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Attomolar Detection of Influenza A Virus Hemagglutinin Human H1 and Avian H5 Using Glycan-Blotted Field Effect Transistor Biosensor

Sho Hideshima,[†] Hiroshi Hinou,[‡,§] Daisuke Ebihara,[∥] Ryosuke Sato,[∥] Shigeki Kuroiwa,[∥] Takuya Nakanishi,[∥] Shin-Ichiro Nishimura,[‡,§] and Tetsuya Osaka*[,†,∥]

[†]Research Institute for Science and Engineering, Waseda University, Okubo 3-4-1, Shinjuku-ku, Tokyo 169-8555, Japan

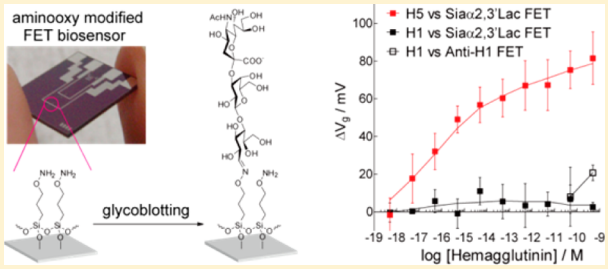
[‡]Graduate School of Life Science, Hokkaido University, N21, W11, Sapporo 001-0021, Japan

[§]Medicinal Chemistry Pharmaceuticals, Co. Ltd., N21, W12, Sapporo 001-0021, Japan

[∥]Graduate School of Advanced Science and Engineering, Waseda University, Okubo 3-4-1, Shinjuku-ku, Tokyo 169-8555, Japan

Supporting Information

ABSTRACT: Influenza virus, through cell invasion and propagation with the interaction between hemagglutinin (HA) present on its surface and glycans on the host cell, causes a rapidly spreading infection throughout the world. In the present investigation, we succeeded for the first time in the attomolar-level sensing and discrimination of influenza A viral HA molecules H1 and H5 by using a glycan-immobilized field effect transistor (FET) biosensor. The small ligand glycans immobilized on the FET device, which make effective use of the charge-detectable region for FET-based detection in terms of Debye length, gave an advantage in the highly sensitive detection of the proteins. Two kinds of trisaccharides receptors terminating in sialic acid-α2,6-galactose (6′-sialyllactose) and in sialic acid-α2,3-galactose (3′-sialyllactose) were conjugated directly with the SiO_2 surface of FET devices by a simple glycoblotting method using the self-assembled monolayer (SAM) of aminooxy terminated silane-coupling reagent, 3-aminooxypropyltriethoxysilane. Furthermore, it was demonstrated that the FETs with densely immobilized glycans, which possess the high capture ability by achieving the glycoside cluster effect, clearly distinguish HA molecules between their subtypes H1 (human) and H5 (avian) at the attomolar level, while the conventional method based on HA antibodies achieves only picomolar-level detection. Our findings indicate that the glycan-immobilized FET is a promising device to detect various pathogenic bacteria and viruses through glycan-protein interaction found ubiquitously in many infectious diseases.



Biosensors that determine the level of proteins at ultralow concentrations provide accurate and rapid biological information related to health-care, contributing to the enhancement in quality of life (QOL). For achieving the degree of specificity and sensitivity required for their practical use in the biomedical field, a field effect transistor (FET) that realizes label-free sensing of proteins is a promising tool, as discussed for the application to tumor marker detection, DNA sequencing, and so on.[1−4] The FET generally detects potential changes on its gate surface in terms of the intrinsic charge of target molecules, which are allowed to react specifically with probe molecules immobilized on the gate surface, such as the threshold voltage shift (ΔV_g) in the gate voltage (V_g)−drain current (I_d) characteristics.[5−8] Yet, the FET-based detection suffers from its limitation in detection because charge detection in electrolytes decay exponentially with distance from the solid−electrolyte interface by ionic screening effects (Debye screening effect).[2,3,5,8] The response of FET is profoundly affected by the screening effect, suggesting that the size of receptors needs to be considered to improve the sensitivity.

