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Enhancement of the Immunogenicity of Synthetic Carbohydrate Vaccines by Chemical Modifications of STn Antigen†

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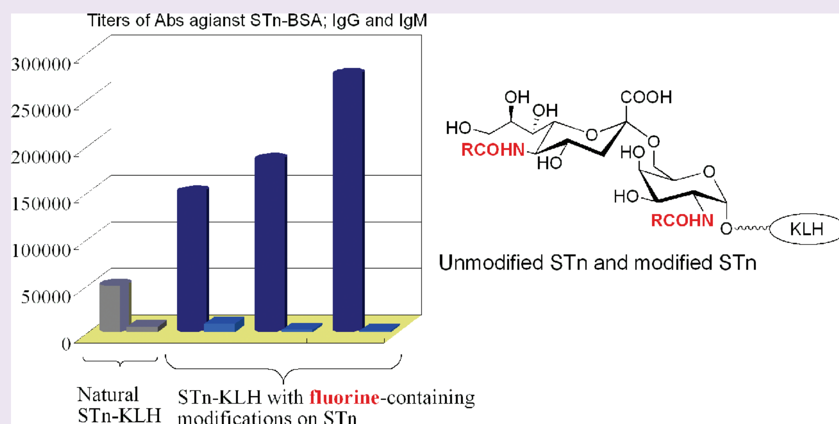
Supporting Information

ABSTRACT: The abnormal glycans expressed on the surface of tumor cells, known as tumor-associated carbohydrate antigens, increase the chance to develop carbohydrate-based anticancer vaccines. However, carbohydrate antigens pose certain difficulties, and the major drawback is their weak immunogenicity. To tackle this problem, numerous structurally modified STn antigens were designed and synthesized in this work. These synthetic antigens were screened *in vitro* by using competitive ELISA method, and the antigens with positive response were conjugated to the protein carrier for vaccination. The vaccination results on mice showed that some fluorine-containing modifications on the STn antigen can significantly increase the anti-STn IgG titers and improve the ratios of anti-STn IgG/IgM. The antisera can recognize the tumor cells expressing the native STn antigen.



Tumor-associated carbohydrate antigens (TACAs) are regarded as important targets for vaccine development.[1−4] Vaccines based on synthetic oligosaccharide antigens against cancers are at different stages of preclinical and clinical studies.[2−4] However, one of the major problems is that carbohydrate antigens possess weak immunogenicity, since they are self-antigens and consequently tolerated by the immune system. Various efforts have been made to break the immunotolerance. Generally speaking, in addition to the use of immunological adjuvants, the low immunogenic properties of carbohydrate antigens can be overcome by conjugation to immunogenic carriers including proteins,[2−5] monoantibodies,[6] peptides,[7−10] or lipopeptides.[11] In addition, the immune efficiency may also benefit from unnatural glycosidic linkages,[12] less immunogenic linkages between antigens and carriers,[13] clustered antigens,[14] or truncated antigens.[15] Although advances have been achieved in this field, approaches to enhancement of the immunogenicity of carbohydrate antigens are still in great demand.

In 1986, Jennings' group reported that the modification of carbohydrate antigen structures (MCAS) could improve the immunogenicity of the vaccine.[16] However, this strategy was seldom applied to other types of structural modifications or other kinds of carbohydrate antigens,[17,18] and the results were undermined by the lack of cross-response of IgG to natural antigens, making the efficiency of the strategy of MCAS uncertain. In recent years, it was reported that MCAS strategy in combination with the technology of metabolic oligosaccharide engineering (MOE) of sialic acid increased the immunoefficiency,[19−24] but the uncertain selectivity of MOE between the tumor cells and normal cells may raise the safety problems, especially when considering the critical biological roles of sialic acid containing glycoconjugates.[19] To tackle this problem, we reasoned that the efficiency of vaccination by MCAS strategy without combination of MOE could hopefully be improved and therefore the risk caused by MOE could be avoided. So we wished to explore the possibilities of structural modifications of TACAs for enhancement of the immunogenicity.

RESULTS AND DISCUSSION

STn antigen (**1** in Figure 1), an *O*-linked disaccharide [NeuAcα-(2−6)GalNAc], was chosen for this purpose, because the expression of STn on normal cells is limited but is abundant on a wide range of tumors such as breast, prostate, pancreas, colorectal, lung, gastric, and ovarian cancers, which makes STn a relatively specific tumor-associated antigen for vaccination.[25] The development of tumor can be efficiently affected by anti-STn-antibodies,[26,27] which makes STn-based vaccine more promising.

