Effect of Clustering on Fluctuations in Binding Activity of Sugar Chains to Influenza Viruses

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***Abstract*—The spread of influenza A virus variants requires continuous monitoring given the high mutation rate. The surveillance of such mutations is essential and therefore highly sensitive sensor systems are needed. Host changes can be observed as changes in the bonding between virus protein and host sugar chains. We have investigated the dispersion of sugar chains such as sialylglycopeptide (SGP) and sialoglycopolymer, to name a few. These represent potential candidate molecules in biosensors for detecting influenza virus host specificity. The clustering of sugar chains might enhance and stabilize the binding activity and the relative errors in binding activity fluctuations will be addressed here.**

***Index Terms*—influenza virus, host specificity, sugar chain, neoglycoprotein, biosensor**

I. Introduction

A worldwide outbreak of influenza is the most feared potential public health emergency of international concern [1]. The spread of influenza A virus variants requires continuous monitoring given the high mutation rate of the virus [2]. Many infections in humans have been reported and this might be due to the potential of these viruses to mutate which results in a change of host from birds to humans [3], [4]. Since the surveillance of such mutations is essential, the development of highly sensitive sensor systems is needed. We have developed a biosensor using nano-carbon materials which can detect the preferred host of the virus [5], [6].

Host changes can be observed as a change in the bonding between virus protein and host sugar chains. We have developed several processes to fabricate biosensor systems. One of these utilizes an immobilizing process for the binding of specific molecules onto the device surface, such as those found in avian and human influenza viruses [7]–[10]. For biosensors with host specificity for influenza viruses, we used sugar chains with different coordination of terminal sialic acid and penultimate galactose, such as α(2-3)-linkage sialic acid for an avian influenza virus and α(2-6)-linkage sialic acid for a human virus.

In this paper, we will discuss about the dispersion of sugar chains such as sialylglycopeptide (SGP) [11], and sialoglycopolymer [12], [13] to name a few. These represent potential candidate molecules for use in biosensors for the detection of influenza viruses, and we have investigated virus host specificity using an enzyme-linked immunosorbent assay (ELISA). Using bovine serum albumin (BSA) localization, we have obtained a stable distribution. The clustering of sugar chains might also enhance and stabilize the binding activity. The relative errors in binding activity fluctuations will also be addressed here. Compared with single sugar chains of SGP, clustered molecules of sialoglycopolymer and SGP-BSA display smaller relative errors.

II. Experiments

Sugar chains of SGP (molecular weight (MW) $\sim$2.8 kDa), sialoglycopolymer (MW $\sim$3500 kDa), and neoglycoprotein of SGP-BSA (MW $\sim$66 kDa) were used. For SGP, glycopeptide

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powder was purified to monomer by reversed-phase HPLC. For sialoglycopolymer, sialyllactosamine was attached to polymer chains of γ-polyglutamic acid by chemoenzymatic synthesis [14]. The exchange ratio of sugar chains was 51.5%, and the structures of the synthesized sialoglycopolymer were confirmed by ^{1}H and ^{13}C NMR analysis [15]. For SGP-BSA, the amino group of SGP was attached to sulfhydryl groups of BSA using *m*-maleimidobenzoyl-*N*-hydroxysulfosuccinimide ester [16]. The average number of SGP groups attached to each BSA molecule was approximately 10, as determined by HPLC analysis of purified fluorescently-labeled *N*-glycan.

