DNA aptamer to sialyllactose

ssDNA	SL-11	SL-1	SL-2	SL-3	SL-5	SL-8
% Binding DNA	93±4	56±7	79±1	71±1	64±3	86±4
ssDNA	SL-11-Natural	J1	J2	J3		
% Binding DNA	64±2	60±9	31±1	42±2		

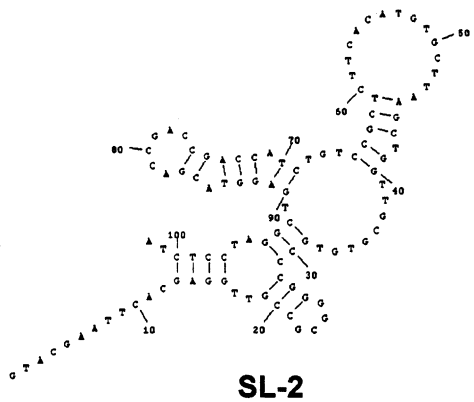
% Binding DNA is expressed by the percentage of the bound DNA to sialyllactose-magnetic particles to the input DNA. Mean value and standard deviation were estimated from two to five determinations.

The secondary structure of the SL-11 clone indicates that it is composed of three kinds of three-way junctions, (J1, J2 and J3) as shown in Figure 4. In order to know the role of each three-way junction in the binding of SL-11 to sialyllactose and to explore a short-sized DNA aptamer for sialyllactose, we prepared three truncated DNAs, (J1, J2 and J3) and measured their binding ability. The J1, J2 and J3 DNAs have binding ability, as shown in Table 2, although the ability is lower than SL-11. Among the truncated DNAs, the shortest 42mer J1 DNA has considerably strong binding ability. The three-way junction structure was also reported to be a binding motif of the DNA aptamer for several low-molecular-weight ligands.[25,26] Further studies will be needed to determine the exact binding site for sialyllactose.

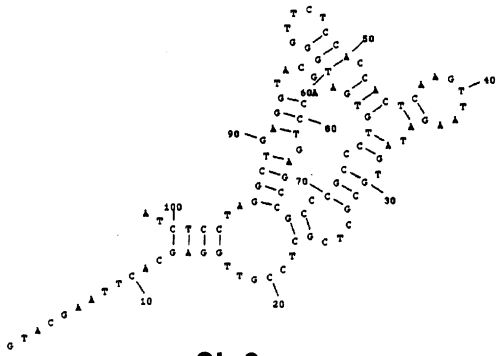
Some sugar molecules can specifically bind to RNA aptamers, as demonstrated by the selection of aminoglycoside-binding RNA aptamers.[27–29] Selection of the RNA aptamers against aminoglycosides could have been relatively easily achieved due to the electrostatic interaction between the protonated amino functionality in aminoglycosides and the negatively- charged phosphate backbones in the RNA molecule. The selection of RNA aptamers that bind to other sugars, such as the



