

Imaging the Substructures of Individual IgE Antibodies with Atomic Force Microscopy

Jing Hu,^{†,‡} Mingyan Gao,^{†,‡} Ying Wang,^{†,‡} Mengnan Liu,^{†,‡} Jianfei Wang,^{†,‡} Jiani Li,^{†,‡} Zhengxun Song,^{†,‡} Yujuan Chen,^{*,†,‡,§} and Zuobin Wang^{*,†,‡,||} 

[†]Ministry of Education Key Laboratory for Cross-Scale Micro and Nano Manufacturing, Changchun University of Science and Technology, Changchun 130022, China

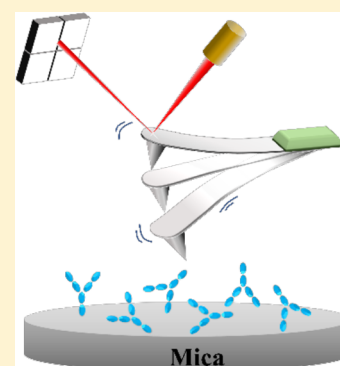
[‡]International Research Centre for Nano Handling and Manufacturing of China, Changchun University of Science and Technology, Changchun 130022, China

[§]School of Life Sciences, Changchun University of Science and Technology, Changchun 130022, China

^{||}JR3CN & IRAC, University of Bedfordshire, Luton LU1 3JU, U.K.

Supporting Information

ABSTRACT: The interactions between antibodies and substrates directly affect their conformations and thus their immune functions. Therefore, it is desirable to study the structures of antibodies at the single molecule level. Herein, the substructures of Immunoglobulin E (IgE) on solid surfaces were investigated. For this purpose, tapping-mode atomic force microscopy (AFM) was applied to observe individual IgE substructures adsorbed onto Mg²⁺ and Na⁺ modified mica substrates in air. As expected, the AFM images revealed that the IgE antibodies exhibited different conformations on the surface of mica substrate consisting of the four basic orientations: three domain, two equivalent domain, two unequal domain, and single domain morphologies. Moreover, the differences in the different orientations in single IgE antibodies were also identified clearly.



■ INTRODUCTION

Proteins have attracted huge interest due to their biological functions such as transportation, catalysis, defense, and regulation.^{1–3} All these properties make protein an indispensable substance in the human body. Antibodies are a kind of the most important proteins in humoral immunity.^{4,5} Immunoglobulin E (IgE) is the fifth antibody isotype (IgG, IgA, IgM, IgD, and IgE) and is least abundant in serum.^{6,7} As shown in Figure 1, the bent Y-shaped IgE shares the same basic

molecular structure as other classes of antibodies, consisting of one crystallizable fragment (Fc) as well as two separate and identical antigen-binding fragments (Fab).^{8,9} The antibody plays an absolutely essential role in allergic responses and can be used for the diagnosis of allergic diseases.^{10–12} In order to sufficiently understand the function of IgE, it would be interesting to acquire the substructure of IgE adsorbed on a substrate.

In recent years, tremendous efforts have been made on single antibodies, but it is still a challenging task to directly visualize the substructure of antibodies on the surface of substrate. There are many methods that have been proposed to deduce more substructure information about antibodies on substrates, such as ellipsometry (ELM), transmission electron microscopy (TEM), and quartz crystal microbalance (QCM-D).^{13–15} However, most of the techniques only provide the macroscopic properties of antibodies, and the sample preparation process is too complex for their practical applications.¹⁶ Therefore, ultrahigh resolution and simple experiment setup are needed to reveal the substructures of isolated antibodies. Nowadays, atomic force microscopy (AFM) has emerged as a powerful technique to understand

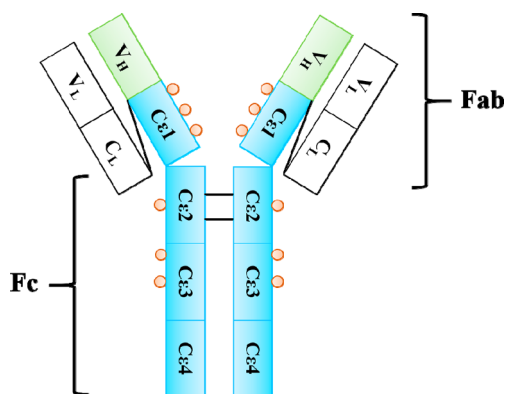


Figure 1. Schematic drawing of a typical IgE.