The properties of the binding between viruses and sugar chains were examined by ELISA. Solutions of sugar chains at concentrations from 1000 ng/mL to 15.6 ng/mL with two-fold serial dilution by PBS were spread onto 96-well plates (Nunc MaxiSorp flat-bottom, Thermo Fisher Scientific) for sialoglycopolymer and SGP-BSA. One thousand nanograms per milliliter of sialoglycopolymer corresponds to 997 pM of sialic acid, and that of SGP-BSA to 279 pM. SGP was used at concentrations ranging from 5 ng/mL to 78 pg/mL, and 5 ng/mL of SGP corresponds to 3.49 pM of sialic acid. Following incubation of the sugar chains for 2 hours, viruses comprising 32 HAU of A/California/07/2009 (H1N1) and 6 HAU of A/mallard/Hokkaido/24/2009 (H5N1), a non-pathogenic avian influenza virus, were fed into the wells. The viruses were adsorbed overnight at 4°C. Bound viruses were inactivated and fixed with 10% formalin in PBS buffer (3.7% formaldehyde) at room temperature for 30 min. Using rabbit anti-human influenza A and B polyclonal antibodies (Takara Bio) and Simple Stai MAX PO (Nichirei Bioscience), the addition of *o*-phenylenediamine (Wako Pure Chemical) produced a yellow color. The binding activity was measured by the absorbance at 490 nm using a Model 680 Microplate Reader (Bio-Rad).

AFM (AFM5010, Hitachi High-Tech Science, Tokyo, Japan) was used for the observation of sugar chains. For the SGP and sialoglycopolymer, the stock sample (1 mg/mL) was diluted to 10 ng/mL or 1 ng/mL with double-distilled water and then 1 μL of the samples was observed on a freshly cleaved mica surface in air using a self-sensing micro-cantilever PRC-DF40P instrument (Hitachi High-Tech Science) with resonance frequency of 477.2 kHz. For the observation of sugar chains of SGP-BSA in buffer solution, the stock sample of the sugar chains (1 mg/mL) was diluted to 1 μg/mL with double-distilled water, and 100 μL of the samples was spread onto a freshly cleaved mica surface attached in the dish. Micro-cantilevers of SI-DF3 (Hitachi High-Tech Science) with resonance frequency of 27 kHz and spring constant of 1.7 N/m were used.

III. Results and Discussion

Figure 1(a) shows the binding activity of SGP to avian influenza virus of H5N1. SGP was directly attached onto the ELISA plate by the terminal amino groups. Avian type receptor of α(2-3)-linked sugar chains has a higher binding activity compared to human type receptor of α(2-6)-linked sugar chains. In this case, however, we observed large fluctuations in

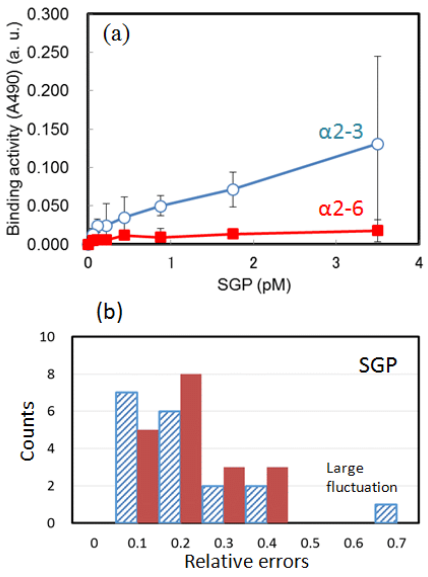

Fig. 1. Binding of viruses to SGP detected by ELISA. (a) Binding activity of avian H5N1 influenza viruses to α(2-3)- and α(2-6)-linked SGP. (b) Relative errors for binding activity of SGP. Red and blue (hatched) bars are for human and avian IFVs, respectively.

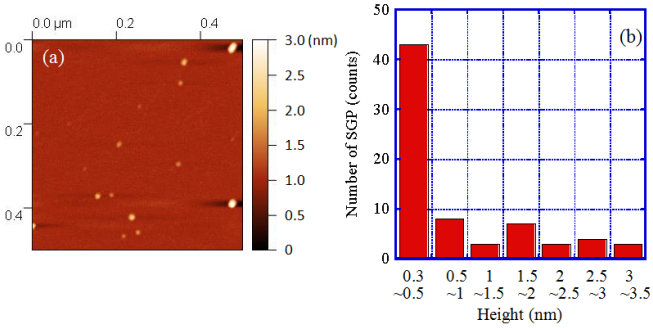

Fig. 2. (a) Representative AFM image and (b) height distribution of SGP.

