Biomolecular Interactions

Direct Observation of Sugar–Protein, Sugar–Sugar, and Sugar–Water Complexes by Cold-Spray Ionization Time-of-Flight Mass Spectrometry**

Shin-Ichiro Nishimura, Noriko Nagahori, Kenji Takaya, Yuki Tachibana, Nobuaki Miura, and Kenji Monde*

The direct observation of noncovalent and specific intermolecular-recognition events such as enzyme–substrate, antigen–antibody, receptor–ligand, carbohydrate–protein, and carbohydrate–carbohydrate interactions is important for understanding the mechanisms behind these biological processes. Electrospray ionization mass spectrometry (ESI MS) is an important tool for investigations of the sensitivity, specificity, and speed of noncovalent complex formation.[1] The advantages of MS over traditional methods such as UV/Vis spectroscopy, fluorescence spectroscopy, surface plasmon resonance (SPR), isothermal titration microcalorimetry, NMR spectroscopy, and X ray crystallographic analysis include the accuracy of mass measurement, speed of analysis, and small sample quantities. However, the harsh conditions of the ionization process in MS are often detrimental to the survival of noncovalent and unstable biomolecular interactions such as those of sugar–protein, sugar–sugar, and sugar–water complexes. Recently, Yamaguchi and co-workers developed cold-spray ionization mass spectrometry (CSI MS), which allows facile and precise characterization of labile self-assembling nanostructures and unstable organometallic complexes in solution.[2] CSI MS may become one of the most promising and versatile tools for characterizing a variety of weak but specific biomolecular interactions, as the ionization at low temperature (−20 °C) allows direct observation of unstable large-scale aggregates of amino acids or nucleosides in organic solvents with magnetic-sector-equipped instruments.[2d,e] It was suggested that a cooled ion spray promotes stable solvation–ionization processes through increased compound polarizability caused by higher dielectric constants at low temperature. Moreover, CSI MS combined with an orthogonal acceleration time-of-flight (oa-TOF) mass analyzer extends the applicability of this method to the characterization of dynamic interactions of biomacromolecules. This is possible through a number of attractive features of TOF MS analyzers, such as their theoretically unlimited mass range, very high spectrum acquisition rates, high ion transmission, high sensitivity, multiplex detection capacity, reasonable mass resolution, and simplicity in instrument design. Our interest is focused on the potential of CSI-TOF MS, with particular respect to specific and weak biomolecular interactions in water at low temperature. Herein we report the feasibility of the CSI-TOF MS method to monitor directly the formation of noncovalent protein–carbohydrate, carbohydrate–carbohydrate, and carbohydrate–water complexes in aqueous solution at 4 °C.

Figure 1a shows the effect of temperature on complex formation between hen egg lysozyme and the chitooligosaccharide, chitotetraose. As anticipated, the intensity of the signal that corresponds to the lysozyme–chitotetraose complex as the major hydrolytic product was gradually enhanced by lowering the ion spray temperature of the reaction mixture from 200 to 4 °C. As shown in Figure 1b, CSI-TOF MS is quite an efficient and simple method for the determination of dissociation constants of protein–ligand interactions, as it does not require the use of molecular probes. Indeed, the dissociation constant K_d of lysozyme with chitotetraose was determined to be 1.6×10^{-5} M, which is in good agreement with data reported previously.[3] Furthermore, CSI-TOF MS can be used to search for a compound that shows the highest affinity for the target protein in a mixture of several oligosaccharides by making a simple "snapshot assay".[4] As illustrated in Figure 2a, lysozyme interacted selectively with chitooligosaccharides $(GlcNAc)_3$, $(GlcNAc)_4$, and $(GlcNAc)_5$ at 4 °C in a compound library containing 23 different oligosaccharide types. Such valuable information could not be obtained by common ESI-TOF MS techniques carried out at 200 °C as shown in Figure 2b. Moreover, when a compound library void of chitooligosaccharides was assayed at 4 °C, lysozyme showed significant affinity toward the maltooligosaccharides $(D\text{-glucose})_{3-7}$ and γ-cyclodextrin. These interactions cannot be detected by general spectroscopic analyses such as UV/Vis, fluorometric, and SPR methods.[5] Figure 3a shows binding between lysozyme and maltotriose ($(Glc)_3$) (m/z 1646.3 $[M+9A]^{9+}$ and m/z 1851.9 $[M+8A]^{8+}$; 'A' = ion adduct). Although the interaction is weak and is considered nonspecific,[5] the CSI-TOF MS data instead indicate specific binding. The results of a competition experiment (Figure 3a and b) suggest that maltotriose binding involves the same lysozyme cavity as for the chitotriose $(GlcNAc)_3$. This is supported by computer-assisted docking simulations carried out in our laboratory (Figure 3c). Notably, CSI-TOF MS can be employed to search for unexpected ligand candidates with "specific and low affinity" for a target protein. Identified ligands could then be applied as scaffold molecules for the design of new drugs (inhibitors) with high potential for success.