Received: August 21, 2019

Revised: October 23, 2019

Published: October 29, 2019

the structures of antibodies due to its unprecedented sensitivity, outstanding signal-to-noise ratio, and minimal sample damage.^{17,18} For instance, Thomson et al.¹⁹ used tapping-mode AFM to image the individual IgG molecules in air at 25 kDa resolution. A different method was developed by Martinez-Martin et al.,²⁰ who combined the frequency modulation AFM with bimodal excitation to image and measure the single IgM flexibility with the molecular resolution. Moreover, the antibodies must be immobilized on a suitable substrate for high resolution AFM imaging.²¹ Thus, a variety of supporting materials including mica, gold, and highly ordered pyrolytic graphite (HOPG) have been utilized to support the antibodies.^{22–24} However, mica has been used as a common support for analyzing antibodies by AFM due to its hydrophilic character and its nature of being flat and pretty clean after stripping off the layers.²⁵

In this work, the substructures of flexible IgE antibodies adsorbed on the modified mica substrate with different cations (Mg^{2+} and Na^+) were visualized by tapping-mode AFM. In addition, the different orientations on the mica substrate caused by the three-dimensional conformations of IgE were also analyzed.

EXPERIMENTAL SECTION

Materials. Human antibodies (IgE isotype, $\text{PI} = 7.3$) were obtained from Solarbio Science & Technology Ltd. (Beijing, China). Magnesium chloride hexahydrate ($\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$) and sodium chloride (NaCl) were supplied by Beijing Chemical Works Ltd. (Beijing, China). Natural mica was purchased from Yaan Kaifeng Electrical Material Ltd. (Sichuan, China). All the reagents were used as received without further purification. Ultrapure water with a minimum resistivity of $18.2 \text{ M}\Omega \cdot \text{cm}$ was used in the experiments.

Sample Preparation. The IgE antibodies (30 ng) were dissolved in 6 mL of water to obtain a fresh antibody solution with a final concentration of 5 ng mL^{-1} . All the IgE solutions were stored at -20°C except those for immediate use.

Atomic Force Microscopy Imaging. In a typical procedure, a 20 μL droplet of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ (50 mM) was first deposited onto a freshly cleaved mica surface (about $1 \text{ cm} \times 1 \text{ cm}$) and incubated for 1 min at room temperature, in which the $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ was used to functionalize the mica surface to enhance the adsorption capacity of antibodies. Then, a 5 μL droplet of IgE (5 ng mL^{-1}) solution was injected into the $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ droplet. After the incubation time of 5 min, the sample was gently rinsed with 1 mL water to remove the weakly adsorbed antibodies. Finally, the sample was dried in a nitrogen atmosphere and imaged by AFM immediately. Alternatively, the NaCl (50 mM) was also employed to functionalize the surface of mica substrate. As a control experiment, a sample without the IgE solution was also prepared under the same conditions.

The morphologies of different samples were measured by an AFM instrument (Nano Wizard III, Germany) in air at ambient laboratory conditions. To minimize the destructive lateral forces of the scanning tip on the IgE protein, AFM was performed in tapping mode. The silicon cantilevers with an aluminum reflex coating on the backside (Tap300AI-G) were used in the experiments. The free resonance frequency and nominal spring constants of the silicon probes were 300 kHz and 40 N m^{-1} , respectively. The images of all dried samples were recorded at a scanning rate of 1 Hz. The height profiles obtained from the AFM images are shown in Figure 2 and Table 1.