the binding activity. For example, the standard deviation of the binding activity at an SGP concentration of 3.49 pM was 0.115 with an average absorbance of 0.131. At times we observed no binding activity of α(2-3)-linked sugar chains to H5N1, and where control experiments confirmed the presence of active viruses. Furthermore, the relative errors as shown in Fig. 1(b) also show a wide distribution, where over 0.4 (40%) relative errors could be observed. The standard deviation of the relative errors is 0.170 for the binding activity of α(2-3)-linked sugar chains to H5N1, and 0.114 for that of α(2-6)-linked sugar chains.

An AFM image of SGP (10 ng/mL) spread onto mica is shown in Fig. 2(a). Several molecules are present with wide height distribution between 0.3 nm and 3.9 nm, as shown in Fig. 2 (b). This might result from molecular conformational differences of the SGP. SGP has multiple amino groups that can potentially bind to the substrate, and differences in the extent of binding could generate the conformational variety

observed. When the molecules adopt a lying conformation, which can be observed with heights of less than *ca.* 1 nm, the active sites of terminal sialic acid seems to be too close to the substrate and the binding could be shielded. The standing conformation of SGP, which can be observed with heights of *ca.* 3 nm, could be associated with greater binding activity. Figure 2(b) shows the spatial size distributions at *ca.* 15 nm and 20 nm. This might result from the conformational flexibility of long molecules of SGP. Thus, SGP shows large fluctuations, which could relate to the observed fluctuations in binding activity.

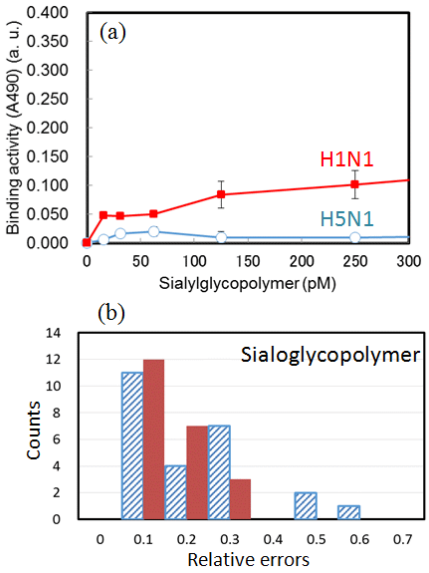

Fig. 3. Binding of viruses to sialoglycopolymer containing α(2-6)-linked sialic acid detected by ELISA is shown in (a). Filled squares denote human influenza virus (H1N1), while open circles denote avian virus (H5N1). (b) Relative errors for binding activity of sialoglycopolymer. Red and blue (hatched) bars are for human and avian IFVs, respectively.

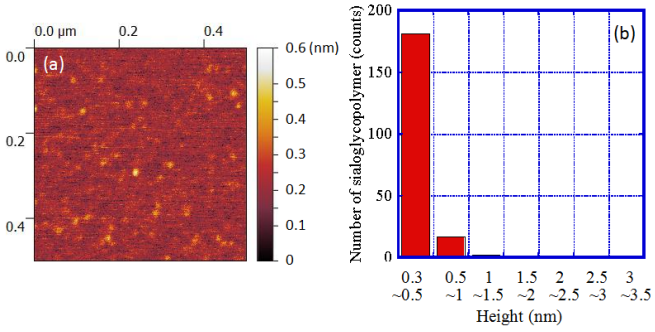

Fig. 4. Representative AFM image of sialoglycopolymer on mica. (a) AFM image and (b) height distribution of sialoglycopolymer.

