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Characterization of the Carbohydrate Binding Specificity and Kinetic Parameters of Lectins by Using Surface Plasmon Resonance

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An accurate, rapid, and sensitive method for characterizing the carbohydrate binding properties of lectins using a BIAcore apparatus and the detection method of surface plasmon resonance is described. As a model study, the sialic acid binding lectins from *Sambucus nigra* and *Maackia amurensis,* which are specific for the epitopes Neu5Ac(α2–6)Gal and Neu5Ac(α2–3)Gal, respectively, were chosen as suitable candidates. Two systems, one for the analysis of oligosaccharides and the other for glycoproteins, were developed after a rigorous analysis and evaluation of such parameters as binding conditions, buffers, and regeneration conditions. The systems take into account nonspecific binding, using the respective denatured lectin as negative blank, and avoid loss of activity: regeneration of the surface using either 10 mM NaOAc (pH 4.3) buffer (oligosaccharide system) or 20 mM HCl (glycoprotein system). The specificity of the lectins is well illustrated, while the kinetics parameters are shown to be sensitive to subtle changes in the recognized epitopes, and to be affected by steric hindrance. Surface plasmon resonance is a suitable technique for the analysis and characterization of lectins.

Key Words: **surface plasmon resonance; lectin–carbohydrate binding; *Sambucus nigra* agglutinin; *Maackia amurensis* agglutinin.**

Lectins recognize and interact with specific carbohydrate epitopes, and so these molecules have proved themselves very valuable for the separation and characterization of carbohydrates (1, 2). Although the specificity of a vast number of lectins is known, many have never been fully characterized, and thus a system which could both rapidly and accurately characterize the specificity of a lectin would be of enormous value in the study of glycoconjugates.

Surface plasmon resonance (SPR)[3] is the detection method used by BIAcore apparatus (3), a device developed for the analysis of biomolecular interactions. This method involves the immobilization of one compound onto the surface of a sensor chip, while another flows over the surface in solution. An interaction between the two compounds results in a change of the surface plasmon resonance of the system.

Although several groups have used SPR for the analysis of lectin–carbohydrate binding (4–10), only one has immobilized the lectin to the surface (4) rather than the oligosaccharide or glycoprotein. However, a rigorous analysis of the conditions with respect to nonspecific binding, loss of activity, or effects of different buffer systems under which the system is optimally operated is still lacking. In addition, a method in which the lectin is bound to the surface, rather than the glycan moiety, would enable rapid screening of a large number of glycan-containing compounds in a relatively short time, and would also be practical when a lectin of unknown specificity requires characterization.

MATERIALS AND METHODS

Instrumentation and reagents. BIAcore 2000 instrument, BIAevaluation software 3.0, sensor chip

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[3] Abbreviations used: SPR, surface plasmon resonance; SNA, *Sambucus nigra* agglutinin; MAA, *Maackia amurensis* agglutinin; b, biotinylated; RU, response unit; K_A, association constant; k_a, association rate constant; k_d, dissociation rate constant.

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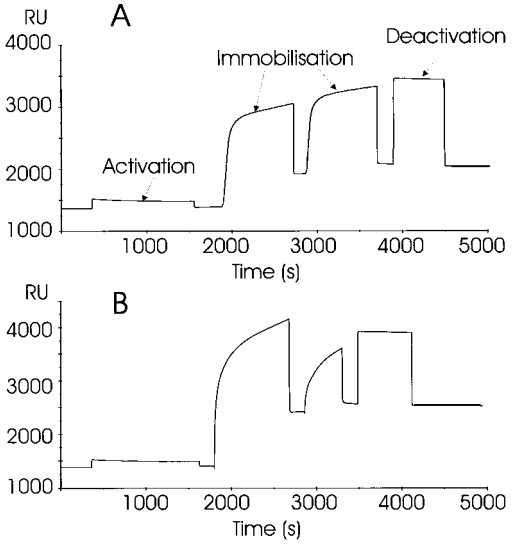


FIG. 1. Sensorgrams obtained by activation, immobilization of lectin, and deactivation of remaining sites: Immobilization of SNA (A) and immobilization of MAA (B) onto sensor chip CM5. During the immobilization, the system utilized a constant flow of Tris buffer (5 μl/min). The chip was activated with a 1:1 mixture (70 μl) of 0.1 M *N*-hydroxysuccinimide and 0.1 M *N*-ethyl-*N'*-(dimethylaminopropyl)carbodiimide. Both SNA (200 μg/ml) and MAA (200 μg/ml) were immobilized in a buffer of 10 mM sodium acetate (pH 4.3). Deactivation utilized a solution (50 μl) of 1 M ethanolamine (pH 8.5).