Contact Angle-Measuring System. The contact angles of different samples were measured with a contact angle-measuring system (Drop shape analyzer-DSA100, Germany) at room temperature. In the experiments, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ or NaCl (20 μL , 50 mM) was placed onto a freshly cleaved mica surface and incubated for 1 min to measure the water contact angle. For comparison, $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$ or NaCl (20 μL , 50 mM) dropped on a freshly cleaved mica surface was

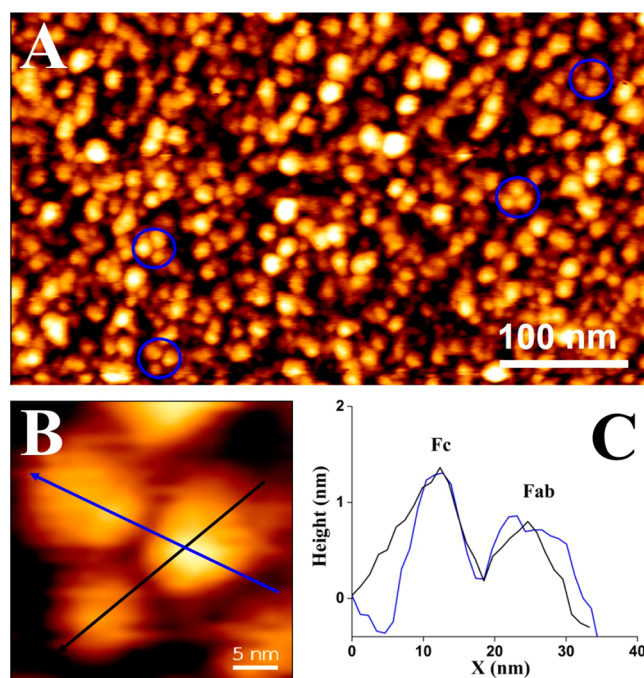


Figure 2. AFM height images of IgE antibodies adsorbed onto the Mg^{2+} modified mica substrate (A). Image of an individual antibody (B). Cross sections along the arrows depicted in Figure 2B (C). The blue circles show the Y-shaped IgE antibodies.

also measured after being dried. During the measurement, the selected water droplet volume was 3 μL .

RESULTS

The antibodies are soft biomolecules that may result in different conformations when they are adsorbed on the surface of substrate. AFM was utilized to investigate the conformations of the IgE antibodies prepared in this study. In order to ensure no artifacts were generated during flushing, the AFM height image of the mica substrate after rinsing by water was first obtained (Figure S1). The AFM height imaging of the IgE antibodies was then performed. The observation of the individual antibodies on the Mg^{2+} cation modified mica substrate indicates that the IgE molecules appear as ellipsoidal particles in most cases (Figure 2A), which is consistent with the previously reported results.¹⁹ Although it is difficult to observe the substructure of the antibodies under this condition, careful identification of a few areas can still provide valuable information about IgE molecules. As shown in Figure 2A, some IgE molecules present the characteristic Y-shaped conformation with three fragments. We further randomly chose an individual Y-shaped IgE molecule for analysis, and its corresponding morphology profiles as well as cross sections along the arrows are displayed in Figure 2B,C, respectively. There are three obvious peaks on the cross sections of the IgE molecule. Two of them have identical heights (about 1.3 nm), and they are larger than the other (around 0.7 nm). The maximum peak is referred to the Fc fragment, while the slightly smaller peaks are ascribed to the Fab fragments, suggesting that the positions of the Fc and Fab fragments can be identified by accurately measuring the heights of different fragments.²⁶ In addition, analogous results were obtained by measuring 9 IgE molecules with the Y-shaped morphology (Table 1).

Table 1. Fragment Height Information of Y-Shaped IgE Molecules on the Mg^{2+} Modified Mica Substrate

Molecules	Height: fragment 1 (nm)	Height: fragment 2 (nm)	Height: fragment 3 (nm)
1	1.5	1.3	1.1
2	1.3	1.1	1.1
3	1.4	0.9	0.8
4	1.2	0.6	0.8
5	1.0	0.6	0.8
6	1.0	0.5	0.6
7	1.5	1.1	1.1
8	1.2	0.8	0.9
9	1.5	1.3	1.0

In addition to the Y-shaped morphology, the other morphologies after IgE adsorption on the Mg^{2+} modified mica substrate were also obtained by careful inspection (Figure S2). In fact, it has been shown that antibody molecules could adopt different orientations on the support due to their three-dimensional morphology and size.²⁷ In this way, Figure 3