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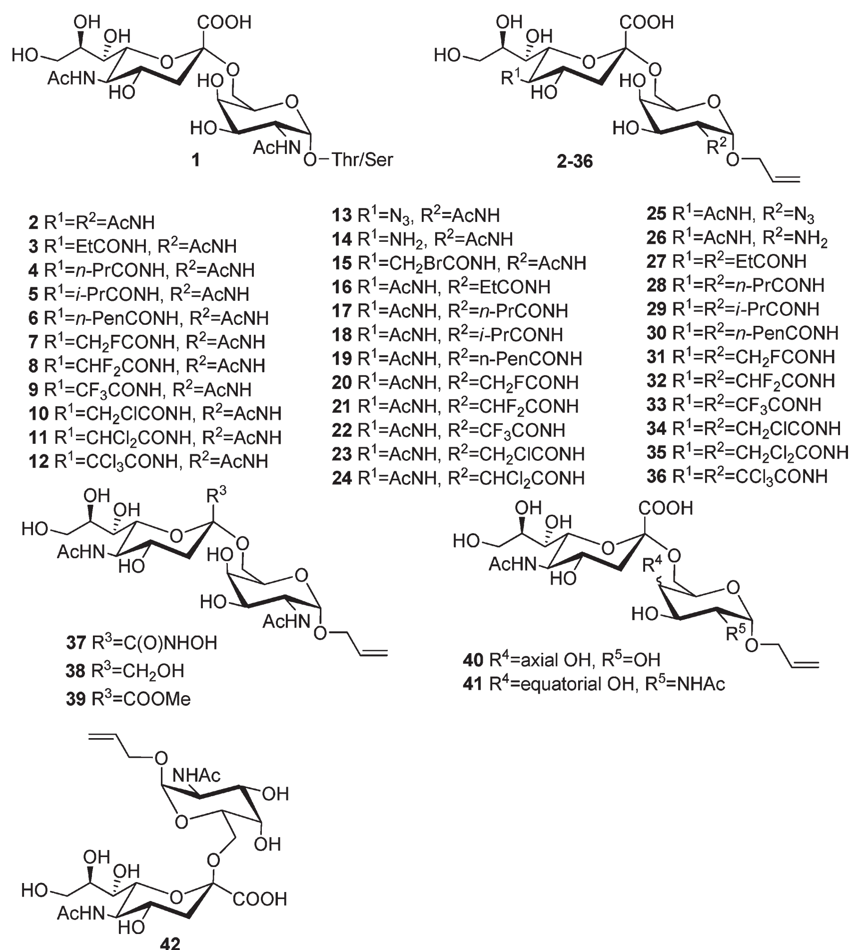

Figure 1. Structures of native antigen and structure-modified antigens.

Another fact is that when Theratope, a STn-KLH (keyhole limpet hemocyanin) conjugation vaccine, was subjected to phase III clinical trial, the result was not so encouraging because it did not induce strong T-cell-mediated immune response in patients, and only modest clinical efficacy was achieved when patients were treated in conjugation with hormone therapy.[25] One possible reason for the partial failure of Theratope could be insufficient enhancement of STn immunogenicity. We hope that the suitable modifications [28] on STn antigen by a chemical approach will enhance the immunogenicity of STn.

Natural STn is linked to a serine or threonine site of protein through an α-*O*-glycosidic bond,[25] and many efforts on the synthesis of STn have been reported.[24,29−31] In our case, allyl alcohol was used as the surrogate of the amino acid residues with the α-*O*-glycosidic bond retained (**2** in Figure 1). The *O*-allyl group also functioned as a protecting group for the anomeric OH and facilitated the consequent conjugation with proteins.[32]

The diversity of structural modifications needs to be augmented to find better modified antigens (Figure 1). The two *N*-acetyl groups of STn could be conveniently replaced by many other acyl groups (**3**−**6**, **10**−**12**, **15**−**19**, **23**−**24**, **27**−**30**, **34**−**36**). Besides these acyl-substituted modifications, the *N*-acetyl group was also replaced by azido or free amino groups (**13**, **14**, **25**, **26**). In addition, the carboxylic group of STn was changed to other groups such as hydroxamic acid (**37**), which is a weaker acid and plays important roles in some inhibitor designs.[33] Other groups such as $-CH_2OH$ (**38**) and −COOMe (**39**) were also possible substitutes for the −COOH. Since the specificity of the recognition of antibodies with different glycoforms is still controversial,[34] altering the glycoform in the antigen could also provide a chance to enhance the immunogenicity. Therefore, the galactosamine moiety (GalNAc) of STn antigen was replaced by similar glycoforms such as galactose (Gal) (**40**) and glucosamine (GlcNAc) (**41**). Furthermore, the anomeric isomer NeuAc*β*-(2−6)GalNAc (**42**) of STn was also designed for the immunological test.