CM5, and amino coupling kit were obtained from Pharmacia Biosensor AB (Uppsala, Sweden). *Sambucus nigra* agglutinin (SNA) and *Maackia amurensis* agglutinin (MAA) were supplied by Sigma, biotinylated *S. nigra* agglutinin (b-SNA) was purchased from Oxford Glycosystems (Oxford, England), and biotinylated *M. amurensis* agglutinin (b-MAA) was supplied by Vector Laboratories (Burlingame, CA). Streptavidin was supplied by Pierce. Bovine fetuin, horse transferrin, and human transferrin were supplied by Sigma. The glycans **1–10** were previously isolated and purified in our laboratory.

Experiments were performed at 25°C in 10 mM Tris-buffered saline (pH 8.0) containing 150 mM NaCl, 2 mM $MgCl_2$, 2 mM $CaCl_2$ and 0.02% (w/v) NaN_3. HBS buffer (10 mM Hepes, pH 7.4, containing 150 mM NaCl, 2 mM $MgCl_2$, and 2 mM $CaCl_2$) was also used with either 0.005 or 0.05% surfactant P20.

Preparation of sensor surfaces. After equilibration of the sensor surface with Tris buffer (used throughout the immobilization) the sensor surface was activated at a flow rate of 5 μl/min with a 14 min pulse of a 1:1 mixture of freshly prepared 0.1 M *N*-hydroxysuccinimide and 0.1 M *N*-ethyl-*N'*-(dimethylaminopropyl) carbodiimide.

Fixation of biotinylated lectins. Streptavidin, at a concentration of 400 μg/ml in 10 mM sodium acetate buffer, pH 5.0, was injected for 7 min, and the remaining *N*-hydroxysuccinimide esters were blocked by the addition of 1.0 M ethanolamine hydrochloride, pH 8.5, for 7 min. The level of bound streptavidin was approximately 7000 response units (RU). b-MAA and b-SNA, at a concentration of 200 μg/ml, in 10 mM sodium acetate buffer, pH 4.5, were injected over the streptavidin layer for 7 min.

Fixation of nonbiotinylated lectins. Nonbiotinylated lectins were bound to the activated surface during 2 $\times$ 14 min pulses of the lectin (200 μg/ml) in 10 mM sodium acetate buffer, pH 4.3. Blockage of the remaining *N*-hydroxysuccinimide esters was performed by the addition of 1.0 M ethanolamine hydrochloride, pH 8.5, for 7 min. The reference "blank" channels were prepared in an analogous manner.

Deactivation of the lectins. For this purpose denatured SNA was immobilized to one flow channel and denatured MAA to the other (sensor chip contains four flow channels). The lectins were denatured either after immobilization onto the sensor chip by injection (2 $\times$ 4 min) of 12 M guanadinium chloride (pH 1.0) or by heating at 90°C for 15 min in 10 mM sodium acetate buffer, pH 4.5, prior to immobilization onto the sensor chip.

SPR detection of oligosaccharides and glycoproteins. All analyses were performed at a flow rate of 5 μl/min using Tris buffer as eluent. Injection times of oligosaccharides were 5 min followed by 7 min of dissociation. Injection times for glycoproteins were 7 min followed by 10 min of dissociation. Regeneration was performed using either a 2-min pulse of sodium acetate, pH 4.3, for removal of glycans, or a 2-min pulse of 0.02 M HCl, for removal of glycoproteins.

Data analysis. Association and dissociation rate constants were calculated by nonlinear fitting of the primary sensorgram data using the BIAevaluation 3.0 software. The closeness of fit for each estimated parameter is described by the statistical value χ^2. The association constant (K_A) was calculated both experimentally from the association (k_a) and dissociation (k_d) rate constants, and by Scatchard analyses based on the equilibrium steady-state kinetics. For the calculation of rate constants, samples were appropriately diluted in Tris buffer, ranging from 1 nM to 10 μM for the oligosaccharides, and from 10 nM to 100 μM for glycoproteins.