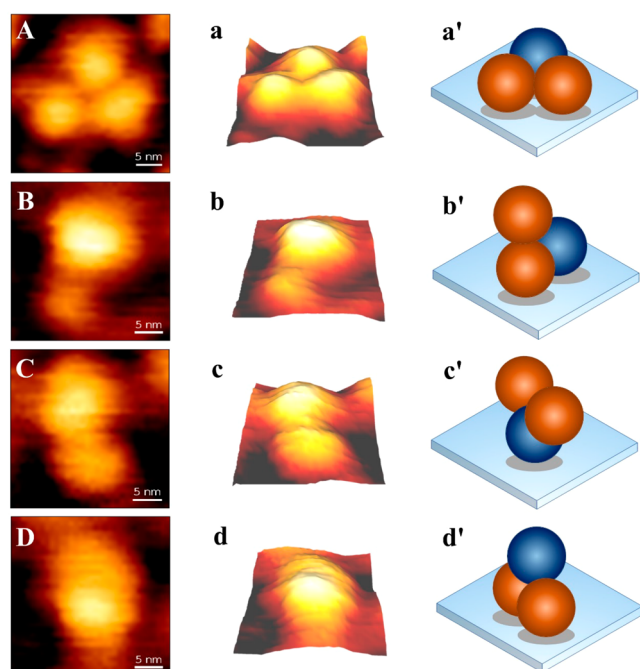


Figure 3. AFM height (A–D), three-dimensional (a–d) images, and spatial (a'–d') images of the four basic orientations of IgE antibodies on the Mg^{2+} modified mica substrate: three domain (A, a and a'), two unequal domain (B, b and b'), two equivalent domain (C, c and c'), and single domain morphologies (D, d and d').

shows the magnified images of the four basic orientations of IgE molecule and their corresponding schematic representations of spatial orientations. Specifically, the Y-shaped antibody (Figure 3A,a,a') at the lowest height (measured from the mica substrate) would correspond to the three fragments resting flat on the substrate of mica. Meanwhile, the molecule could also be located on one side, in which one of the Fab and Fc fragments is attached to the surface and the other Fab fragment protrudes from the support. The AFM image of the antibody with this adopted orientation would produce an image as indicated in Figure 3B,b,b'. Furthermore, Figure 3C,c,c' would represent the antibody adsorbed on the mica substrate by its Fc fragment, and the two Fab fragments stick out of the surface. On the contrary, Figure 3D,d,d' would likely be

referred to the antibody deposited on the substrate via its Fab fragments, while the Fc fragment protrudes from the surface. Furthermore, the relative weights among the four orientations and their corresponding column diagrams were obtained from more than 130 molecules, as shown in Figure 4. The results

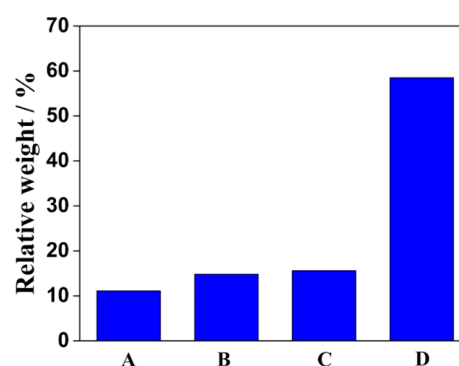


Figure 4. Relative weights among the three domain (A), two unequal domain (B), two equivalent domain (C), and single domain (D) morphologies of IgE antibodies on the Mg^{2+} modified mica substrate.

show that the maximum proportion on the single domain of IgE antibodies reaches 58%, which is ca. 5.3, 3.9, and 3.6 times of the three domain (11%), two unequal domain (15%), and two equivalent domain (16%) morphologies, respectively.