Fluorinated compounds are frequently used in medicinal chemistry and have led to a large number of highly effective drugs.[35] However, the fluorine substitution strategy has not been examined for improving the immunogenicity of vaccines.[36,37] The fluorinated modifications might provide hopeful opportunities for vaccine development, for fluoro-substituted structures may be highly immunogenic as a result of the absence of fluorine in most organisms, whereas the similar atom radius and lipophilicity compared to the hydrogen atom may satisfy the structural and functional requirements. So the fluorinated modifications (**7**−**9**, **20**−**22**, **31**−**33**) of STn were designed.

Starting from D-*N*-acetylgalactosamine (**43**), the allyl group was introduced as a tether to produce the allyl-linked compound **44** *via* the Fischer glycosylation[38] (Figure 2). To improve the solubility of the compound **44** toward glycosylation reactions, 3-*O*-benzoyl (Bz) protected acceptor **46** was prepared *via* the intermediate **45** by functional group transformations. It should be noted that the 3-*O*-Bz group was able to migrate under basic conditions when using base to neutralize the acid employed for

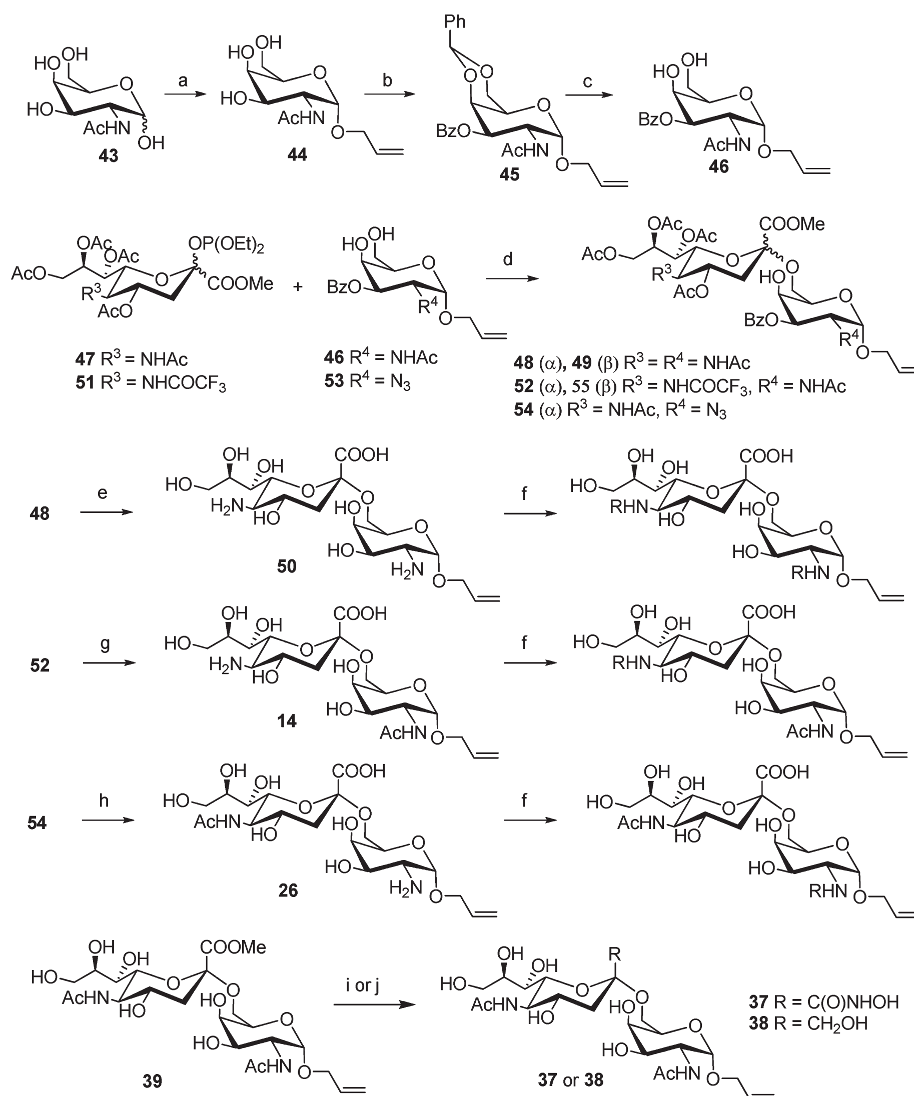

Figure 2. Synthesis of antigens. Reagents and conditions: (a, b) ref 38; (c) strong acid resin in acid form, MeOH, reflux, 97%; (d) TMSOTf, −72 °C in THF, for **48** (**46** + **47**): 84%, α/β = 1/1.2; for **52** (**46** + **51**): 81%, α/β = 2.6/1; for **54** (**47** + **53**): 58%, pure α isomer; (e) (i) NaOMe/MeOH, (ii) 1 N NaOH, (iii) 2 N NaOH, 90 °C; (f) acylation; (g) NaOMe/MeOH, then 2 N NaOH, 86%; (h) (i) NaOMe/MeOH, then 1 N NaOH, (ii) H_2S/pyridine/triethylamine, 67%; (i) for **37**, NH_2OH, KCN, THF, ref 41, 50%; (j) for **38**, $NaBH_4$ in MeOH, 94%.