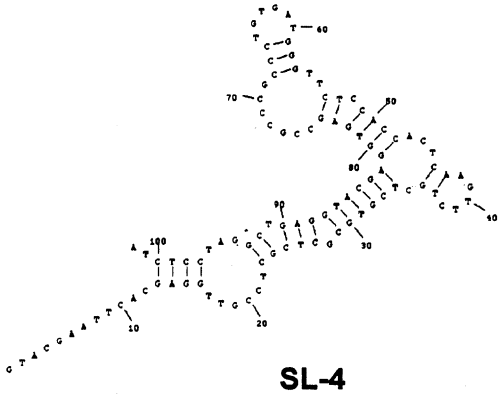
SL-1



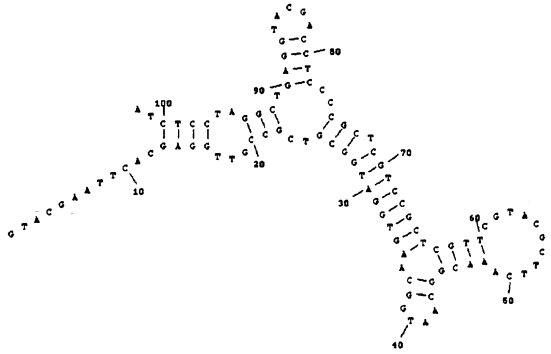
SL-2



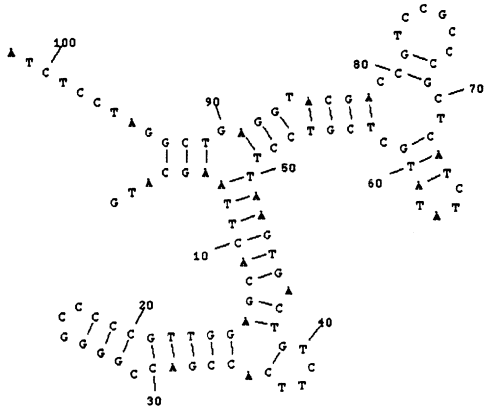
SL-3



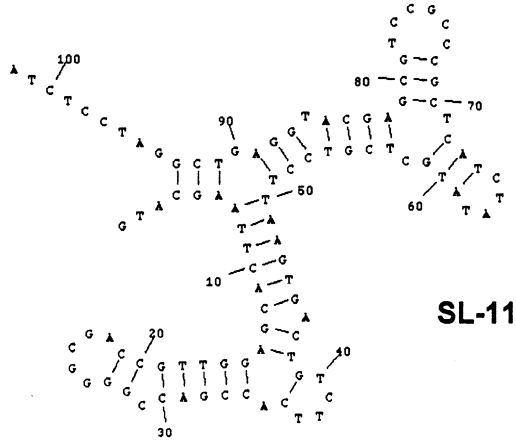
SL-4



SL-5



SL-8



SL-11

Figure 3. Secondary structures of selected aptamers.