Sequentially, the imaging experiments of IgE molecules were further performed by AFM on the Na^+ cation modified mica substrate. As shown in Figure 5, the IgE antibodies with various structural conformations are found to be individually dispersed on the mica substrate. The presence of the structures might be ascribed to the different orientations of flexible IgE molecules adsorbed on the mica substrate.²⁸ It is notable that the mica substrate coverage of IgE antibodies was significantly

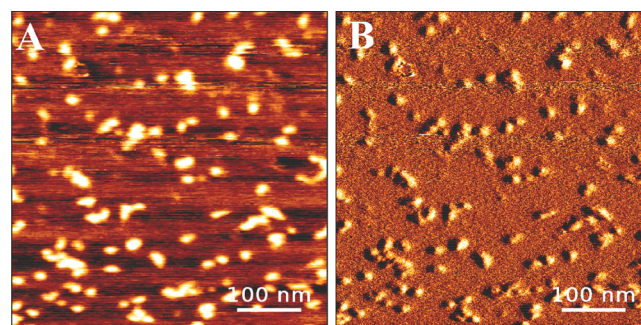


Figure 5. AFM height (A) and amplitude (B) images of IgE antibodies adsorbed onto the Na^+ modified mica substrate.

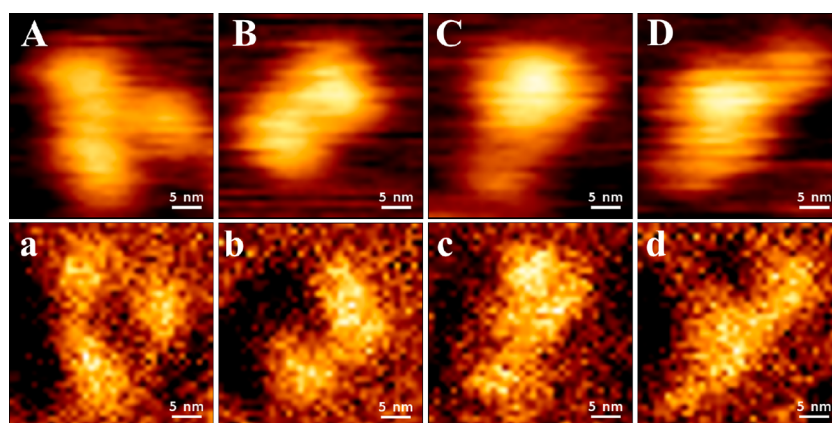


Figure 6. AFM height (A–D) and amplitude (a–d) images of the four basic orientations of IgE antibodies on the Na⁺ modified mica substrate.

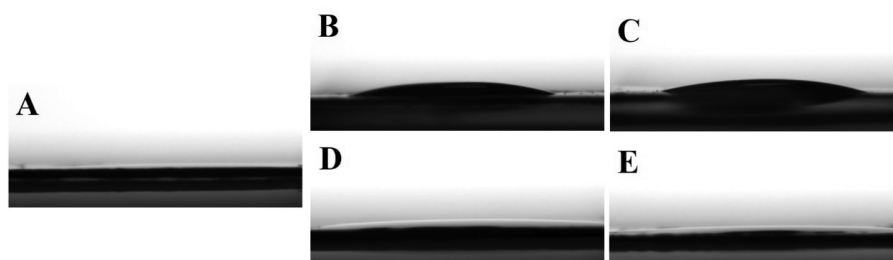


Figure 7. Contact angle of different samples: freshly cleaved mica substrate (A), Mg²⁺ (B) and Na⁺ (C) dried on the freshly cleaved mica substrates, Mg²⁺ (D) and Na⁺ (E) incubated on the freshly cleaved mica substrates for 1 min.

reduced after Mg²⁺ was replaced by Na⁺. The phenomenon may be related to Na⁺ decreasing the binding of IgE to the mica substrate.

Compared with Mg²⁺, Na⁺ reduces the chances of IgE binding to the mica substrate, thereby reducing the total amount of IgE antibodies adsorbed on the mica substrate. However, it gives us an opportunity to better identify individual antibody molecules because the interaction between proteins is minimized. The similar AFM imaging of the IgE antibodies has shown that the four basic orientations can be found in the Na⁺ modified mica substrate. Figure 6A–D,a–d shows the AFM height and amplitude images of IgE in four orientations, respectively. Although the softness of IgE may slightly compromise the resolution of the height images, the IgE substructures can be identified on the AFM amplitude micrographs in each of IgE orientations. Afterward, we also obtained the relative weights among the four orientations by calculating more than 50 IgE molecules on the Na⁺ modified mica substrate (Figure S3). As presented in Figure S4, the relative weight of single domain of IgE molecules is 68%, which is higher than the corresponding values of the three domain (2%), two unequal domain (17%), and two equivalent domain (13%) morphologies.