the deprotection of the benzylidene group, and this problem was avoided by using the resin-based acid without neutralization. The sialylation reactions in acetonitrile[39,40] gave products in low yields (∼20%) due to the low solubility of acceptors **46** and **53**. However, when tetrahydrofuran (THF) was used as solvent instead of acetonitrile, the sialylation efficiency was greatly improved, providing disaccharides **48**, **52**, and **54** in good isolated yields.

Deprotection of the *O*-acyl groups in disaccharide **48** by MeONa/MeOH gave compound **39** in 95% isolated yield, which was further saponified under 1 N NaOH aqueous conditions producing compound **2**. Similarly, the β-isomer **42** was obtained from the corresponding precursor (Supporting Information). Treatment of the methyl ester **39** with NH_2OH[41] yielded the hydroxamic acid derivative **37**, whereas the reduction of **39** provided the primary alcohol derivative **38**. As a result of the activation of methyl ester in compound **39** by its anomeric atom, convenient conditions such as $NaBH_4$/MeOH provided the reduction product in 94% yield.

With the stronger basic conditions (2 N NaOH, 90 °C), the two *N*-acetyl groups in compound **2** were hydrolyzed to yield the key intermediate **50** in which the free amino functionalities were subjected to derivations. For the modifications of different acyl groups, the acylation conditions differed. Generally, simple fatty acylations were performed with the corresponding anhydrides, but this protocol did not work well for halogen-containing acylations, and the conversion efficiency dropped dramatically despite various solvents and bases employed. Therefore, for the monochloride-containing acylation (preparation of **34**), it proved to be efficient to use the corresponding carboxylic acid in the presence of the coupling reagent *O*-(benzotriazol-1-yl)-*N*,*N*,*N*′,*N*′-tetramethyluronium hexafluorophosphate (HBTU), whereas for other halogen-containing acylations, the methyl esters of carboxylic acids were used to obtain acylated products by the condensation of methyl esters and amine **50**. Thus, many diacylated STn derivatives (**27**−**36**) were prepared by the modifications of different acyl groups. To prepare the monoacylated STn derivatives, disaccharide **52**, formed in high yield and with better stereoselectivity by the

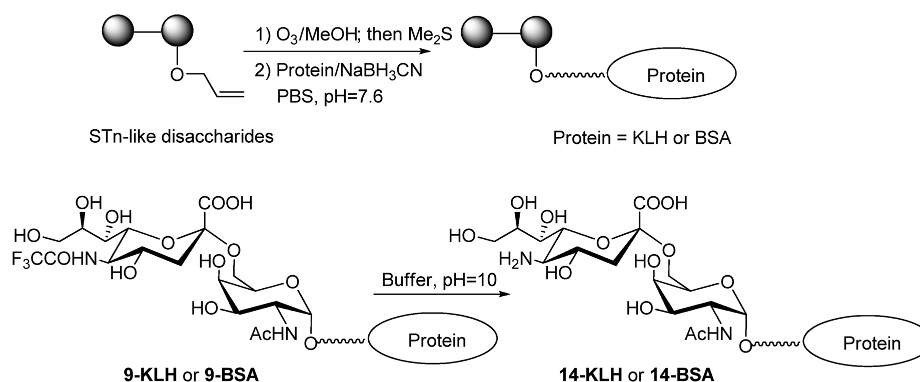

Figure 3. Preparation of the glycoconjugates.

coupling reaction of trifluoroacetyl (TFA)-substituted sialic acid donor **51**[42,43] and acceptor **46**, was treated with bases at RT, providing the selectively deprotected product **14** with the *N*-acetyl group in GalNAc moiety intact (Figure 2). The free amino group of **14** was then modified by different acyl groups to produce various monoacylated products **3**−**12** and **15**. Azido-substituted product **13** was also prepared *via* the modified diazo-transfer procedure.[44] On the other hand, after deprotection of the ester groups in disaccharide **54**, the azido functionality was reduced to a free amino group, yielding compound **26**, which was also subjected to modifications in the same way as mentioned above to obtain the monomodified STn derivatives **16**−**25**. In addition, the glycoform-replaced analogues, NeuAcα-(2−6)Gal (**40**) and NeuAcα-(2−6)GlcNAc (**41**), were prepared in the similar manner as the synthesis of compound **2**. To sum up, 40 modified STn derivatives were obtained.