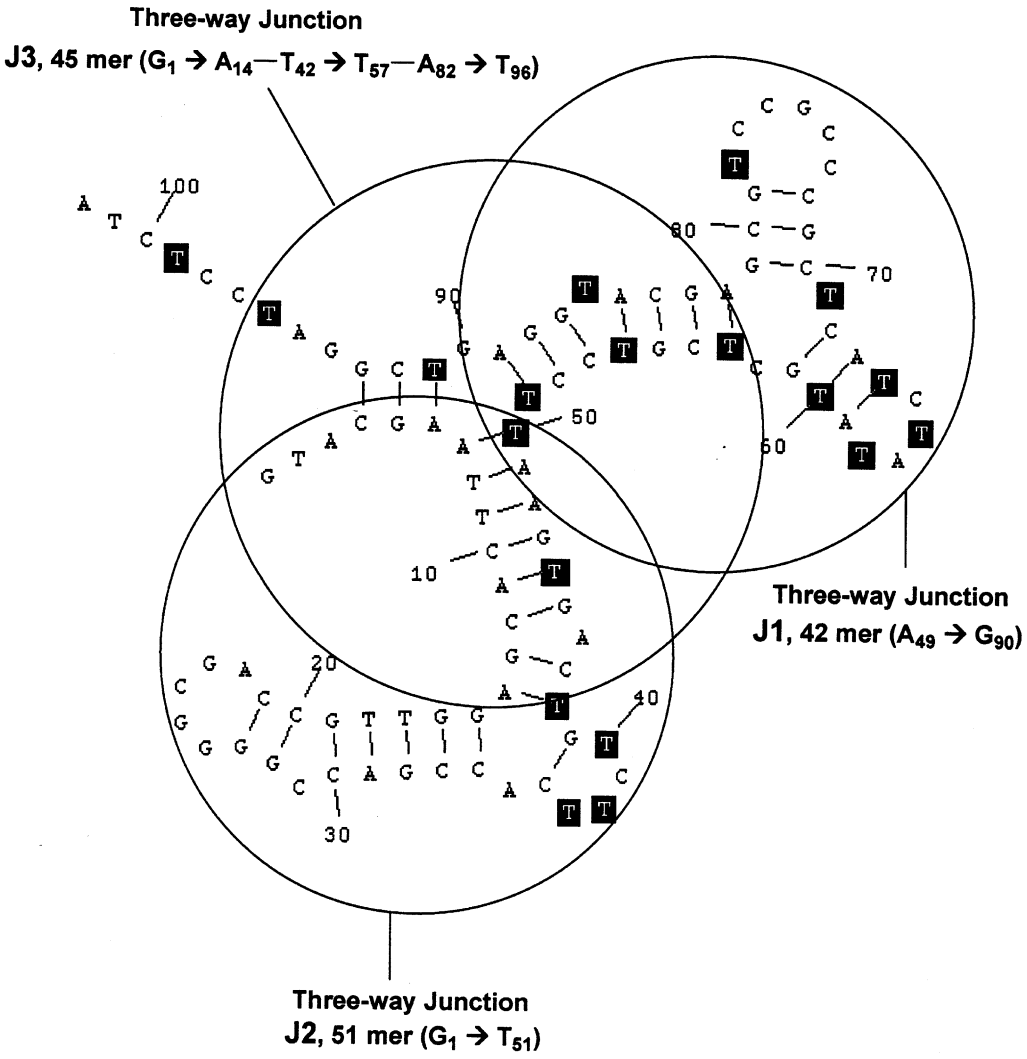


Figure 4. Secondary structure of SL-11, J1, J2 and J3 aptamers. Modified Ts are highlighted by black square box.

cellobiose unit of cellulose[30] and Sialyl Lewis X,[31] and DNA aptamers that bind to a neutral polysaccharide chitin have been also reported.[32] However, the interaction of DNA molecules with the carbohydrate in the cell surface has been rarely studied, in spite of their potency as therapeutic agents. In this study, we utilized the magnetic particle-based SELEX method to develop a novel modified DNA aptamer for sialyllactose of the cell surface. After 13 rounds of selection we selected 22 clones as sialyllactose-binding DNA aptamers containing modified substrate. Among the clones of the sialyllactose-binding aptamers, SL-11 has the strongest binding ability and its dissociation constant was 4.9 μM. We are currently investigating the precise sequence requirements of the selected sialyllactose binding aptamers, their specificity to other sugars and the cell-adhesion inhibitory activity.

3. Conclusion

We could obtain a specific aptamer for sialylylactose from a library of modified DNA bearing a cationic-charged amino group using SELEX. The DNA aptamer forms a three-way junction structure that could be a binding site for siallyllactose. The three-way junction and stem regions contain modified thymidines bearing a positively-charged amino group at the C5 position, that could enhance the binding ability for sialyllactose which has a negatively-charged carboxyl group. To our knowledge, this is the first report on the selection of a modified DNA aptamer that has micromolar affinity for sialyllactose, the cell surface carbohydrate. The high-affinity sialyllactose-specific DNA aptamer might be useful for studies of cell-adhesion and the development of a lead molecule for cell-adhesion-blocking anti-viral therapy.

4. Experimentals

4.1. Materials

Sialyllactose (α-Neu5Ac-[2-3]- and-[2-6]-β-D-Gal-[1-4]-D-Glc; sodium salt from human milk) was purchased from Sigma. Biotin-$(AC_5)_2$-Osu, 5-[5-(*N*-succinimidyloxycarbonyl)pentylamido]hexyl D-biotinamide, from Dojindo Laboratories and streptavidin magnetic parti-

cles from Roche. Templates and primers for the synthesis of DNA by PCR were purchased from Sawady Technology Co and Japan Bio Service Co. KOD Dash DNA polymerase was from Toyobo and Taq DNA polymerase from Takara. 5′-Triphosphate of 5-*N*-(6-aminohexyl)carbamoylmethy-2′-deoxyuridine ($pppT^{HM}$, Figure 2) was prepared as reported previously.[33,34] Linear polyacrylamide (LPA) was from Sigma, dialysis bag (Spectra/Por® molecular porous membrane tubing, MWCO: 6-8,000) from Spectrum Laboratories, Inc., glass fiber filter paper from Advantec and Biomax-5K NMWL membrane centrifugal filter device from Millipore. For cloning and sequencing, a ligation kit from Takara, a gene-elute plasmid mini-prep kit from Sigma, and a transformation and sequencing kit from Invitrogen. All other chemicals were reagent grade and used without further purification.