DISCUSSION

With the rapid advancement of nanomeasurement technologies, people have begun to explore the physical properties of immobilized antibodies at the molecular level by AFM.^{29,30} However, most of the research on antibodies through AFM focuses on IgM and IgG, and little is known about the information of IgE.^{31,32}

It is known that the orientations of the immobilized antibodies are usually determined by the protein–surface

interactions and their characteristics. Therefore, numerous functional methods have been used to effectively modify proteins and surfaces.^{33–35} In this study, the Mg²⁺ and Na⁺ cations were employed to functionalize the mica substrate to distinctly observe individual IgE molecules. In the process of sample preparation, the dispersibility of the IgE solution is an important factor affecting the subsequent individual molecule observation. According to the results of contact angle measurement (Figure 7), the contact angle has obvious changes after Mg²⁺ and Na⁺ were dried on freshly cleaved mica substrates, and their contact angles are nearly 27° and 17°, respectively. However, it should be emphasized that the contact angles after Mg²⁺ and Na⁺ were incubated on freshly cleaved mica substrates for 1 min were almost unchanged. This is because metals usually have high surface energy, resulting in a smaller contact angle on the hydrophilic surface.^{36,37} However, the presence of the contact angle leads to incomplete spreading of the IgE droplets, which interferes with the observation of the IgE substructures. Therefore, the method adopted in this study would not affect the diffusion of the IgE solution, which lays a solid foundation for the observation of IgE antibodies. Besides, since the IgE molecules and freshly cleaved mica substrates are negatively charged at neutral pH, Mg²⁺ and Na⁺ cations could weakly bind IgE molecules to the mica surface through electrostatic interaction,³⁸ which provides favorable conditions for the further identification of the substructures of IgE molecules.

As expected, the IgE antibodies on the mica substrates modified by different cations were successfully observed (Figure 2 and Figure 5). The fact that the mica substrate was covered by different amounts of IgE antibodies suggests that the Mg²⁺ mediation of the attractive interaction between the mica substrate and IgE molecules was relatively stronger

than that of Na^+ . Additionally, the results from AFM confirmed that the four basic orientations of the IgE antibodies were formed on the divalent (Mg^{2+}) and monovalent (Na^+) cations modified mica substrates (Figure 3 and Figure 6). We also confirmed that the four basic orientations of IgE antibodies exist on the mica substrates modified by Ni^{2+} and K^+ cations (Figure S5 and Figure S6), respectively. Moreover, it should be noted that the single IgE antibody is surrounded by a vague pattern, which causes the IgE molecule to be larger in size than it really is. The unclear morphology of an individual IgE molecule adsorbed on the modified mica surface can be explained as follows. First, the tip with a radius of curvature (approximately 10 nm) moves on the compact IgE molecule making the image of IgE less visible. Second, the readsorption of water to the modified mica surface at ambient laboratory conditions also reduces the resolution of image during the imaging period.

CONCLUSIONS

In summary, the substructures of IgE molecules on the Mg^{2+} and Na^+ cations modified mica substrates were investigated in air by tapping-mode AFM. We found that an individual IgE molecule showed four basic orientations on different mica substrates. It is worth noting that the relatively strong binding of IgE molecules mediated by Mg^{2+} and Na^+ cations on the mica substrate enables us to visualize the substructures of isolated IgE molecules. This work is the basis for the identification of individual protein substructures by AFM.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.langmuir.9b02631.