[*] Prof. S.-I. Nishimura, Dr. N. Nagahori, K. Takaya, Dr. Y. Tachibana, Dr. N. Miura, Dr. K. Monde
Division of Biological Sciences, Graduate School of Science
Frontier Research Center for Post-Genome Science and Technology
Hokkaido University
N21, W11, Sapporo 001-0021 (Japan)
Fax: (+81) 11-706-9042
E-mail: shin@glyco.sci.hokudai.ac.jp

[**] This work was supported in part by a grant from the National Project on Functional Glycoconjugate Research Aimed at Developing for New Industry by the Ministry of Education, Science, and Culture of Japan. A portion of this study was presented at the 84th Annual Meeting of the Chemical Society of Japan on March 29, 2004 in Nishinomiya, Japan. We acknowledge Prof. K. Yamaguchi of Tokushima Bunri University for valuable suggestions and comments on CSI mass spectrometry.

Supporting information for this article is available on the WWW under http://www.angewandte.org or from the author.

 DOI: 10.1002/ange.200461867

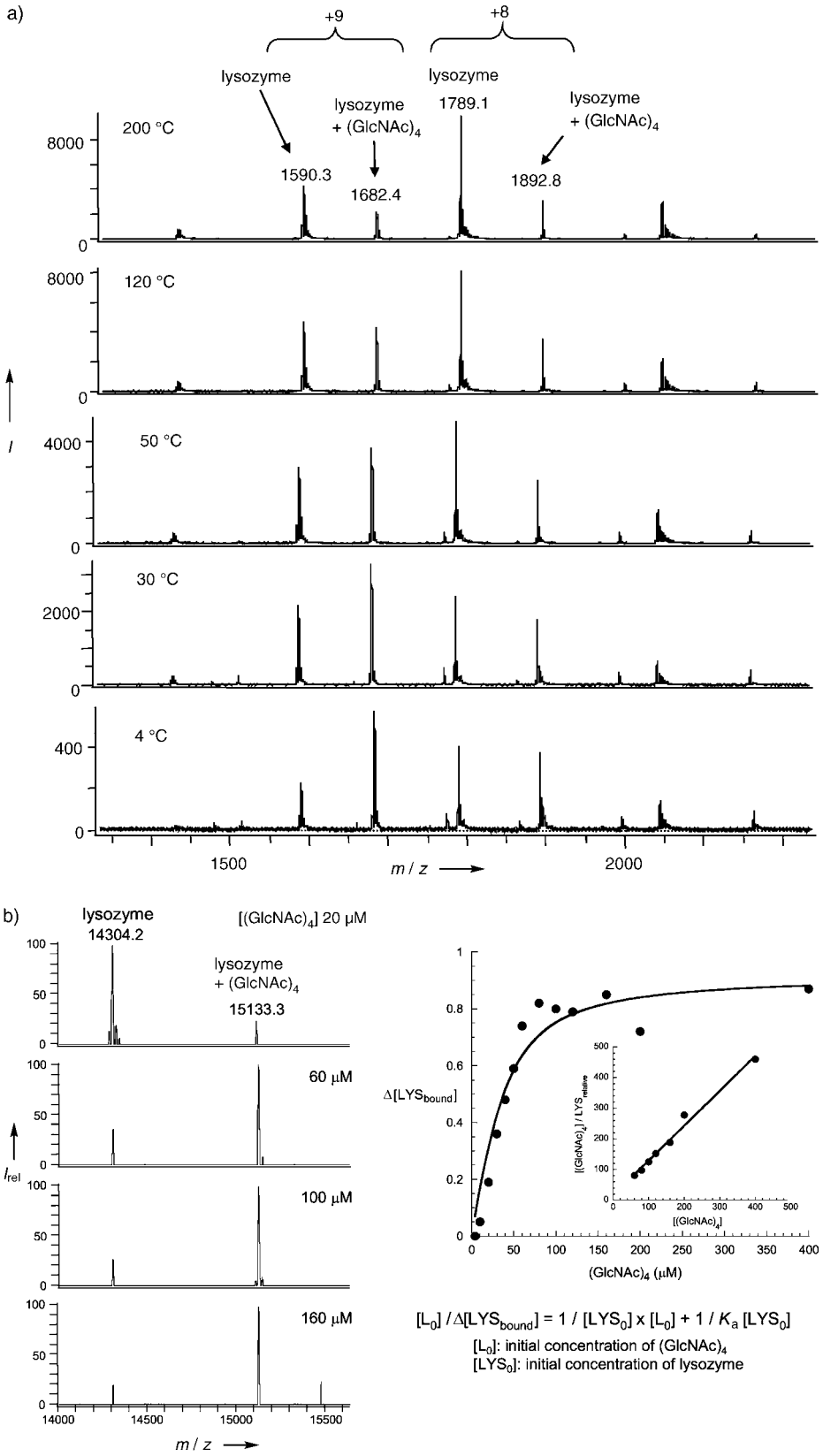

Figure 1. Interaction of hen egg lysozyme with chitooligosaccharides. a) Temperature dependence of signal intensities of the lysozyme–chitotetraose ($(GlcNAc)_4$) complex in ESI (CSI) MS. Spray temperatures are indicated. [Lysozyme] = 35 µM, $[(GlcNAc)_4]$ = 200 µM. b) The binding of lysozyme with $(GlcNAc)_4$ at 4 °C. [Lysozyme] = 35 µM; the $(GlcNAc)_4$ concentrations are indicated. The peak intensities from mass spectra were used to determine the relative amount of bound lysozyme as a function of $[(GlcNAc)_4]$, and a sample K_d determination is shown at the bottom. GlcNAc = *N*-acetyl-D-glucosamine.