4.2. Method

ESI-Mass spectra were recorded on a MDS-Sciex API-100 mass spectrometer from Applied Biosystem. HPLC on an ODS silica gel column (4×250 mm) was carried out with a linear-gradient elution (7–70%) of acetonitrile in 50 mM triethylammonium acetate (pH 7.0) over 40 min at a flow rate of 1 mL/min using a JASCO PU-2080 Plus HPLC machine. The UV spectrum was measured using a Shimadzu UV-1200 spectrometer. Sequencing was carried out using an ABI PRISM™ 310 Genetic analyzer (Applied Biosystem) according to ABI's sequencing protocol. Quantification of DNA on the gel was done by a Molecular imager® FX (Bio-Rad).

4.3. Synthesis of a sialyllactose–biotin conjugate

The sialyllactose–biotin conjugate was prepared as shown in Scheme 1. At first, sialyllactose was converted to sialyllactosylamine by the published procedure.[22] Sialyllactose, 17 mg in saturated aqueous ammonium carbonate (1%, w/v) was stirred at room temperature for 5 days. During the course of the reaction, solid $(NH_4)_2CO_3$ [ca. 40 mg/(mg of sialyllactose)] was added to the solution in fractions to ensure saturation. The reaction was monitored by TLC (silica gel K 60, 1-propanol/ethyl acetate/water, 6:1:3, detection with orcinol and ninhydrin reagents). After the conversion, samples were lyophilized for 3 days. The crude sialyllactosylamine was dissolved in warm methanol (ca. 70 °C) and stirred to remove excess solid $(NH_4)_2CO_3$. Methanol was slowly evaporated after termination of CO_2 evolution and the residual material was dried in a rotary evaporator producing sialyllactosylamine, 16 mg (94%). The product was confirmed by mass spectra and compared with that of sialyllactose {sialyllactosylamine, 631.3 (M−H)−; sialyllactose, 632.3 (M−H)−}.

The solution of sialyllactosylamine (16 mg, 1 eq.) and biotin-$(AC_5)_2$-OSu (28 mg, 2 eq.) in DMSO (375 μL) was stirred for 2 h at room temperature. The reaction was monitored by TLC (silica gel K 60, 1-propanol/ethyl acetate/water, 6:1:3, detection with orcinol and ninhydrin reagents). After complete conversion, the product was precipitated with acetone/ether (1:2, 6 mL). Following centrifugation, the residue was washed with acetone/ether (1:1, 6 mL) and dried by a rotary evaporator. Mass spectra of the resulting residue showed that it contained by-product (**4**) in addition to the target compound **3**. We purified **3** by preparative HPLC and checked it by mass spectrum {1083.4 (M−H)−}. The yield of **3** was 6 mg (22.5%). Similarly, we prepared lactose–biotin conjugate (**7**), 2 mg (12.5%) (Scheme 2) and checked it by mass spectrum {792.4 (M−H)−}.

4.4. Conjugation of sialyllactose-biotin to streptavidin magnetic particles

After 1 mL of the streptavidin magnetic particles (10 mg/mL) suspension was washed three times (1 mL×3) with the binding buffer (50 mM Tris–HCl, pH7.6, 250 mM NaCl, 5 mM $MgCl_2$). 6 μg (5.5 nmol) of sialyllactose-biotin was added (10 mg streptavidin magnetic particles can bind with 2 nmol biotin compound) and the mixture was agitated for 3 h at room temperature. Then the mixture was washed 3 times (1 mL×3) with the binding buffer using a magnetic particle separator and collected by careful siphoning and kept at 4 °C. To determine the amount of unbound **3**, we constructed a calibration curve for quantification of a biotin compound by the titration method.[23] 1 mL of a solution of avidin-HABA (4-hydroxyazobenzene-2′-carboxylic acid) complex (0.3 mg avidin/mL of 0.25 mM HABA in 0.1 M sodium phosphate buffer, pH 7) was taken into a 1-cm cuvette. Biotin was added in small aliquots (0.05 μg/5 μL) each time with a Hamilton microsyringe, and the absorbance was measured at 500 nm, and a calibration curve for absorbance versus the amount of biotin was constructed (data is not shown). After measuring the amount of the unbound **3**, we found that 2 μg (1.8 nmol) of **3** was bound to the 10 mg of streptavidin magnetic particles. Similarly, we prepared conjugates of streptavidin magnetic particles with **4** and **7** (10 mg streptavidin magnetic particles bound with 2 nmol of **4** and **7**, respectively).