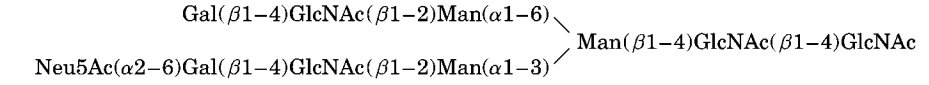
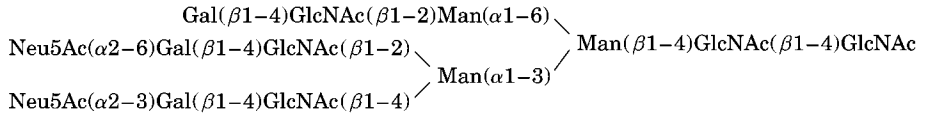
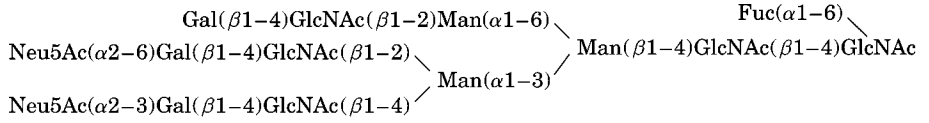
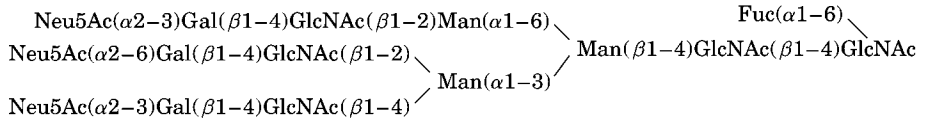
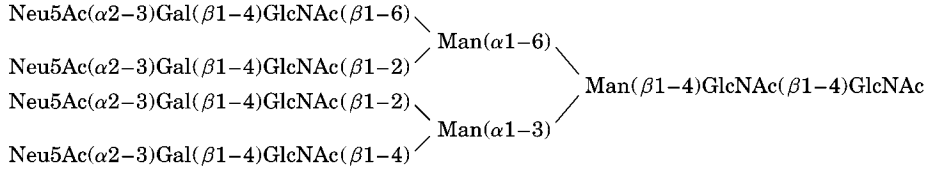
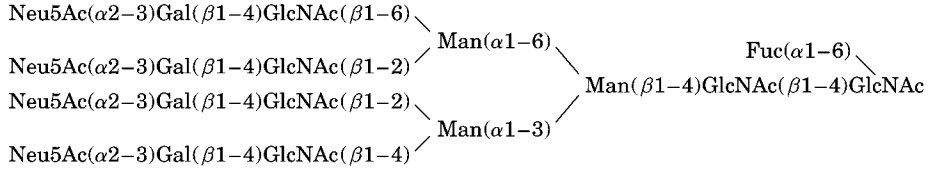
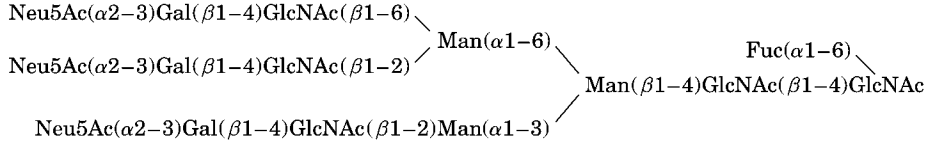
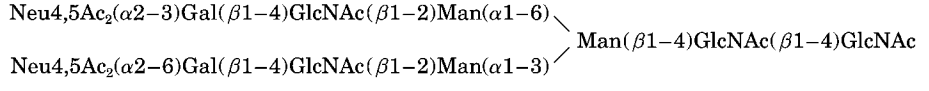
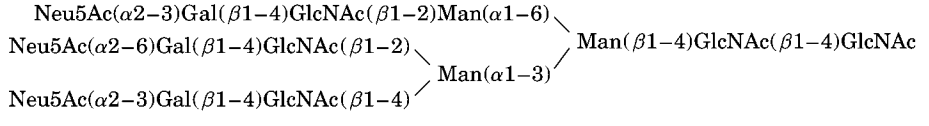
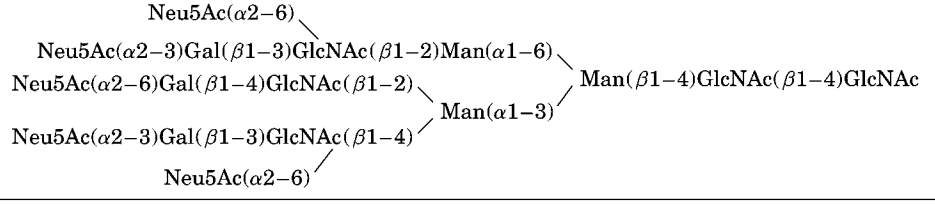
RESULTS AND DISCUSSION