With various modified antigens in hand, a preliminary screening was performed based on an *in vitro* test. It is necessary to check whether the modified antigens can be recognized by the antibodies elicited by the native STn antigen. So the competitive enzyme-linked immunosorbent assay (ELISA) was carried out,[12,15,16,45] in which the antibodies induced by the native STn-based vaccine were employed and the unmodified type STn-BSA (bovine serum albumin) as coating antigen. After *in vitro* screening (Supporting Information), there were a total of 28 synthetic antigens having comparable antibody-recognition abilities, and these compounds were allowed to conjugate with the carrier protein for further *in vivo* testing.

Compound **2** and 28 modified carbohydrate antigens needed to be conjugated to protein carriers for vaccination and analysis (Figure 3). All of the conjugates except for **14**-KLH (or **14**-BSA) were prepared through a two-step procedure.[32] That is, each STn derivative was treated with ozone in methanol to selectively oxidize the carbon−carbon double bond of the anomeric *O*-allyl group, producing an aldehyde intermediate; the aldehyde intermediate was subsequently coupled with carrier proteins including KLH and BSA by reductive amination in the aqueous buffer solutions, accomplishing the synthesis of glycoconjugates. For the conjugation of antigen **14**, the above procedure was not applicable, so another approach was adopted. Thus, **9**-KLH (or **9**-BSA) conjugate was treated with basic aqueous buffer (pH = 10) solution to afford **14**-KLH (or **14**-BSA), which was detected by ^{19}F NMR. The epitope ratios of glycoconjugates (including carbohydrate-KLH and carbohydrate-BSA) were determined by estimating sialic acid content using the resorcinol method described by Svennerholm [46] and protein content by BCA assay.[47] (For more details, see "Determination of protein and sialic acid contents of carbohydrate-KLH and carbohydtate-BSA conjugates" in the Supporting Information; the results are shown in Supplementary Table 1.)

In the following vaccination, the use of immunological adjuvants was omitted, because the object of this work was to compare the relative immunological efficacy of the modified STn-based vaccines with the unmodified STn-based vaccine. Thus, groups of six female BALB/c mice were vaccinated four times at biweekly intervals with unmodified or modified STn-KLH conjugates. The anti-STn antibody titers of the mouse sera were determined by ELISA with the plate coated by unmodified STn-BSA conjugate (Supporting Information). Very fortunately, it was found that 3 modified STn-based vaccines (**20**, **21**, and **31**) among 28 antigens displayed outstanding results. As shown in Table 1 and Supplementary Figure 14, vaccination based on the synthetic antigens **20**, **21**, and **31** elicited anti-STn IgG antibody titers higher than that of **2**. The superiority was further confirmed by determination of individual titers for IgG, and the results proved to be statistically significantlt different (Supplementary Tables 6 and 7). Meanwhile, the anti-modified-STn antibody titers were also determined by ELISA with the plates coated by the corresponding modified-STn-BSA conjugates instead, and the results reflected the immunogenicity of the modifications (Supporting Information). The division of anti-STn antibody titers by anti-modified-STn antibody titers gave the cross-recognition efficiency of the antibodies against unmodified STn. Modified STn antigens **20**, **21**, and **31** produced high anti-modified-STn antibody titers with reasonable cross-recognition efficiency, so effective anti-STn IgG antibodies were induced. In antigens **20**, **21**, and **31**, with one or two fluoro-substituents per acetyl group, cross-recognition was obtained, which might benefit from the similar atom radius and lipophilicity of fluorine atom compared to the hydrogen atom. By contrast, some other modifications such as *n*-butanoyl analogue **4**, azido analogue **13**, and hexanoyl analogue **30** did lead to high anti-modified-STn antibody titers. Compared with the unmodified **2**-KLH conjugate, the modified **4**-KLH, **13**-KLH, and **30**-KLH conjugates resulted in 7.8, 8.6, and 6.1 times anti-modified-STn IgG antibody titers, respectively, which indicated the immunogenicity was significantly increased. However, their anti-unmodified-STn (**2**-BSA) IgG antibody titers were very low. This sharp contrast reflected poor cross-recognition efficiency of the antibodies induced by **4**-KLH, **13**-KLH, and **30**-KLH conjugates. In addition, there were other substitutions such as some halogen-containing analogues (**7**, **8**, **9**, **22**, **23**, **32**, **33**, **34**) and some fatty acyl analogues (**3**, **16**, **17**), which gave weak immune response.