Our attention shall now turn to the clustered structures such as sialoglycopolymer and SGP-BSA. The binding activity of α(2-6)-linked sialoglycopolymer to influenza viruses is shown in Fig. 3 (a). Only the human influenza viruses of H1N1 have high activity. The fluctuation in binding activity seems to be lower with small error bars. Figure 3(b) shows the relative errors of binding activity. The standard deviation of the relative errors is 0.071 for the binding activity of α(2-6)-linked sugar chains to H1N1, and 0.169 for H5N1. Therefore, the standard deviation of the binding activity between H5N1 and α(2-6)-linked sugar chains is reduced to 0.071 compared to SGP (0.114).

Figure 4(a) shows an AFM image of sialoglycopolymer (1 ng/mL) on mica. Uniform dispersion can be achieved. Furthermore, the small side chains of sialyllactosamine can easily adopt a standing conformation. As a result, most of the molecules have a height of *ca.* 0.6 nm as shown in Fig. 4(b). Partially clustered sugar chains might have higher binding activity, and there is the possibility that many free sugar chains would remain during the binding experiments.

Finally, we show the binding activity of SGP-BSA in Fig. 5(a). As the range in the number of sialic acids is the same in Fig. 3 (a), the binding activity could be enhanced in SGP-BSA. The fluctuations in binding activity seem to be reduced with small error bars. Figure 5(b) shows the relative errors of the binding activity. The standard deviation of the relative errors is 0.046 for the binding activity of α(2-6)-linked sugar chains to H1N1, and 0.084 for H5N1. Therefore, the standard deviation of the binding activity between H5N1 and α(2-6)-linked sugar chains is reduced to 0.046. Highly concentrated clusters could have higher binding activity with smaller fluctuations.

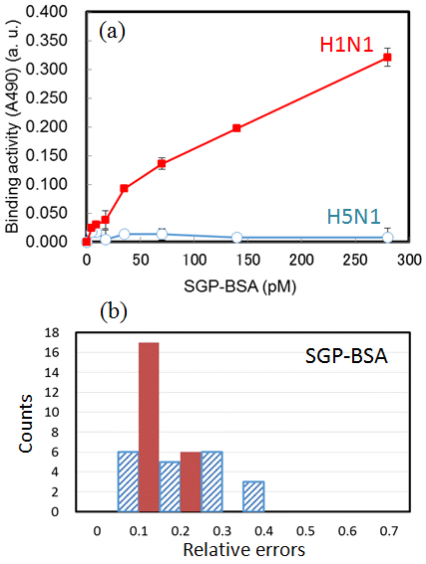

Fig. 5. Binding of viruses to SGP-BSA containing α(2-6)-linked sialic acid detected by ELISA is shown in (a). Filled squares denote human influenza virus (H1N1), and open circles denote avian virus (H5N1). (b) Relative errors for binding activity of SGP-BSA. Red and blue (hatched) bars are for human and avian IFVs, respectively.

Figure 6(a) shows an AFM image of SGP on mica. The sparse dispersion can be observed, with the sugar chains being highly concentrated on BSA. The height distribution is shown in Fig. 6(b) and spreads over the range between 4 nm and 9 nm. Fluctuations in sugar chains on BSA is evident. As a result of

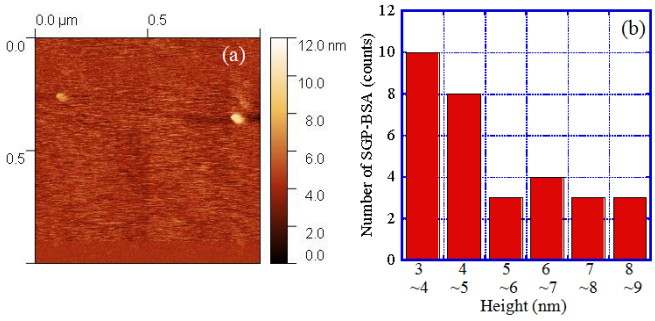

Fig. 6. Representative AFM image of SGP-BSA in a liquid. (a) AFM image and (b) height distribution of SGP-BSA.

the scaffolding provided by the BSA molecules, the clustered sugar chains on BSA with fluctuation can work effectively [17]. Therefore, we achieved effective use of sugar chains and high binding activity with fewer free sugar chains. In general, when the density of viruses is low under actual working conditions, the high clustering and effective use of sugar chains seems to be important for the binding molecules. With this in mind, SGP-BSA appears to be a potential candidate for use in initial reactions of influenza virus sensors.