Optimal system characteristics. Two systems were developed for characterizing the sialic-acid binding lectins from SNA and MAA, one more suitable for the analysis of glycans and the other for glycoproteins.

CM5 sensor chips were activated using the standard procedure for amine coupling. The best level of activation (~200 RU increase) was reached following activa-

TABLE 1

Structure of the Oligosaccharides Used in the Study

1	
2	
3	
4	
5	
6	
7	
8	
9	
10	

tion for 14 min with freshly prepared carbodiimide and hydroxysuccinimide buffers, used immediately after mixing. Although biotinylated lectins are used in system 2 (glycoprotein analysis), these could be replaced by the native lectins which give identical results.

The sensor chip for the analysis of glycan–lectin binding was prepared as follows: Native SNA was bound to channels 1 (Fig. 1A) and 3 (6500 RU each), and native MAA was bound to channels 2 (Fig. 1B) and 4 (11,000 RU each). The SNA bound to channel 3 and the MAA bound to channel 4 were denatured by flowing 12 M guanadinium chloride, pH 1.0, across these channels. This treatment caused no change in the level of SNA bound to the surface, but a reduction (−2500 RU) in the amount of MAA was observed: 8000 RU of MAA remained. The system utilized Tris-buffered saline (pH 8.0), containing Ca^{2+} and Mg^{2+} (2 mM each) for conservation of the activity of the lectins, and regeneration was performed with a 2-min injection of 10 mM sodium acetate, pH 4.3, conditions suitable for removal of glycans but not glycoproteins. The sensor chip was stored at 4°C in the presence of silica gel.

For the binding of glycoprotein to the lectins the sensor chip was prepared as follows: Channels 1 and 3 were treated with streptavidin, onto which b-SNA (4500 RU) was fixed to channel 1 and deactivated (heated in 10 mM sodium acetate buffer, pH 4.5) b-SNA (1000 RU) to channel 3. Channels 2 and 4 were used for the addition of native MAA (5000 RU, channel 2) and deactivated (heated in 10 mM sodium acetate buffer, pH 4.5) MAA (1000 RU, channel 4). The system utilized Tris-buffered saline (see above), and regeneration of the chip used 20 mM HCl for 2 min. The sensor chip was stored at 4°C in the presence of silica gel.

Choice of lectin. Both biotinylated and native lectins are suitable for immobilization to sensor chips, although biotinylated MAA became inactivated following immobilization.

Choice of blank reference channel. To measure the level of nonspecific binding, a blank with either identical or very similar properties to those of the active surfaces had to be implemented. The choice of a good blank channel is critical for an accurate and meaningful analysis of the data.

By screening a number of possibilities, it was evident that neither the native channel, the activated-deactivated channel, a biotin–streptavidin-containing layer, nor the use of another lectin provided a sufficient blank for either of these lectins. In each case the blank behaved in a manner different from the active channel, leading in the most extreme case to a higher level of nonspecific binding than specific binding. This was particularly the case in experiments with oligosaccharides.

The most suitable reference was shown to be the respective deactivated lectin. The better method for denaturing the lectins is the use of guanadinium chloride, since it is possible to immobilize more of the denatured lectin in this way than if the lectin is denatured beforehand by boiling.