4.5. DNA pool

For the initial selection, synthetic 102 mer oligonucleotides with a random region of 60 nucleotides, 5′-GTA CGA ATT CAC GAG GTT GCC $(N)_{60}$ AGC ATG GAG TCG GAT CCT CTA-3′, were amplified over 10 cycles of PCR (94 °C, 30 s; 60 °C, 30 s; 74 °C, 1 min) using primer 1, 5′-GTA CGA ATT CAC GAG GTT GCC-3′ and primer 2, 5′-TAG AGG ATC CGA CTC CAT GCT-3′. We used modified $pppT^{HM}$ instead of natural TTP during PCR. 200 μL of the PCR mixture contained 1 nM template, 0.2 mM dNTPs (modified $pppT^{HM}$ instead of natural TTP), 0.4 μM primers, 5 units of KOD Dash DNA polymerase, 20 μL 10× buffer for KOD Dash (pH 8.8). The dsDNA containing the PCR mixture (200 μL PCR mixture) was used as a template for the synthesis of ssDNA by an additional 25 cycles of asymmetric PCR using only primer 1 (1 μM). 2 mL of the PCR mixture contained 200 μL PCR-DNA mixture as a template, 1 μM P1, 0.2 mM dNTPs (modified $pppT^{HM}$ instead of natural TTP), 50 units of KOD

Dash DNA polymerase and 200 μL 10× buffer for KOD Dash (pH 8.8). The thermal cycling was the same as that for the dsDNA synthesis described above. The PCR products were precipitated with 100% ethanol, 3 M sodium acetate (pH 5.2) and linear polyacrylamide according to the manufacturer's protocol. The precipitated ssDNA mixtures were purified on a 10% non-denaturing polyacrylamide gel. The bands were visualized by UV-shadowing and cut out. Then the ground gels were put into a dialysis bag with 1 mL of 0.5×TBE buffer and the ssDNA was eluted from the gel after gel electrophoresis in 0.5×TBE buffer for 3 h. The eluted ssDNA was collected from the dialysis bag and filtered through glass fiber filter paper to remove the ground gels. Then the ssDNA solution was dialyzed overnight again in water using a dialysis bag to remove the buffer. Finally we obtained (~1.5 OD = 1.5 nmol) ssDNA and used this for selection.

4.6. Magnetic particle-based SELEX

For each round of selection, the 102mer ssDNA (~1.5 nmol) with a random insert of 60 nucleotides was denatured in 400 μL of the binding buffer at 95 °C for 5 min and then allowed to cool at room temperature for 30 min. The folded ssDNA was loaded onto 400 μL of sialyllactose-biotin (0.8 nmol) conjugated streptavidin magnetic particles in the binding buffer. The mixture was incubated for 2 h at room temperature with agitation. Then the unbound ssDNA was removed by washing 3 times (1 mL×3) with the binding buffer using a magnetic particle separator and careful siphoning. The magnetic particles were resuspended in 100 μL×2 autoclaved deionized water and heated to 95°C for 5 min to elute the bound ssDNA from the sialyllactose-biotin (**3**) attached to the magnetic particles. The magnetic particles were re-collected with the help of a separator, and the supernatant containing the bound ssDNA was separated. The amounts of bound and unbound ssDNA were estimated by the UV absorbance at 260 nm. The volume of the bound ssDNA was reduced to 100 μL and used as a template to synthesize dsDNA (200 μL PCR mixture) by PCR and then by asymmetric PCR (2 mL PCR mixture) to synthesize ssDNA as before. Purified ssDNA was used as the input for the next round of selection. Negative selection was carried out with a conjugate of magnetic particles with **4** in the 5th round and a conjugate of magnetic particles with **7** in rounds 7, 8, 10, 12 and 13, before positive selection with **3** conjugated with the magnetic particles to remove the ssDNA which binds to **4** and **7** attached to the magnetic particles, respectively. After 13 rounds of selection we found that about 0.4 nmol ssDNA was bound to sialyllactose (0.8 nmol) attached to the magnetic particles.