Nowadays, influenza viruses undergo mutation, facing the constant danger of multiple deaths resulting from pandemic.[9−11] In preparation for the pandemic, rapid and highly sensitive detection of the influenza viruses is a crucial step in preventing the spreading of the infection. For the detection of influenza virus using FET biosensors, commonly used probes, such as antibodies, are relatively large (4−14 nm) in size, resulting in a large space occupied by the probe in the Debye length region even in low ionic strength solution (~7.5 nm in 0.01× phosphate buffered saline solution), suggesting that the signals caused by target adsorption will become weak. Antigen binding fragment (Fab) and aptamer, which are smaller than the antibody, may be useful, but these receptors are incapable of detecting the change in hemagglutinins (HAs) from various host viruses. Here we propose the use of small glycoligands, sialylated trisaccharide structures, to realize an efficient application of the ideal region for highly sensitive detection of viral HAs by using FET-based biosensor (Figure 1A). Glycans, their length ranging from angstroms to a few nanometers, play an essential role as ligands in the cell specific

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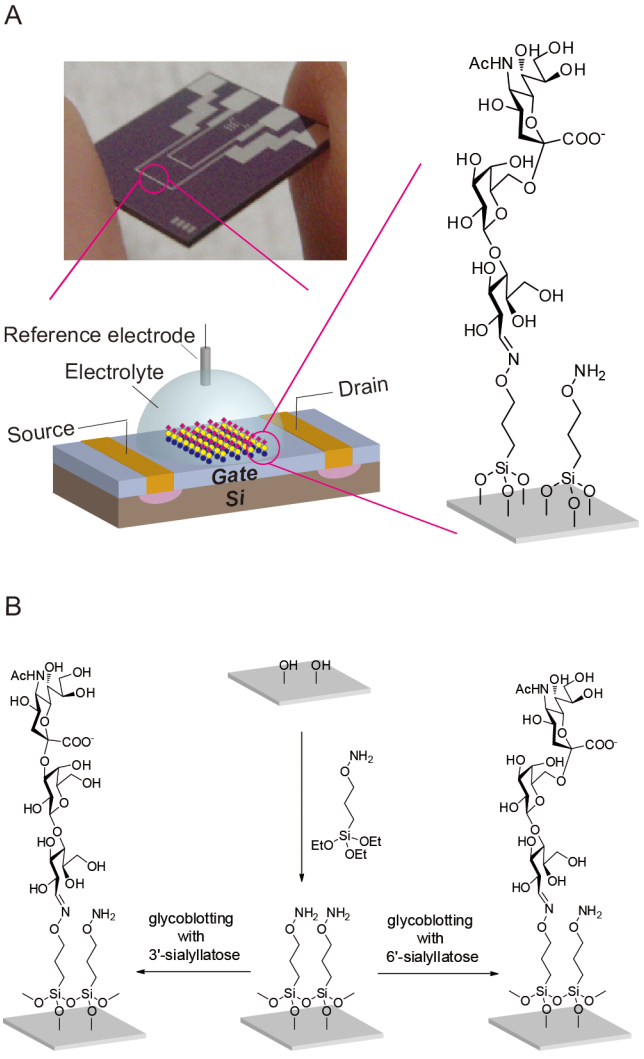

Figure 1. Glycan-immobilized field effect transistor (FET) biosensor. (A) Optical image of an FET chip and schematic illustration of glycan-immobilized FET for highly sensitive and specific detection of influenza virus hemagglutinin (HA). (B) One step functionalization of the SiO_2 surface of FET devices with aminooxy terminated trialkoxysilane reagent followed by glycoblotting with unmodified glycans.

recognition by various HA variants of the influenza viruses in influenza virus infection to host cells.

When the influenza virus invades epidermal cells, HAs bind with sialic-acid-containing oligosaccharides such as α2,3- and/or α2,6-sialyl *N*-acetyllactosamine units of glycoconjugates.[12,13] Since human adaptation of avian H5N1 virus HA is determined by a characteristic glycosidic linkage between sialic acid and galactose residues,[10,14] it is expected that rapid, highly sensitive and convenient detection and characterization of viruses can be achieved by using the FET biosensor immobilizing the above-mentioned two sialyl oligosaccharide ligands. Thus, host cell surface-mimetic glycan-immobilized platform may allow highly sensitive and specific recognition of the HA proteins on the virus surfaces. We considered that the glycoblotting method using an aminooxy-terminated self-assembled monolayer (SAM) is an ideal and versatile technology for the immobilization of complex oligosaccharides on the sensitive FET surfaces (Figure 1B).[15,16] The glycoblotting reaction does not require any additional coupling reagents and proceeds smoothly under mild acidic conditions to permit direct immobilization of unmodified glycans (see the Supporting Information). In addition, glycoblotting on SAM is expected to lead to its high density and efficient orientation, which have a great advantage in capturing the target proteins by the glycoside cluster effect.[17]