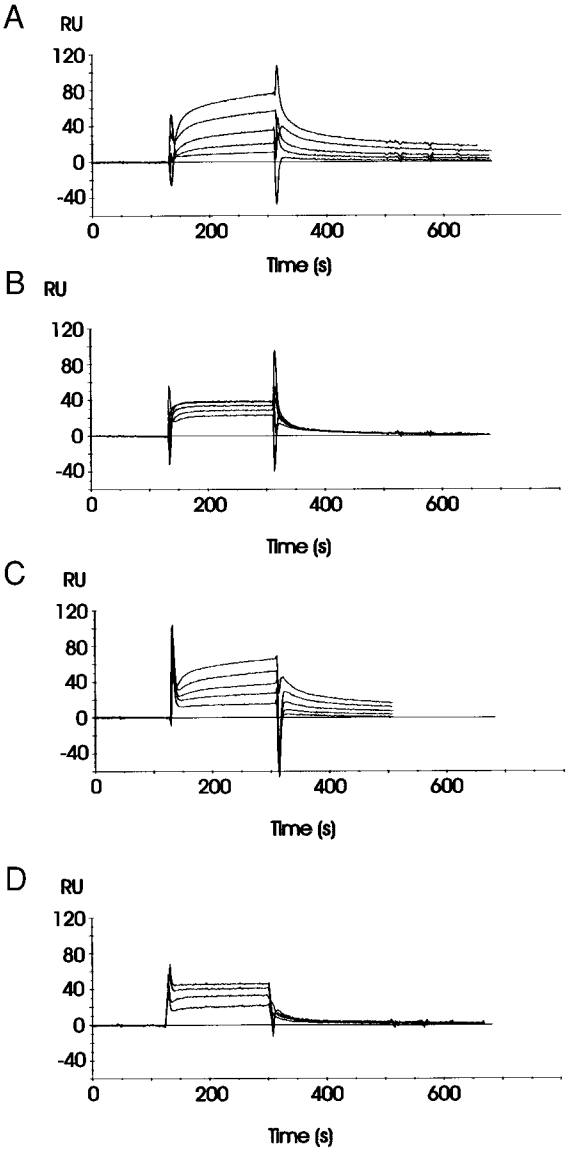


FIG. 2. Relative response (RU) versus time for the binding of oligosaccharides **2** (A and B) and **3** (C and D) to the immobilized lectins (MAA, A and C; SNA, B and D). Concentrations of oligosaccharide (from top to bottom): 10, 5, 2.5, 1.25, 0.625, and 0 μM.

Buffer. Several buffer systems were screened for their suitability in such a system. It was found that most buffers containing NaCl, Ca^{2+}, and Mg^{2+} ions and a pH between 7.0 and 8.0 were effective. However, the use of a high concentration of surfactant P20 (0.05%) or EDTA (3.0 mM) inactivated the lectin membrane, and so should be avoided. The chosen buffer system con-

TABLE 2

Kinetics Data for the Binding of Oligosaccharides **2–10** to *Maackia amurensis* Agglutinin (MAA)

Oligosaccharide	k_a (M^{-1} s^{-1})	k_d (s^{-1})	k_a/k_d (M^{-1})	K_A (M^{-1}) (Scatchard analysis)
2	2.52×10^3	7.49×10^{-3}	3.36×10^5 [1.04]	3.83×10^5 [2.94]
3	1.23×10^3	5.76×10^{-3}	2.14×10^5 [0.31]	2.92×10^5 [0.16]
4	1.05×10^4	5.70×10^{-3}	1.84×10^6 [1.07]	1.42×10^6 [0.71]
5	1.06×10^4	4.40×10^{-3}	2.41×10^6 [2.09]	1.96×10^6 [2.29]
6	3.43×10^3	4.68×10^{-3}	7.33×10^5 [1.00]	8.27×10^5 [0.68]
7	6.32×10^3	4.97×10^{-3}	1.27×10^6 [0.70]	1.12×10^6 [1.61]
8	2.57×10^3	5.49×10^{-3}	4.68×10^5 [0.40]	6.73×10^5 [5.80]
9	1.54×10^4	6.14×10^{-3}	2.51×10^6 [0.26]	2.40×10^6 [2.46]
10	8.90×10^2	3.66×10^{-3}	2.43×10^5 [0.36]	3.92×10^5 [5.33]

Note. Average values from five experiments, error between 10 and 20% for each result. The closeness of fit is indicated by the value of χ^2 in brackets after the calculated association constants.

taining 10 mM Tris–HCl, pH 8.0, reduced dramatically the solvent bulk effect.