4.7. Cloning and sequencing

After 13 rounds of selection, the ssDNA bound to sialyllactose were amplified by KOD Dash DNA polymerase using primer 1, primer 2 and natural dNTPs. Then the amplified DNA (102 bp) was purified on a 10% non-denaturing polyacrylamide gel, as described above. The purified dsDNA was further amplified by PCR using Taq DNA polymerase, primer 1, primer 2 and natural dNTPs. The amplified DNA was cloned into a T vector (pCR® 2.1 vector) for sequencing according to the manufacturer's protocol. Plasmid DNAs were prepared from 22 different clones and isolated by alkaline lysis using a gene-elute plasmid miniprep kit. Then their sequences were determined by the dye termination method using a genetic analyzer. The sequence match analysis of the selected ssDNA and the prediction of the ssDNA secondary structure by free-energy minimization were done by GENETYX-MAC Software (version 8.0).

4.8. Binding assay of the isolated aptamers

We checked the binding ability of the selected clones from different groups. For this purpose, each clone (0.01 OD) was dissolved in 200 μL of binding buffer, heated at 95 °C for 5 min and allowed to cool at room temperature. Then, 200 μL of the **3**-conjugated magnetic particle (0.4 nmol) suspension was taken for each clone and the magnetic particles were separated with the help of a separator. The ssDNA solution was added to each tube containing the magnetic particles and the mixtures were kept at room temperature for 2 h with agitation. The unbound DNA was separated and compared with the amount of input DNA for binding after gel electrophoresis on a 10% non-denaturing polyacrylamide gel. The DNA on the gel was stained with SYBR® Gold and quantified by a Molecular imager® FX. The ratio of the bound DNA was determined after subtraction of the background.

4.9. Dissociation constant (K_d) determination

The affinity of sialyllactose with ssDNA was analyzed by the equilibrium filtration method.[24] We selected clone SL-11 for dissociation constant (K_d) determination because it represents the sequences of 12 clones, except for the 1-4 base difference and showed stronger binding ability than the other clones. Sialyllactose-biotin (2.7 nmol) was added to the ssDNA (0.93 nmol) of clone SL-11 in the binding buffer (100 μL) and the mixture was incubated for 5 min at 25 °C. Then the mixture was placed on a Biomax-5K NMWL membrane centrifugal filter device and centrifuged for 2 min at 10,000×g, allowing 30–35 μL of solution to flow through the membrane. The concentration of sialyllactose-biotin in the filtrate and retentate was determined by the titration method, using the calibration curve for the biotin compound. The equilibrium-dissociation constant (K_d) of SL-11 was calculated from the following equation:

$$\begin{aligned} K_d &= \left[(Lf_1/V_1) \times (DNA)_f/V_1\right]/[(DNA \cdot L)V_1] \\ &= \left[Lf_1 \times (DNA)_f\right]/[V_1 \times (DNA \cdot L)] \\ &= \left[Lf_1\left\{(DNA)_t - (DNA \cdot L)\right\}\right]/[V_1 \times (DNA \cdot L)] \\ &= \left[Lf_1\left\{(DNA)_t\text{-}(L_t\text{-}Lf_1\text{-}Lf_2)\right\}\right]/[V_1 \times (L_t\text{-}Lf_1\text{-}Lf_2)] \end{aligned} \quad (1)$$

where V_t, V_1 and V_2 are the total volume, the volume of the retentate and the volume of the filtrate, respectively. L_t, Lf_1 and Lf_2 are the quantities of ligand in the total volume, the retentate and the filtrate, respectively. (DNA·L) is the quantity of the ligand bound DNA, $(DNA)_f$ is the quantity of unbound free DNA, and $(DNA)_t$ is the quantity of total DNA.