RESULTS AND DISCUSSION

Specificity of the Glycan-Immobilized Surface to HA Protein. We investigated the specificity of the interaction between proteins and glycans immobilized on the FET surface using atomic force microscopy (AFM). AFM images show clear differences in surface morphologies after the immersion of the glycan-immobilized surface in protein-containing solutions, revealing high specificity of the directly immobilized glycans to a target protein (Figure S1 in the Supporting Information). It should be noted that the nonspecific adsorption of HSA was suppressed after the optimization of the reaction time and the concentration of the glycan solution (Figure S2 in the Supporting Information). This result clearly indicates that the sialylated glycan-immobilized FET surface possesses high specificity to the influenza virus HA, and the nonspecific adsorption can be depressed by controlling the glycan density on the FET surface. Characterization using X-ray photoelectron spectroscopy was also performed (Figure S3 in the Supporting Information).

Cross-Reactivity of Glycan-Immobilized FETs to Human H1 HA and Avian H5 HA. To test cross-reactivity of the present glycan-immobilized surface of the FETs, we evaluated the response of FET in the presence of HA and HSA solutions (Figure 2). For the FET with the Siaα2,6′Lac-

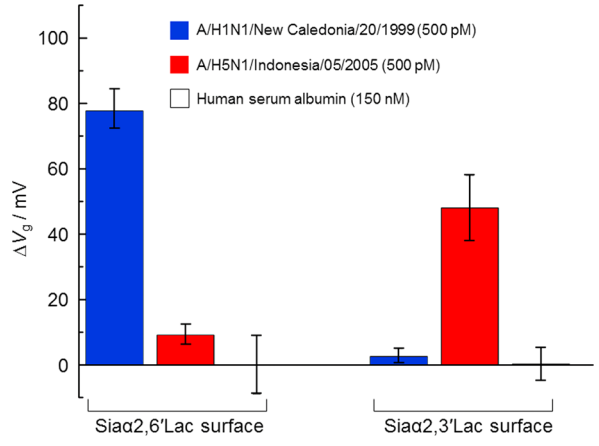

Figure 2. Cross-reactivity of glycan-immobilized FETs with human influenza virus H1 HA and avian influenza virus H5 HA. The FETs, prepared with Siaα2,6′Lac and Siaα2,3′Lac, were tested by using HA derived from A/H1N1/New Caledonia/20/1999 and A/H5N1/Indonesia/05/2005 (500 pM, respectively). The control experiment was conducted by using HSA (150 nM). Error bars represent the standard deviation (N = 3–4).

immobilized surface, a clear response of FET to human H1 HA (A/H1N1/New Caledonia/20/1999, 500 pM) was observed. In this system, the binding of HA (pI = 6.85)[18] negatively charged in 0.01× PBS (pH 7.4) to the gate surface causes a change of FET characteristics as a shift ΔV_g in the positive direction (Figure S4 in the Supporting Information). On the contrary, no significant shifts were detected when the FETs were immersed in avian influenza virus H5 HA (A/H5N1/

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Indonesia/05/2005, 500 pM), suggesting that the adsorption of H1 HA was specific against the dense Siaα2,6′Lac-immobilized surface. As anticipated, essentially no shift was observed when HSA solution (150 nM) was used instead of HA solution. These results were in good agreement with the AFM observations showing no aggregates found on the glycan-immobilized surface after HSA immersion as described above. In addition, the FET with the Siaα2,3′Lac-immobilized surface showed a high specificity with H5 HA and little cross-reaction with H1 HA. Thus, the difference in the linkages between Siaα2,6Gal and Siaα2,3Gal emerged in the cross-reactivity tests using H1 HA and H5 HA. These results indicated that the immobilized glycans maintain its original function as ligands for influenza virus HAs.[12,13]