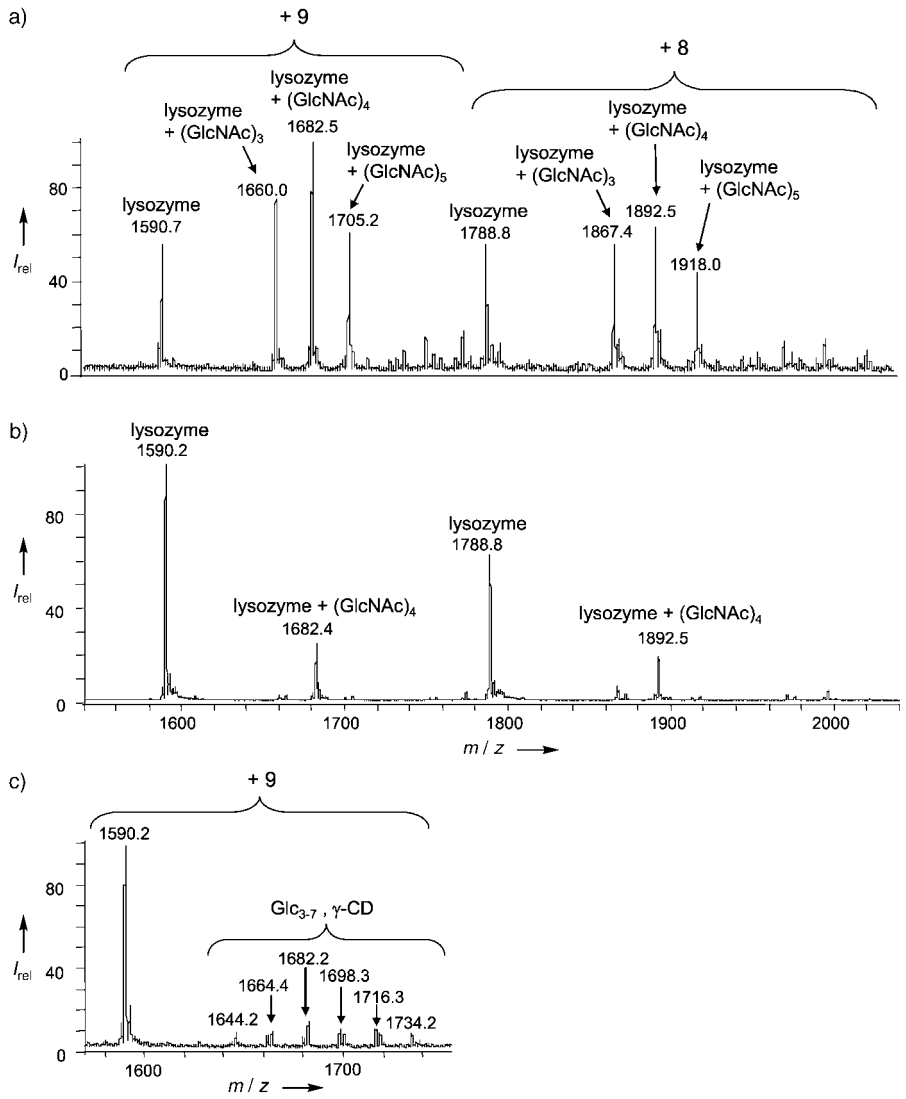

Figure 2. Oligosaccharide–protein complex screening. The interaction of lysozyme with an oligosaccharide library containing GlcNAc, $(GlcNAc)_2$, $(GlcNAc)_3$, $(GlcNAc)_4$, $(GlcNAc)_5$, Glc, $(Glc)_3$, $(Glc)_4$, $(Glc)_5$, $(Glc)_6$, $(Glc)_7$, cellobiose (β-Glc-[1→4]-Glc), lactose (β-Gal-1[→4]-Glc), methyl α-mannoside, $(Man)_5$, D-glucosamine, L-arabinose, lactose, xylose, L-gluconolactone, α-CD, β-CD, and γ-CD (all at 35 μM) was monitored by a) CSI-TOF MS at 4 °C and b) ESI-TOF MS at 200 °C. c) CSI-TOF MS analysis (4 °C) of lysozyme with an oligosaccharide library lacking chitooligosaccharides $(GlcNAc)_2$, $(GlcNAc)_3$, $(GlcNAc)_4$, and $(GlcNAc)_5$. [Lysozyme] = 35 μM. Glc = D-glucose; Gal = D-galactose; Man = D-mannose; CD = cyclodextrin.

Our attention was next directed toward monitoring the weak but specific noncovalent interactions of glycoconjugates in nature. Carbohydrate–carbohydrate interactions may be crucial to intercellular interactions that lead to cellular differentiation, growth, and malignancy. Lewis^X trisaccharide (Le^X) has been reported to self-assemble through carbohydrate–carbohydrate interactions in the presence of Ca^{2+} ions.[6] As shown in Figure 4, CSI-TOF MS spectra measured at 4 °C clearly demonstrate that Le^X trisaccharides preferentially dimerize by binding to Ca^{2+} ions, and that in the absence of Ca^{2+}, Le^X trisaccharides form a range of larger aggregates of up to 10 trisaccharide units (*m*/*z* 2670.0 $[10\,Le^X + 2\,Na]^{2+}$) (Figure 4b). Several ion peaks shown in Figure 4a are evidence for the preferential Le^X–Ca^{2+} complex formation such as $[Le^X + Ca - H]^+$, $[Le^X + Ca - H + H_2O]^+$, and $[Le^X + CaCl]^+$. The ion peaks of $[2\,Le^X + Ca]^{2+}$, $[2\,Le^X + 2\,Ca - 2\,H + 2\,H_2O]^{2+}$, and $[2\,Le^X + 2\,CaCl]^{2+}$ provide valuable information that reveals the mechanism of Le^X–Le^X dimerization in the presence of Ca^{2+} ions.