4.10. Three-way junctions of SL-11 and their binding assay

The secondary structure of SL-11 showed that it contains three three-way junctions (J1, J2, J3) as shown in Figure 4. We synthesized each three-way junction. For J1 and J2, SL-11-Natural (102mer) was used as a template. The sequence of SL-11-Natural was exactly the same as that of SL-11 and did not contain any modified T (T^{HM}). SL-11-Natural was amplified over 15 cycles of PCR (94 °C, 30 s; 60 °C, 30 s; 74 °C, 1 min) using the two primers. For J1, the primers were primer 3, 5′-CCA GCC ACT TCT GTC AGT GrAA-3′ and primer 4, 5′-CTC CAT GCT CGC AGG CG-3′. For J2, primer 1, 5′-GTA CGA ATT CAC GAG GTT GCC-3′ and primer 5, 5′-AAT TCA CTG ACA GAA GTG GCT GG-3′. 100 μL of the PCR mixture contained 1 nM template, 0.2 mM dNTPs (modified $pppT^{HM}$ instead of natural TTP), 0.4 μM primers, 2.5 unit of KOD Dash DNA polymerase, 10 μL 10× buffer for KOD Dash (pH 8.8). The dsDNA containing the PCR mixture (100 μL PCR mixture) was used as a template for the synthesis of ssDNA by an additional 25 cycles of asymmetric PCR, using only primer 3 for J1, and primer 1 for J2. 1 mL of the PCR mixture contained 100 μL PCR-DNA mixture as a template, 1 μM primer, 0.2 mM dNTPs (modified $pppT^{HM}$ instead of natural TTP), 25 unit of KOD Dash DNA polymerase, 100 μL 10× buffer for KOD Dash (pH 8.8). The thermal cycling was the same as that for dsDNA synthesis, as described above. For J3, synthetic 45mer oligonucleotides, 5′-GTA CGA ATT CAC GAT CAG TGA ATT CCT GCT AGC ATG GAG TCG GAT-3′ were amplified over 10 cycles of PCR (94 °C, 30 s; 50 °C, 30 s; 74 °C, 1 min) using primer 6, 5′-GTA CGA ATT CAC GAT CAG T-3′ and primer 7, 5′-ATC CGA CTC CAT GCT AG-3′. The scales for the PCR reaction mixture were the same as those for J1 and J2. The dsDNA containing the PCR mixture (100 μL PCR mixture) was used as a template for the synthesis of ssDNA of J3 by an additional 25 cycles of asymmetric PCR using only primer 6 (1 μM). The scales for the PCR reaction mixture were the same as for J1 and J2. The thermal cycling was the same as that for the dsDNA synthesis described above. Each ssDNA, for J1 (62mer), J2 (51mer) and J3 (45mer), was purified from the PCR mixture, as described in the section on DNA pool synthesis. The ssDNA obtained for J1 by PCR was 62mer. But the target ssDNA for J1 is 42mer. Primer 3 for J1 contains one ribonuclotide, rA, at 20 bases from the 5′ position, and the ssDNA 62mer of J1 also contains ribonucleotide, rA, at 20 bases from the 5′ position. Thus, the 62mer ssDNA for J1 was hydrolyzed with 1 N NaOH at 95 °C for 5 min to cleave the ribonucleotide position yielding the 42mer ssDNA. The 42mer ssDNA of J1 was purified, as described previously. The ssDNA, J1 (42 mer), J2 (51 mer), J3 (45 mer) and SL-11-Natural were subjected to the DNA binding assay described above.

Acknowledgements

This work was partly supported by a Grant-in-Aid for Scientific Research from the Ministry of Education, Science and Culture of Japan.

References and notes

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