Table 1. Immunological Results after Vaccination with Synthetic Carbohydrate Conjugates[a]

	ELISA titer anti-STn after vaccination and ratio of IgG/IgM after third and fourth								ELISA titer anti-modified-STn	
	after second		after third			after fourth			after third	
vaccine	IgG	IgM	IgG	IgM	IgG/IgM	IgG	IgM	IgG/IgM	IgG*	IgG/IgG* (%)
2-KLH	910	<1000	50,144	5763	8.70	120,918	20,686	5.85	50,144	100
20-KLH	11000	<1000	185,354	2581	71.81	241,990	4944	48.95	511,964	36
21-KLH	1266	<1000	150,504	9236	16.30	245,932	16,987	14.48	249,307	60
31-KLH	20000	<1000	276,162	1804	153.08	279,506	3370	82.94	799,854	35

[a] IgG is the IgG titer against STn-BSA (**2**-BSA), whereas IgG* is the titer against the corresponding modified STn-BSA. Mouse serum IgG obtained from pre-immune could not be detected when diluted 1:250.

The specificity of the antibodies elicited by **20**-KLH, **21**-KLH, and **31**-KLH was further confirmed by three methods. In the first two experiments, sialosides **40** and **41** were chosen to compare with unmodified STn (compound **2**) because of the similarity of their chemical structures. First, after immunization with **20**-KLH, **21**-KLH, and **31**-KLH, the antibodies titers against **40**-BSA and **41**-BSA were shown to be much lower than that against **2**-BSA (Supplementary Table 8). Second, competitive inhibition ELISA was performed on the binding of antibodies to **2**-BSA by using **2**, **40**, and **41** as inhibitors, respectively. As shown in Supplementary Figure 15, the native STn antigen **2** significantly inhibited the binding of the antibodies elicited by **20**-KLH, **21**-KLH, and **31**-KLH to **2**-BSA, whereas the modified STn antigens **40** and **41** exhibited little inhibitory effects. This nearly negative recognition of the antibodies with **40**, **41**, or their BSA-conjugates also suggested that the allyl linker used in our work had minimal influence on the antibody−antigen recognition. Third, since the disaccharide units, NeuAcα-(2−6)GalNAcβ- and NeuAcα-(2−6)Galβ-, are found to exist in the termini of *N*-glycans of some human glycoproteins such as leucocyte lactoferrin,[48] lutropin,[49] and glycodelin,[50] it is necessary to determine whether the antibodies elicited by **20**-KLH, **21**-KLH, and **31**-KLH recognize these glycoproteins. Thus bovine lactoferrin,[51] which expresses both NeuAcα-(2−6)GalNAcβ- and NeuAcα-(2−6)Galβ- units, was employed for this purpose. The experimental results showed that the antibodies did not recognize the protein (see Supplementary Table 9 and Supplementary Figure 16), further confirming the specificity of the antibodies.

The ratio of IgG/IgM is also regarded as another important factor for success of vaccination, and the improvement of this ratio has been a major challenge for carbohydrate vaccine.[3,15] As can be seen, after the third/fourth vaccinations, the IgG/IgM ratios of the modified carbohydrate conjugates (**20**, **21**, and **31**) were increased by 8.3/8.4 times (**20**-KLH), 1.9/2.5 times (**21**-KLH), and 17.6/14.2 times (**31**-KLH), respectively, compared with the unmodified STn conjugate (Table 1).

To ensure that the mouse sera after vaccination were able to recognize the native STn antigen present on cancer cells, binding of the sera to LS-C human colon cancer cells expressing STn antigen[52] was examined by flow cytometry (Figure 4). The experimental results demonstrated that the antisera elicited against the antigens (**20**, **21**, **31**) reacted strongly with the STn-positive tumor cells. In contrast, when LS-B cells that do not express the STn antigen were used, much weaker binding was observed.

In summary, numerous structurally modified STn antigens were designed and synthesized. These synthetic antigens were screened *in vitro* by using a competitive ELISA method, and the antigens with positive response were conjugated to the protein carrier for vaccination. The vaccination results on mice showed that some fluorine-containing modifications (**20**, **21**, and **31**) on STn antigen significantly improved the antigenicity. Not only IgG titers but also the ratios of IgG/IgM were increased by several folds. These two values are both regarded as key marks for tolerance-breaking in vaccines and will benefit long-term immunity memory. Our experiments also demonstrated the antisera reacted strongly with the STn-positive tumor cells, implying the potential as anticancer vaccines. By avoiding the MOE technology, the risk in modification of sialic acid on normal cells would be eliminated. The strategy based on antigen modifications with fluoric or other suitable substitutions might find applications in the development of carbohydrate-based and peptide-based vaccines.