Surprisingly, it also appears that the minimal active structure of the antifreeze glycopeptide (*sy*$AFGP_3$)[7] forms specific complexes with three water molecules as shown in Figure 5, whereas the inactive monomeric AFGP (*sy*$AFGP_1$) produced neither the complex with water nor the self-aggregation behavior found with the Le^X trisaccharide. Hydration of *sy*$AFGP_3$ at 4 °C (*m*/*z* 949.4 $[M + 3\,H_2O + 2\,H]^{2+}$, *m*/*z* 960.4 $[M + 3\,H_2O + H + Na]^{2+}$, and *m*/*z* 968.4 $[M + 3\,H_2O + H + K]^{2+}$) by CSI-TOF MS may become an important way to investigate AFGP binding with the ice (water) lattice, and hence the mechanism of its antifreeze

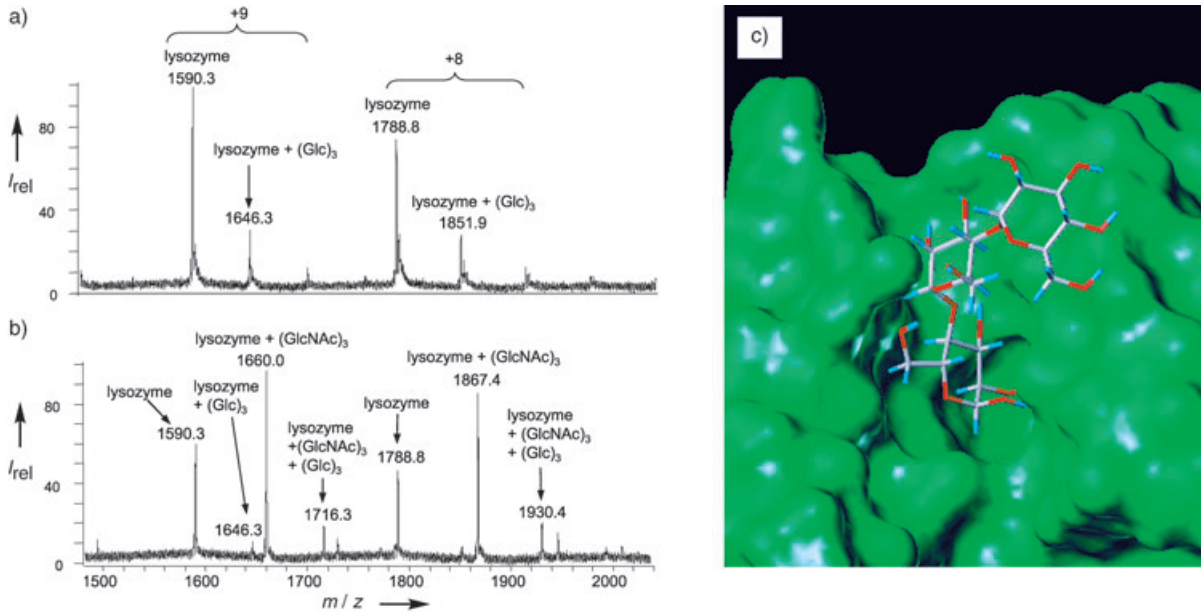

Figure 3. CSI-TOF MS (4 °C) evaluation and computer simulation of "nonspecific" interactions. a) The interaction between lysozyme (35 μM) and $(Glc)_3$ (100 μM). b) Competitive assay between lysozyme (35 μM) and $(GlcNAc)_3$ (100 μM). (c) Docking simulation of maltotriose ($(Glc)_3$) with the subsite of lysozyme calculated with the SYBYL FlexX module.[8]