Regeneration conditions. The most commonly used regeneration conditions for Biacore studies involve dilute hydrochloric acid. Although 20 mM HCl was found to be a suitable solution for complete regeneration of the lectin surface, it appeared that some of the activity may be lost, especially on the streptavidin-containing layer (10 mM is the recommended maximal HCl concentration for streptavidin-containing chips: BIAcore), and is particularly apparent when analyzing smaller glycans.

Attempts to regenerate the lectin surface using either monomeric sialic acid (25 mM) or lactose (1 M), the latter being a solution commonly used for removing glycoconjugates from SNA and MAA, were not successful due to only partial removal of the bound material.

The activity of the lectin-containing sensor chip to glycoproteins is largely unaffected by several hundred regeneration steps of 20 mM HCl for 2 min at 5 μl/min. To prevent the loss of sensitivity and activity of the sensor chip to oligosaccharides, which occurs when using dilute HCl as a regeneration agent, a buffer of 10 mM sodium acetate, pH 4.3, was used as an alternative. It removed all bound glycan from the lectins, and after equilibration, the loss of material from the chip per injection was negligible ($\leq$1 RU), whereas regeneration with 20 mM HCl resulted in a loss of $\sim$10 RU per injection. Glycoproteins are not removed from the lectin surface using these milder conditions, and this was the main reason for developing two systems.

Storage conditions. Storage of the sensor chip overnight at 4°C resulted in >50% loss of lectin activity, as did storage of the chip in running buffer. The best condition for storage of a sensor chip containing lectins is a moisture-free environment at 4°C.

Application of the systems. The specificity and accuracy of the systems were tested by analyzing both oligosaccharides and glycoproteins containing terminal Neu5Ac(α2–3)Gal(β1– and/or Neu5Ac(α2–6)Gal(β1– fragments. Kinetic data were calculated and compared. Since it was not possible to accurately screen oligosaccharides and glycoproteins in the same system, one was used for analyzing oligosaccharides and the other for glycoproteins.

TABLE 3

Kinetics Data for the Binding of Oligosaccharides **1–4, 9,** and **10** to *Sambucus nigra* Agglutinin (SNA)

Oligosaccharide	k_a (M^{-1} s^{-1})	k_d (s^{-1})	k_a/k_d (M^{-1})	K_A (M^{-1}) (Scatchard analysis)
1	3.71×10^4	1.40×10^{-3}	2.65×10^6 [0.15]	1.14×10^6 [0.15]
2				1.58×10^6 [0.41]
3	3.97×10^4	2.09×10^{-2}	1.90×10^6 [0.20]	1.36×10^6 [0.054]
4				7.88×10^5 [0.74]
9	4.71×10^4	1.37×10^{-3}	3.44×10^7 [0.20]	1.86×10^6 [0.34]
10	3.33×10^4	4.11×10^{-3}	8.10×10^6 [0.05]	1.31×10^6 [5.33]

Note. Average values from five experiments, error between 10 and 20% for each result. The closeness of fit is indicated by the value of χ^2 in brackets after the calculated association constants.