Discrimination of Influenza A Virus Hemagglutinin Human H1 and Avian H5 Using Glycan-Immobilized FET Biosensor. Surprisingly, it was revealed that the glycan-immobilized FETs allowed quantitative sensing of HAs ranging from 50 aM to 5 nM. Figure 3A shows the sensor responses from 3 or 4 different FET devices displaying Siaα2,6′Lac residues with respect to HA concentration. Increasing the concentration of H1 HA (A/H1N1/New Caledonia/20/1999) molecules increased the negative charge of adsorbed HAs within the Debye length, resulting in the enhancement of the magnitude of ΔV_g. We also investigated the influence of different buffer ionic strengths on device sensitivity (Figure S5 in the Supporting Information). Conversely, the control experiments performed by the incubation with H5 HA (A/H5N1/Indonesia/05/2005) did not yield any significant signals, indicating that the nonspecific adsorption of avian source HA within the same concentration range was suppressed. This result suggests that the FET-gate surface modified with glycans can discriminate between human and avian influenza viruses with high sensitivity and specificity. It should be emphasized here that the sensitivity of the glycan-immobilized FET in the detection of H1 HA (>50 aM) was much higher than that of the antibody (monovalent Fab)-immobilized FETs (>50 pM). It is clear that the difference in size between glycans and antibodies greatly influence the sensitivity of the FET-based detection. Well-oriented glycans as small as ~2 nm in length offers two distinct advantages in the sensitive detection based on FET devices: an effective use of the charge-detectable region and an increase in the number of recognition sites for target binding. The detectable region of the charge in target proteins is defined as the Debye length, approximately 7.5 nm in height from the gate surface in the present experimental conditions. Considering the size of antibodies ranging from 4 to 12 nm in general, glycans have the merit of effective use of the interfacial region for the detection. The size of immobilized molecule is also reflected in its occupation area on the gate surface, from which the number of potential sites for target binding could be higher in the case for glycans than antibodies. Because the glycoside cluster effect of the glycan-blotted SAM can also greatly contribute to enhance the HA−glycan interaction, the immobilized glycans capture more HA oligomers than the immobilized antibodies which are bound to the HA molecules in the monovalent form, suggesting the enhancement of sensitivity (see the Supporting Information for details).

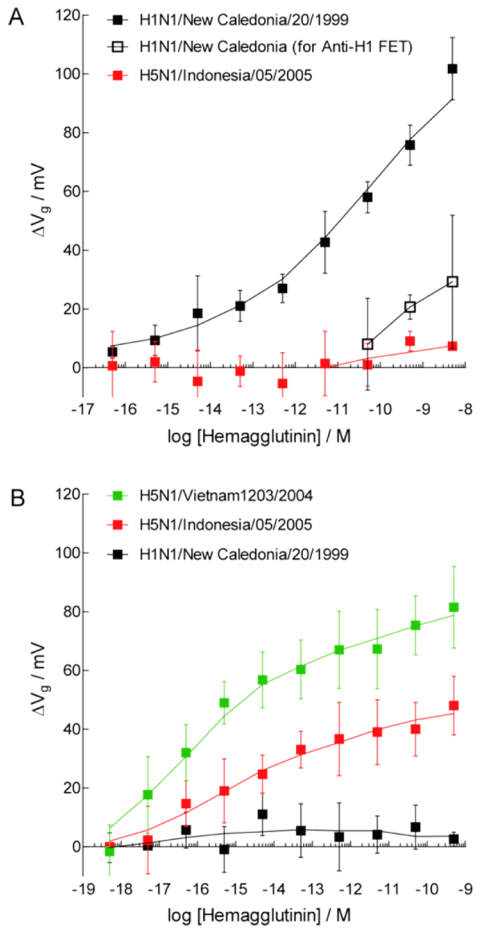

Figure 3. Quantitative determination of HAs using glycan-immobilized FETs. Detection of human influenza virus H1 HA and avian H5 HA for (A) Siaα2,6′Lac-immobilized FETs (50 aM−5 nM) and anti-H1 HA mAb immobilized FET (50 pM−5 nM) and (B) Siaα2,3′Lac-immobilized FETs (500 zM−500 pM). Error bars represent the standard deviation (N = 3−4).