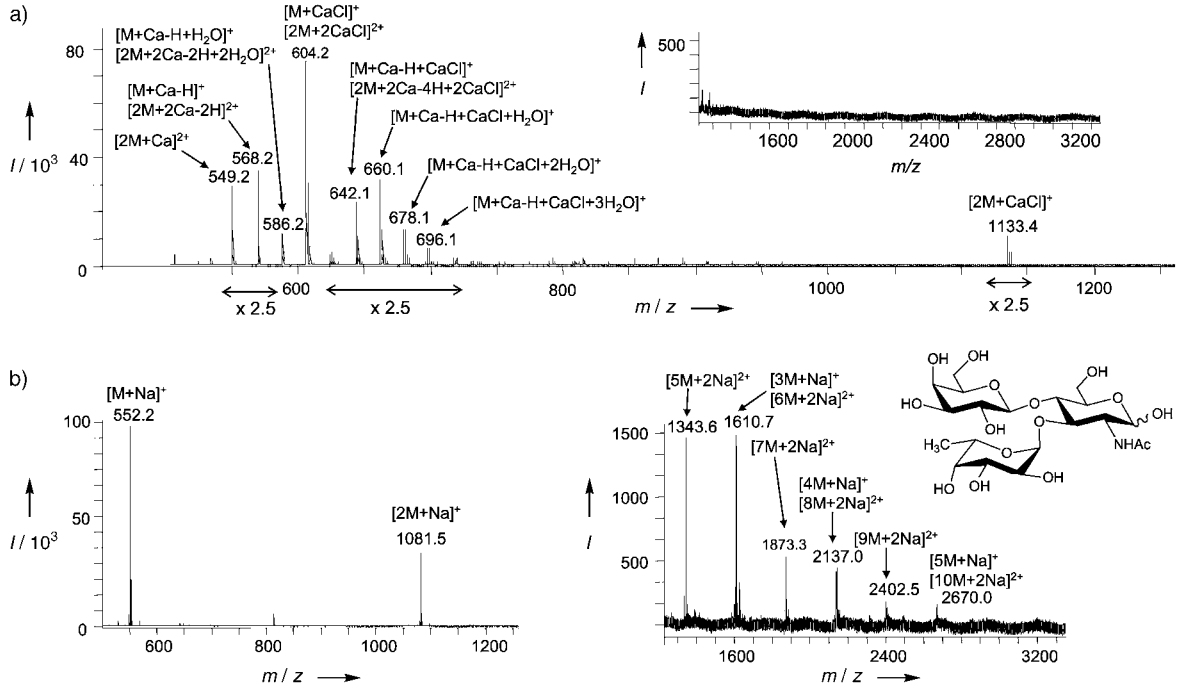

Figure 4. CSI-TOF mass spectra of Le^X self-assembly at 4 °C a) in the presence and b) in the absence of Ca^{2+}. The experiments were performed by injecting 20 μL $Le^X/CaCl_2$ stock solution ($[Le^X] = 190$ μM (3.8 nmol Le^X) and $[CaCl_2] = 1.0$ mM). Le^X = Lewis^X trisaccharide, with structure shown.

activity.[9] As illustrated in Figure 5c, when fully deuterated *sy*AFGP$_3$ with D_2O as solvent were observed, an analogous spectrum with the "water signals" shifted by 20 Da instead of 18 Da was obtained. This is evidence that the binding of three water molecules is favored over the binding of just one or two.

In conclusion, we found that CSI-TOF MS is a highly sensitive and reliable method for analyzing specific non-covalent complexes of glycoconjugates under nondisruptive conditions. The results reported herein demonstrate the ability and versatility of CSI-TOF MS as a means of direct observation and characterization of weak and specific carbohydrate-related interactions in aqueous solution at low temperature. The merits of this novel technique are evident as it will provide not only fundamental insight into the