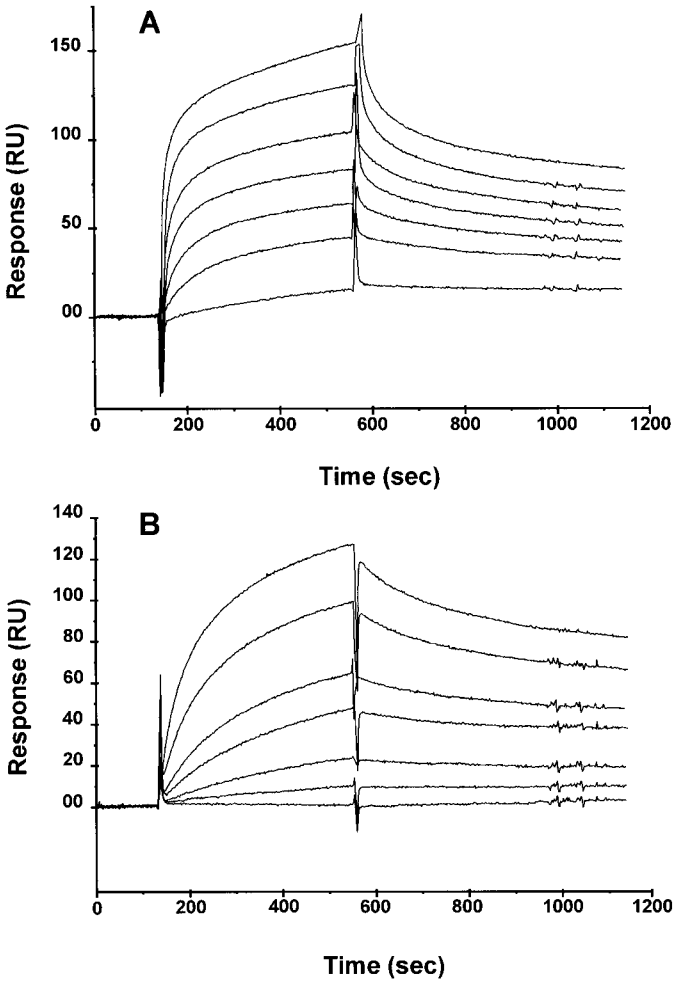


FIG. 3. Relative response (RU) versus time for the binding of fetuin to immobilized SNA (A) and MAA (B). Concentrations of fetuin (from top to bottom): 10, 5, 2.5, 1.25, 0.625, 0.3125, and 0 μM.

Analysis of oligosaccharides. Oligosaccharides (Table 1, structures **1–10**) were used for the measurement and comparison of association and dissociation kinetics. The kinetics of binding were calculated by using the BIAevaluation software 3.0 from experiments using a variety of glycan concentrations. MAA only bound to oligosaccharides containing Neu5Ac or Neu4,5Ac$_2$ (α2–3) linked to Gal, whereas SNA only bound Neu5Ac or Neu4,5Ac$_2$ (α2–6) linked to Gal. The binding of every oligosaccharide with MAA produced similar sensorgrams (Figs. 2A and 2C) as did those sensorgrams of SNA (Figs. 2B and 2D). The concentrations used for this analysis varied from 10 to 0.3125 μM, following a set of accurate twofold dilutions of the 10 μM sample. An injection of buffer without oligosaccharide was used as an internal standard to compensate for any small differences (usually $\leq$5 RU) between the active and denatured lectin surfaces. This result was subtracted from the active results for each flow cell before subtraction of denatured flow cell from the active one.

An overview of the results is given in Tables 2 and 3. Calculation of K_A either by Scatchard analysis or derived from the experimental values for the active rate constants k_a and k_d produced values which were in good agreement with each other, although in some cases modeling of the active kinetics for SNA, because of rapid association and dissociation (Figs. 2B and 2D), was difficult (e.g., samples **9** and **10**). In these cases we relied more on the Scatchard analysis results.

The K_A values for both SNA and MAA are as expected for lectin–carbohydrate binding. It should be noted that upon reaching saturation of the lectin surface, any further increase in the concentration of oligosaccharide results in a slow gradual decrease of the response. This could be due to removal of bound oligosaccharide by the excess carbohydrate.

An analysis of the kinetic constants revealed several interesting trends, particularly for MAA (Table 2). It would appear that glycans containing the Neu5Ac(α2–3)Gal epitope at the terminal position of a tri- or tetrasaccharide unit branching from the 6-position of a mannose residue (see Table 1: Structures **4, 5, 6, 7,** and **9**) possess a larger K_A and therefore bind more strongly than those without this epitope: [$k_a/k_d = 1.75 \times 10^6 \pm 1.02 \times 10^6$ and $K_A = 1.54 \times 10^6 \pm 8.6 \times 10^5$ (**4, 5, 6, 7,** and **9**)]. Those molecules without a Neu5Ac(α2–3)Gal epitope in such a position, or in molecules where this epitope is sterically hindered, e.g., by Neu5Ac(α2–6)GlcNAc in oligosaccharide **10** or by O-acetylation of the Neu5Ac residue in oligosaccharide **8,** have lower K_A

TABLE 4

Kinetics Data for the Binding of Three Glycoproteins to *Maackia amurensis* Agglutinin (MAA)

Glycoprotein	k_a (M^{-1} s^{-1})	k_d (s^{-1})	k_a/k_d (M^{-1})	K_A (M^{-1}) (Scatchard analysis)
Fetuin	1.09×10^3	2.01×10^{-3}	5.43×10^5 [6.76]	2.78×10^5 [4.30]
Human transferrin	**No binding**			
Horse transferrin	125	1.2×10^{-3}	1.04×10^5 [2.45]	4.01×10^5 [1.30]

Note. The closeness of fit is indicated by the value of χ^2 in brackets after the calculated association constants.