Some additional information on HA specificity for the alternative isomer, Siaα2,3′Lac, was also obtained. When HAs were incubated with the glycan (Siaα2,3′Lac) immobilized on FET, changes in the magnitude of ΔV_g were detected specifically only for avian H5 HAs in the wide range of 500 zM to 500 pM (Figure 3B). Interestingly, we discovered that this device can discriminate H5 HAs of two viral strains, A/H5N1/Vietnam/1203/2004 and A/H5N1/Indonesia/05/2005, in terms of the difference of the substitution of H5 HA in the binding affinity with the Siaα2,3′Lac-immobilized FET surface,[19] while little response was observed for human influenza virus HA (A/H1N1/New Caledonia/20/1999). The higher affinity observed in H5 HAs with Siaα2,3′Lac-immobilized FET than that of H1 HA with Siaα2,6′Lac residues might be due to the less hindered α2,3-linkage, in which a linear glycan topology remarkably influences the binding profile with individual HA cavities.[14] In view of its binding specificity, these results are in good agreement with the knowledge reported in previous literature on the glycan-binding specificity of HAs using glycan microarray.[20] The merit of the present method is evident because the new class of glycan-immobilized FET devices allowed for the first time ultrahigh sensitive analyses of both human and avian influenza virus HAs within the clear binding specificity based on the difference in the glycoside bond between the terminal sialic acid and galactose residues.

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CONCLUSIONS

We established a novel strategy for the development of FET-based biosensor device to detect and discriminate human and avian influenza virus HAs (H1 and H5) at attomolar-level sensitivity. It was demonstrated that the glycan-immobilized FETs (Siaα2,6′Lac-immobilized FET and Siaα2,3′Lac-immobilized FET) prepared directly by the glycoblotting reaction made possible the effective use of the crucial interface region for the detection of charge by FET in terms of Debye length. The clear specificity of the FETs to the HAs, observed in the present study, is attributed to the uniform orientation of glycan directly immobilized on the gate through aminooxy-terminated SAM. The present glycan-immobilized FET devices were found to detect 60 H5 HA molecules and 6000 H1 HA molecules in 20 μL samples, respectively. In other words, this sensor device can capture even a single influenza viral particle displaying H5 molecules and 12 particles displaying H1 molecules, since a single virus particle carries approximately 500 HA molecules on its surface membrane as the principal constitution protein.[21,22] It is important to note that antibody-based detection requires at least 1×10^7 HA molecules per 20 μL test solution. The short-glycan-immobilized FET could detect the influenza virus particle directly in clinical application similar to other sensitive detection methods.[23] When avian influenza viruses are passed between humans, the viruses are considered to possess the HA binding affinity not only with Siaα2,3Gal but also with Siaα2,6Gal linkages on the cell surface.[24,25] The present method clearly facilitates a protocol for rapid monitoring and identification of the generation of mutant viruses exhibiting significant affinity toward Siaα2,6Gal structure from avian influenza viruses. We believe that the glycan-immobilized FETs reported here are promising devices to provide valuable information for the protection of pandemic.

ASSOCIATED CONTENT

Supporting Information

Materials, experimental details, AFM observation, merit of glycoblotting method, XPS analyses, and effect of the ionic concentrations. This material is available free of charge via the Internet at http://pubs.acs.org.

AUTHOR INFORMATION

Corresponding Author

*Phone: +81 3 5286 3202. Fax: +81 3 3205 2074. E-mail: osakatets@waseda.jp.

Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Bergveld, P. *Sens. Actuators, B* **2003**, *88*, 1−20.
(2) Stern, E.; Vacic, A.; Rajan, N. K.; Criscione, J. M.; Park, J.; Ilic, B. R.; Mooney, D. J.; Reed, M. A.; Fahmy, T. M. *Nat. Nanotechnol.* **2010**, *5*, 138−142.
(3) Vedala, H.; Chen, Y. A.; Cecioni, S.; Imberty, A.; Vidal, S.; Star, A. *Nano Lett.* **2011**, *11*, 170−175.
(4) Elnathan, R.; Kwiat, M.; Pevzner, A.; Engel, Y.; Burstein, L.; Khatchtourints, A.; Lichtenstein, A.; Kantaev, R.; Patolsky, F. *Nano Lett.* **2012**, *12*, 5245−5254.
(5) Hideshima, S.; Einati, H.; Nakamura, T.; Kuroiwa, S.; Shacham-Diamand, Y.; Osaka, T. *J. Electrochem. Soc.* **2010**, *157*, J410−J414.
(6) Nakamura, T.; Sakurai, Y.; Hideshima, S.; Kuroiwa, S.; Osaka, T. *Chem. Lett.* **2010**, *39*, 1245−1247.
(7) Rothberg, J. M.; Hinz, W.; Rearick, T. M.; Schultz, J.; Mileski, W.; et al. *Nature* **2011**, *475*, 348−352.
(8) Hideshima, S.; Sato, R.; Kuroiwa, S.; Osaka, T. *Biosens. Bioelectron.* **2011**, *26*, 2419−2425.
(9) Bautista, E.; Chorpitayasunondh, T.; Gao, Z. C.; Harper, S. A.; Shaw, M.; et al. *N. Engl. J. Med.* **2010**, *362*, 1708−1719.
(10) Yamada, S.; Suzuki, Y.; Suzuki, T.; Le, M. Q.; Nidom, C. A.; et al. *Nature* **2006**, *444*, 378−382.
(11) Nicholson, K. G.; Wood, J. M.; Zambon, M. *Lancet* **2003**, *362*, 1733−1745.
(12) Rosenthal, P. B.; Zhang, X. D.; Formanowski, F.; Fitz, W.; Wong, C. H.; Meier-Ewert, H.; Skehel, J. J.; Wiley, D. C. *Nature* **1998**, *396*, 92−96.
(13) Stevens, J.; Blixt, O.; Tumpey, T. M.; Taubenberger, J. K.; Paulson, J. C.; Wilson, I. A. *Science* **2006**, *312*, 404−410.
(14) Chandrasekaran, A.; Srinivasan, A.; Raman, R.; Viswanathan, K.; Raguram, S.; Tumpey, T. M.; Sasisekharan, V.; Sasisekharan, R. *Nat. Biotechnol.* **2008**, *26*, 107−113.
(15) Nishimura, S.-I.; Niikura, K.; Kurogochi, M.; Matsushita, T.; Fumoto, M.; Hinou, H.; Kamitani, R.; Nakagawa, H.; Deguchi, K.; Miura, N.; Monde, K.; Kondo, H. *Angew. Chem., Int. Ed.* **2005**, *44*, 91−96.
(16) Ohyanagi, T.; Nagahori, N.; Shimawaki, K.; Hinou, H.; Yamashita, T.; Sasaki, A.; Jin, T.; Iwanaga, T.; Kinjo, M.; Nishimura, S.-I. *J. Am. Chem. Soc.* **2011**, *133*, 12507−12517.
(17) Lee, Y. C.; Lee, R. T. *Acc. Chem. Res.* **1995**, *28*, 321−327.
(18) http://www.fludb.org/brc/home.do?decorator=influenza.
(19) Suenaga, E.; Mizuno, H.; Penmetcha, K. K. R. *Biosens. Bioelectron.* **2012**, *32*, 195−201.
(20) Liao, H. Y.; Hsu, C. H.; Wang, S. C.; Liang, C. H.; Yen, H. Y.; et al. *J. Am. Chem. Soc.* **2010**, *132*, 14849−14856.
(21) Skehel, J. J.; Schild, G. C. *Virology* **1971**, *44*, 396−408.
(22) Ausborn, S. G.; Schoen, P.; Bartoldus, I.; Wilschut, J.; Stegmann, T. *J. Virol.* **2000**, *74*, 2714−2720.
(23) Su, L.-C.; Chang, C.-M.; Tseng, Y.-L.; Chang, Y.-F.; Li, Y.-C.; Chang, Y.-S.; Chou, C. *Anal. Chem.* **2012**, *84*, 3914−3920.
(24) Herfst, S.; Schrauwen, E. J. A.; Linster, M.; Chutinimitkul, S.; de Wit, E.; et al. *Science* **2012**, *336*, 1534−1541.
(25) Imai, M.; Watanabe, T.; Hatta, M.; Das, S. C.; Ozawa, M.; et al. *Nature* **2012**, *486*, 420−428.