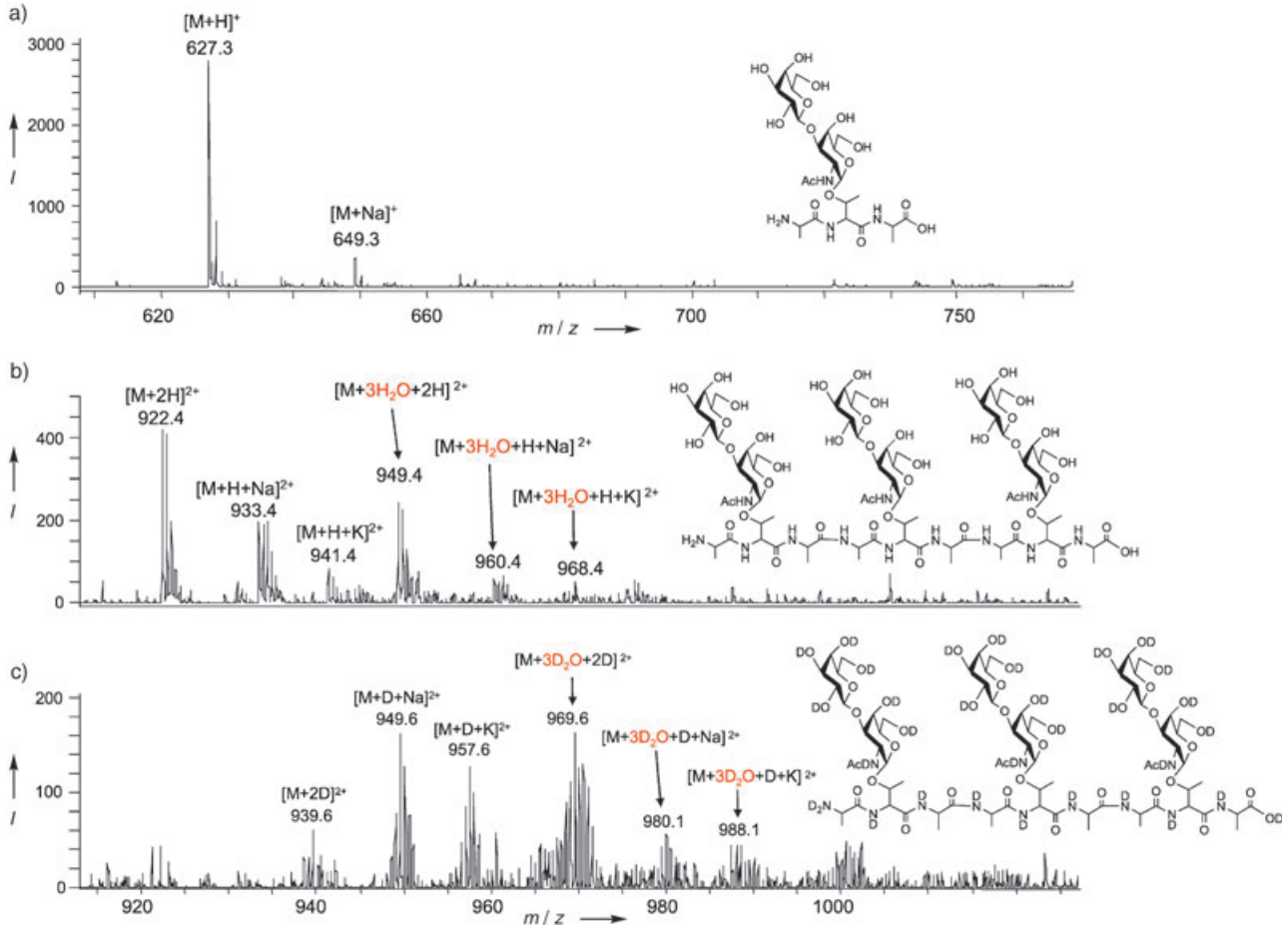

Figure 5. Direct observation of the specific interaction between *sy*AFGP$_3$ and water (D_2O) at 4 °C. CSI-TOF MS of a) monomeric AFGP (*sy*AFGP$_1$) and b) trimeric AFGP (*sy*AFGP$_3$) in water, and c) *sy*AFGP$_3$ in D_2O. AFGP injection volumes (20 μL) contained 320 pmol AFGP. [AFGP] = 16 μM.

mechanisms of biomolecular recognition, but also valuable information for high-throughput molecular screening for bioactive compounds.