TABLE 5

Kinetics Data for the Binding of Three Glycoproteins to *Sambucus nigra* Agglutinin (SNA)

Glycoprotein	k_a (M^{-1} s^{-1})	k_d (s^{-1})	k_a/k_d (M^{-1})	K_A (M^{-1}) (Scatchard analysis)
Fetuin	7.96×10^3	4.14×10^{-3}	1.92×10^6 [5.37]	8.82×10^5 [5.00]
Human transferrin	2.97×10^3	3.81×10^{-3}	7.80×10^5 [1.03]	5.78×10^5 [5.46]
Horse transferrin	8.66×10^3	4.15×10^{-3}	2.09×10^6 [0.84]	8.68×10^5 [1.67]

Note. The closeness of fit is indicated by the value of χ^2 in brackets after the calculated association constants.

and k_a [$k_a/k_d = 3.15 \times 10^5 \pm 1.53 \times 10^5$ and $K_A = 4.35 \times 10^5 \pm 2.38 \times 10^5$ (**2, 3, 8,** and **10**)], although the dissociation rate constant k_d is unaffected by the position of the active epitope.

Since the Neu5Ac(α2–6)Gal epitope is in a similar chemical environment in each of the oligosaccharides, it could be expected that the association kinetics for these molecules would be similar. This appears to be the case for K_A derived from Scatchard analysis, in which all the values fall within a small range of each other: $1.34 \times 10^6 \pm 5.5 \times 10^5$.

Analysis of glycoproteins. Fetuin, human transferrin, and horse transferrin were the glycoproteins chosen for this analysis. The glycoprotein concentrations used for the analyses ranged from 100 μM to 10 nM.

The most obvious observation of this analysis (see Fig. 3) was that fetuin contained both (α2–3) and (α2–6) linked sialic acid residues, whereas both human and horse transferrin contained either no or very little (α2–3)-linked sialic acid residues.

As can be seen from Tables 4 and 5 the values of the different kinetic constants are in general lower than those recorded in the oligosaccharide experiments, and in addition the values of K_A derived from active kinetics and steady-state data appear to differ, although they are of the same order of magnitude. This is not particularly surprising since several factors such as number of glycosylation sites, level of glycosylation, and the assortment of glycan structures have not been taken into account: the kinetics are based on the concentration of glycoprotein, and not of the glycan concentration. Kinetic data obtained for glycoproteins using this system can at present only be used as a fingerprint for identification of that molecule. Statistically meaningful results will only be able to be calculated once the level and variety of glycosylation is known. Practically, it appears either that glycoproteins bind more strongly to lectins than oligosaccharides or that once they are bound are more difficult to remove since harsher regeneration conditions are needed, although this is not apparent from the calculated k_d values.

CONCLUSION

Surface plasmon resonance is an ideal technique for characterizing both lectins and specific carbohydrate epitopes by using lectins immobilized to a carboxymethylated dextran-coated sensorchip. Lectins are easily immobilized onto the surface of a sensor chip, and a sensitive and automated screening system can be developed very quickly. A good choice of buffer, regeneration conditions, and blank leads to reproducible results, and accurate and rapid characterization of the lectin specificity. In systems which contain fast association, fast dissociation, or both, the value of K_A should be derived from the equilibrium steady-state response, as opposed to the active kinetic rate constants since it is very difficult to accurately measure fast kinetics which commonly appear as square-like pulses, e.g., oligosaccharide binding to SNA (Fig. 2 and Table 3). However, when this is not the case, the values obtained for K_A derived from steady-state data and active kinetics are in good agreement with each other, as can be seen from the identical K_A values for every oligosaccharide binding to MAA, within the error of the system (10–20%). The systems developed within this work are now being employed for the profiling of glycans from natural resources using an array of lectins.

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