IV. Conclusion

We have discussed several clustered sugar chains that engage in binding to influenza viruses. α(2-6)-linked sugar chains have higher binding activity to human influenza viruses compared to α(2-3)-linked sugar chains. Single sugar chains of SGP display conformational variability due to the presence of multiple amino groups as binding sites. As a result, SGP shows large fluctuations in binding activity. On the other hand, clustered sugar chains such as sialoglycopolymer and SGP-BSA show small fluctuations in binding activity. The standard deviation of the relative errors could be reduced to 0.071 for sialoglycopolymer and 0.046 for SGP-BSA, while that for SGP was 0.114. Thus, we have succeeded in reducing the fluctuations in binding activity by clustering. For SGP-BSA, the high clustering using scaffold molecules could enhance the effective use of sugar chains to yield high binding activity with fewer free sugar chains.

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References

[1] H. Heesterbeek, R. M. Anderson, V. Andreasen, S. Bansal, D. D. Angelisl, C. Dye, K. T. D. Eames, W. J. Edmunds, S. D. W. Frost, S. Funk, S. Funk, T. D. Hollingsworth, T. House, V. Isham, P. Klepac, J. Lessler, J. O. Lloyd-Smith, C. J. E. Metcalf, D. Mollison, L. Pellis, J. R. C. Pulliam, M. G. Roberts, C. Viboud, and Isaac Newton Institute IDD Collaboration, "Modeling infectious disease dynamics in the complex landscape of global health," Science, vol. 347, aaa4339, Mar. 2015.

[2] S. Yamada, Y. Suzuki, T. Suzuki, M. Q. Le, C. A. Nidom, Y. Sakai-Tagawa, Y. Muramoto, M. Ito, M. Kiso, T. Horimoto, K. Shinya, T. Sawada, M. Kiso, T. Usui, T. Murata, Y. Lin, A. Hay, L. F. Haire, D. J. Stevens, R. J. Russell, S. J. Gamblin, J. J. Skehel, and Y. Kawaoka, "Haemagglutinin mutations responsible for the binding of H5N1 influenza A viruses to human-type receptors," Nature, vol. 444, pp. 378–382, Nov. 2006.

[3] Y. Watanabe, M. S. Ibrahim, Y. Suzuki, and K. Ikuta, "The changing nature of avian influenza A virus (H5N1)," Trends Microbiol., vol. 20, pp. 11–20, Jan. 2012.

[4] Y. Watanabe, M. S. Ibrahim, and K. Ikuta, "Evolution and control of H5N1," EMBO Rep., vol. 14, pp. 117–122, Nov. 2013.

[5] T. Kawahara, H. Hiramatsu, Y. Suzuki, S. Nakakita, Y. Ohno, K. Maehashi, K. Matsumoto, K. Okamoto, T. Matsuba, and R. Utsunomiya, "Development of Nano-Carbon Biosensors Using Glycan for Host Range Detection of Influenza Virus," Condens. Matter, vol. 1, 7, Dec. 2016.

[6] T. Ono, T. Oe, Y. Kanai, T. Ikuta, Y. Ohno, K. Maehashi, K. Inoue, Y. Watanabe, S. Nakakita, Y. Suzuki, T. Kawahara, and K. Matsumoto, "Glycan-functionalized graphene-FETs toward selective detection of human-infectious avian influenza virus," Jpn. J. Appl. Phys., vol. 56, 030302, Feb. 2017.