Received: September 2, 2004
Revised: October 23, 2004
Published online: December 13, 2004

Keywords: aggregation · carbohydrates · enzymes · glycoproteins · mass spectrometry

[1] a) J. B. Fenn, *Angew. Chem.* **2003**, *115*, 3999 – 4024; *Angew. Chem. Int. Ed.* **2003**, *42*, 3871 – 3894; b) J. B. Fenn, M. Mann, K. Meng, S. F. Wong, C. M. Whitehouse, *Science* **1989**, *246*, 64 – 70; c) B. Ganem, Y. T. Li, J. D. Henion, *J. Am. Chem. Soc.* **1991**, *113*, 6294 – 6296; d) V. Katta, B. T. Chait, *J. Am. Chem. Soc.* **1991**, *113*, 8534 – 8535; e) M. Baca, S. B. H. Kent, *J. Am. Chem. Soc.* **1992**, *114*, 3992 – 3993; f) P. Kebarle, L. Tang, *Anal. Chem.* **1993**, *65*, 972A – 986A.

[2] a) S. Sakamoto, M. Fujita, K. Kim, K. Yamaguchi, *Tetrahedron* **2000**, *56*, 955 – 964; b) Y. Yamanoi, Y. Sakamoto, T. Kusukawa, M. Fujita, S. Sakamoto, K. Yamaguchi, *J. Am. Chem. Soc.* **2001**, *123*, 980 – 981; c) S. Sakamoto, T. Imamoto, K. Yamaguchi, *Org. Lett.* **2001**, *3*, 1793 – 1795; d) M. Kunimura, S. Sakamoto, K. Yamaguchi, *Org. Lett.* **2002**, *4*, 347 – 350; e) S. Sakamoto, K. Nakatani, I. Saito, K. Yamaguchi, *Chem. Commun.* **2003**, 788 – 789; f) S. Sakamoto, K. Yamaguchi, *Angew. Chem.* **2003**, *115*, 933 – 936; *Angew. Chem. Int. Ed.* **2003**, *42*, 905 – 908; g) K. Yamaguchi, *J. Mass Spectrom.* **2003**, *38*, 473 – 490.

[3] For more information regarding K_d for the interaction between lysozyme and chitotetraose ($K_d = 5.6 \times 10^{-6}$ M), see: M. Schindler, Y. Assaf, N. Sharon, D. M. Chipman, *Biochemistry* **1977**, *16*, 423 – 431.

[4] An ESI MS based diffusion measurement can also be used to screen for noncovalent complexes of lysozyme and maltotriose: S. M. Clark, L. Konermann, *Anal. Chem.* **2004**, *76*, 1257 – 1263.

[5] Excellent methods and applications of nanoelectrospray fourier transform ion cyclotron resonance (FT-ICR) mass spectrometry have recently been reported: a) W. Wang, E. N. Kitova, J. S. Klassen, *Anal. Chem.* **2003**, *75*, 4945 – 4955; b) R. Daneshfar, E. N. Kitova, J. S. Klassen, *J. Am. Chem. Soc.* **2004**, *126*, 4786 – 4787.

[6] a) I. Eggens, B. Fenderson, T. Toyokuni, B. Dean, M. Stroud, S. Hakomori, *J. Biol. Chem.* **1989**, *264*, 9476 – 9484; b) G. Siuzdak, Y. Ichikawa, T. J. Caulfield, B. Munoz, C.-H. Wong, K. C. Nicolaou, *J. Am. Chem. Soc.* **1993**, *115*, 2877 – 2881.

[7] Y. Tachibana, G. L. Fletcher, N. Fujitani, S. Tsuda, K. Monde, S.-I. Nishimura, *Angew. Chem.* **2004**, *116*, 874 – 880; *Angew. Chem. Int. Ed.* **2004**, *43*, 856 – 862.

[8] T. Ohta, N. Miura, N. Fujitani, F. Nakajima, K. Niikura, R. Sadamoto, C.-T. Guo, T. Suzuki, Y. Suzuki, K. Monde, S.-I. Nishimura, *Angew. Chem.* **2003**, *115*, 5344 – 5347; *Angew. Chem. Int. Ed.* **2003**, *42*, 5186 – 5189.

[9] M. Matsumoto, S. Saito, I. Ohmine, *Nature* **2002**, *416*, 409 – 413.