METHODS

General Sialylation Procedure. Typically, a mixture of glycosyl acceptor **46** (1.07 g, 2.93 mmol, 1.00 equiv), donor **47** (2.06 g, 3.38 mmol, 1.15 equiv), and activated 4 Å molecular sieves (0.8 g) in dry THF (40 mL) under a nitrogen atmosphere was stirred for 0.5 h at RT before it was cooled to −72 °C. Trimethylsilyl trifluoromethanesulfonate (TMSOTf) (70 μL, 0.36 mmol, 0.11 equiv) was then added to the mixture in three batches. The reaction mixture was stirred for 2−4 h until TLC analysis indicated that the reaction was completed. Triethylamine (1.0 mL) was added to the mixture. The precipitate was filtered off, and the filtrate was concentrated under reduced pressure. The residue was purified by column chromatography on silica gel (petroleum ether/acetone 1.5/1) to afford the corresponding disaccharides **48** (α isomer, 0.99 g) and **49** (β isomer, 1.19 g) as white foams. Yield: 84%; α/β = 1/1.2.

General Procedure for Acylation with Fluoro-Containing Substituents. To a solution of the disaccharide (**14**, **26**, or **50**, 10−30 mg, 0.02−0.06 mmol) in methanol (5.0 mL) were added methyl fluorine containing ester (2.0 mL) and triethylamine (1.0 mL) under nitrogen atmosphere. The mixture was stirred at RT to 50 °C for 1−4 h until TLC analysis indicated that the reaction was complete. The solvent was removed under reduced pressure. The residue was purified by C-18 reverse-phase column chromatography (H_2O or H_2O/MeOH as eluent) and Biogel P-2 column chromatography (H_2O as eluent) to yield the products.

Competitive Inhibition ELISA Assay. An ELISA plate was coated with 100 μL of STn-BSA (including 0.02 μg of STn) overnight at 4 °C (0.1 M bicarbonate buffer). After being washed three times with PBST, microwells were blocked with BSA. The anti-STn antibody (1:4000 dilution) was mixed with various concentrations of native-STn or modified-STn antigens for 1 h at 37 °C. Then the mixture was added to the microwells and incubated for 1 h at 37 °C. The plate was washed and incubated with a 1:5000 dilution of horseradish peroxidase conjugated goat anti-rabbit IgG for 1 h at 37 °C. The plate was washed, developed with *o*-phenylenediamine (OPD) substrate in the dark for

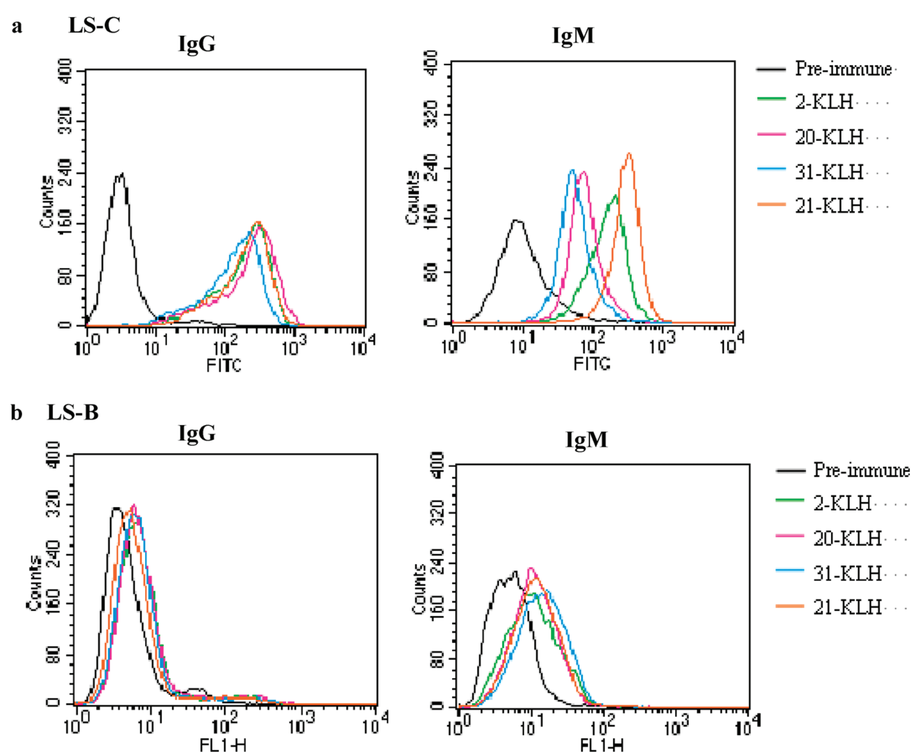

Figure 4. Serological IgG and IgM analysis results on LS-C cells (a) and LS-B cells (b) after the fourth immunization with **2-KLH**, **20-KLH**, **31-KLH**, and **21-KLH** by flow cytometry.