[7] T. Ito, Y. Suzuki, T. Suzuki, A. Takada, T. Horimoto, K. Wells, H. Kida, K. Otsuki, M. Kiso, H. Ishida, and Y. Kawaoka, "Recognition of *N*-Glycolylneuraminic Acid Linked to Galactose by the α2,3 Linkage Is Associated with Intestinal Replication of Influenza A Virus in Ducks," J. Virol., vol. 74, pp. 9300–9305, Oct. 2000.

[8] Y. Suzuki, T. Ito, T. Suzuki, R. E. Holland Jr., T. M. Chambers, M. Kiso, H. Ishida, and Y. Kawaoka, "Sialic Acid Species as a Determinant of the Host Range of Influenza A Viruses," J. Virol., vol. 74, pp. 11825–11831, Dec. 2000.

[9] A. Chandrasekaran, A. Srinivasan, R. Raman, K. Viswanathan, S. Raguram, T. M. Tumpey, V. Sasisekharan, and R. Sasisekharan "Glycan topology determines human adaptation of avian H5N1 virus hemagglutinin," Nat. Biotechnol., vol. 26, pp. 107–113, Jan. 2008.

[10] M. Matrosovich, A. Tuzikov, N. Bovin, A. Gambaryan, A. Klimov, M. R. Castrucci, I. Donatelli, and Y. Kawaoka, "Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals," J. Virol., vol. 74, pp. 8502–8512, Sep. 2000.

[11] B. Sun, W. Bao, X. Tian, M. Li, H. Liu, J. Dong, and W. Huang, "A simplified procedure for gram-scale production of sialylglycopeptide (SGP) from egg yolks and subsequent semi-synthesis of Man3GlcNAc oxazoline," Carbohydr. Res., vol. 396, pp. 62–69, Jul. 2014.

[12] K. Totani, T. Kubota, T. Kuroda, T. Murata, K.I. Hidari, T. Suzuki, Y. Suzuki, K. Kobayashi, H. Ashida, and K. Yamamoto, "Chemoenzymatic synthesis and application of glycopolymers containing multivalent sialyloligosaccharides with a poly(L-glutamic acid) backbone for inhibition of infection by influenza viruses," Glycobiology, vol. 13, pp. 315–326, May 2003.

[13] N. Sriwilaijaroen, S. Nakakita, S. Kondo, H. Yagi, K. Kato, T. Murata, H. Hiramatsu, T. Kawahara, Y. Watanabe, Y. Kanai, T. Ono, J. Hirabayashi, K. Matsumoto, and Y. Suzuki, "*N*-glycan structures of human alveoli provide insight into influenza A virus infection and pathogenesis," FEBS J., vol. 285, pp. 1611–1634, May 2018.

[14] S. Sabesan and J. C. Paulson, "Combined chemical and enzymatic synthesis of sialyloligosaccharides and characterization by 500-MHz and proton and carbon-13 NMR spectroscopy," J. Am. Chem. Soc., vol. 108, pp. 2068–2080, Apr. 1986.

[15] M. Ogata, T. Murata, K. Murakami, T. Suzuki, K. I. P. J. Hidari, Y. Suzuki, and T. Usui, "Chemoenzymatic synthesis of artificial glycopolypeptides containing multivalent sialyloligosaccharides with a γ-polyglutamic acid backbone and their effect on inhibition of infection by influenza viruses," Bioorg. Med. Chem., vol. 15, pp. 1383–1393, Feb. 2007.

[16] S. Nakakita and J. Hirabayashi, "Microarray Technology Methods and Applications," in Methods in Molecular Biology, vol. 1368, P. C. H. Li, A. Sedighi, and L. Wang, Eds. New York: Springer, 2016, pp. 225–235.

[17] M. D. McDonnell and D. Abbott, "What Is Stochastic Resonance? Definitions, Misconceptions, Debates, and Its Relevance to Biology," PLoS Comput. Biol., vol. 5, e1000348, May 2009.