15 min, terminated with 2 M H_2SO_4, and then read at 490 nm. Percentage inhibition was calculated as the difference in absorbance between the uninhibited and inhibited serum as in eq 1. Every concentration was triplicate wells. The procedure of competitive inhibition ELISA assay of **40**, **41**, and **2** was performed as the same mentioned above (except antibodies were sera obtained from mice and the second antibody was horseradish peroxidase conjugated goat anti-mice IgG). The competitive ELISA assays were conducted separately twice.

$$\% \text{ inhibition of binding} = \frac{A_{\text{without}} - A_{\text{with}}}{A_{\text{without}}} \times 100 \qquad \text{(eq 1)}$$

where A_{without} is the absorbance of serum without inhibitor and A_{with} is the absorbance of serum with inhibitor.

Preparation of the Glycoconjugates. A solution of aldehyde-containing disaccharides (5 mg) prepared from ozonization of the allyl-containing disaccharides, KLH (5 mg), and $NaBH_3CN$ (5 mg) in PBS (0.4 mL, 0.1 M, pH = 7.6) was gently shaken in the dark for 12−16 h at RT and then dialyzed against PBS at 4 °C (molecular weight cutoff value 14,000 Da, 6 changes of PBS). The epitope ratios of the glycoconjugates (including carbohydrate-KLH and carbohydrate-BSA) were determined by estimating sialic acid content using the resorcinol method described by Svennerholm [46] and protein content by BCA assay.[47]

Immunization of Mice. Groups of six mice (female pathogen-free BALB/c, age 6−8 weeks, from Department of Laboratory Animal Science, Peking University Health Science Center) were immunized four times at 2-week intervals with STn-KLH or modified-STn-KLH glycoconjugates (each containing 2 μg of carbohydrate in PBS). The vaccines were administered intraperitoneally. Mice were bled prior to the initial vaccination, 13 days after the second and the third vaccinations, and 14 days after the fourth vaccination. Blood was clotted to obtain sera, which were stored at −80 °C.

Serological Assays. The total antigen-specific antibody titers of the sera were assessed by means of ELISA. An ELISA plate was coated with 100 μL of STn-BSA (including 0.02 μg of STn) overnight at 4 °C (0.1 M bicarbonate buffer, pH = 9.6). After being washed three times with PBST, microwells were blocked with BSA. After the plate was washed, serially diluted sera were added to microwells (100 μL/well) and incubated for 1 h at 37 °C. The plate was washed and incubated with a 1:5000 dilution of horseradish peroxidase conjugated goat anti-mouse IgG (γ-chain specific) or IgM (μ-chain specific) (Southern Biotechnology Associates, Inc., Buckingham, AL) for 1 h at 37 °C. The plate was washed, developed with *o*-phenylenediamine (OPD) substrate in the dark for 15 min, terminated with 2 M H_2SO_4, and then read at 490 nm. The antibody titer was defined as the highest dilution showing an absorbance of 0.1, after subtracting background. Meanwhile, the anti-modified-STn antibody titers (sera from 13 days after the third vaccination) were determined by ELISA, with the plate coated by the corresponding modified-STn-BSA conjugates instead.

Flow Cytometry. LS-C (STn positive) and LS-B (STn negative) human colon cancer cells were obtained from Dr. Steven H. Itzkowitz at Mount Sinai School of Medicine.[52] Single-cell suspensions of 5 × 10^5 cells/tube were washed with 3% fetal calf serum (FCS) in PBS and incubated with 25 μL of 1:20 diluted test sera (sera from preimmunization and 14 days after the fourth vaccination) for 30 min on ice. After two washes with 3% FCS in PBS, 25 μL of 1:15-diluted goat anti-mouse IgG (γ-chain specific) or IgM (μ-chain specific) labeled with FITC (Southern Biotechnology Associates, Inc., Birmingham, AL) was added, and the mixture was incubated for 30 min on ice. After a final wash, fixed with 1% formaldehyde and assayed using a FACScan (Becton Dickinson).

■ ASSOCIATED CONTENT

S Supporting Information. Full experimental information including methods and results of compound preparation, methods and results on immunity, and copies of NMR spectra for all compounds. This material is available free of charge *via* the Internet at http://pubs.acs.org.

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Author Contributions

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DEDICATION

†Dedicated to Professor Henry N. C. Wong on the occasion of his 60th birthday.

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