AFM height and amplitude images of mica substrate after rinsing by water, AFM height images of the four basic orientations obtained from more than 130 and 50 IgE antibodies on the Mg^{2+} and Na^+ modified mica substrates, relative weights among the four basic orientations of IgE antibodies on the Na^+ modified mica substrate, AFM height and amplitude images of the four basic orientations of IgE antibodies on the Ni^{2+} and K^+ modified mica substrate (PDF)

AUTHOR INFORMATION

Corresponding Authors

*E-mail: wangz@cust.edu.cn. Tel/Fax: +86-431-85582341.

*E-mail: chenjujuan@cust.edu.cn. Tel/Fax: +86-431-85582341.

ORCID

Zuobin Wang: 0000-0002-7168-0918

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

We are grateful to the National Key R&D Program of China (No.2017YFE0112100), EU H2020 Program (MNR4SCCELL No.734174), Jilin Provincial Science and Technology Program (Nos. 20160623002TC, 20180414002GH, 20180414081GH, 20180520203JH, and 20190702002GH), and "111" Project of

China (D17017). This work was also partly supported by Changli Nano Biotechnology (China).

REFERENCES

- (1) Chico, J. M.; Fernandez-Barbero, G.; Chini, A.; Fernandez-Calvo, P.; Diez-Diaz, M.; Solano, R. Repression of Jasmonate-Dependent Defenses by Shade Involves Differential Regulation of Protein Stability of MYC Transcription Factors and their JAZ Repressors in Arabidopsis. *Plant Cell* **2014**, 26 (5), 1967–1980.
- (2) Wang, W. Q.; Zhang, L. W.; Xue, H.; Yi, L. Cheese Whey Protein Recovery by Ultrafiltration Through Transglutaminase (TG) Catalysis Whey Protein Cross-Linking. *Food Chem.* **2017**, 215, 31–40.
- (3) Shamshad, C.; Padinjat, R. Phospholipid Transport Protein Function at Organelle Contact Sites. *Curr. Opin. Cell Biol.* **2018**, 53, 52.
- (4) Clementi, N.; Cappelletti, F.; Criscuolo, E.; Castelli, M.; Mancini, N.; Burioni, R.; Clementi, M. Role and Potential Therapeutic use of Antibodies Against Herpetic Infections. *Clin. Microbiol. Infect.* **2017**, 23 (6), 381–386.
- (5) Bratanis, E.; Lood, R. A Novel Broad-Spectrum Elastase-Like Serine Protease From the Predatory Bacterium *Bdellovibrio Bacteriovorus* Facilitates Elucidation of Site-Specific IgA Glycosylation Pattern. *Front. Microbiol.* **2019**, 10, 971.
- (6) Dullaers, M.; De Bruyne, R.; Ramadani, F.; Gould, H. J.; Gevaert, P.; Lambrecht, B. N. The Who, Where, and When of IgE in Allergic Airway Disease. *J. Allergy Clin. Immunol.* **2012**, 129 (3), 635–645.
- (7) Zhai, G. T.; Wang, H.; Li, J. X.; Cao, P. P.; Jiang, W. X.; Song, J.; Yao, Y.; Wang, Z. C.; Wang, Z. Z.; Wang, M. C.; Liao, B.; Feng, Q. M.; Lu, X.; Wang, H.; Gao, P.; Liu, Z. IgD-Activated Mast Cells Induce IgE Synthesis in B Cells in Nasal Polyps. *J. Allergy Clin. Immunol.* **2018**, 142 (5), 1489–1499.
- (8) Gould, H. J.; Sutton, B. J. IgE in Allergy and Asthma Today. *Nat. Rev. Immunol.* **2008**, 8 (3), 205–217.
- (9) Ido, S.; Kimiya, H.; Kobayashi, K.; Kominami, H.; Matsushige, K.; Yamada, H. Immunoactive Two-Dimensional Self-Assembly of Monoclonal Antibodies in Aqueous Solution Revealed by Atomic Force Microscopy. *Nat. Mater.* **2014**, 13 (3), 264–270.
- (10) Graham, M. T.; Nadeau, K. C. Lessons Learned from Mice and Man: Mimicking Human Allergy Through Mouse Models. *Clin. Immunol.* **2014**, 155 (1), 1–16.
- (11) Mukai, K.; Tsai, M.; Starkl, P.; Marichal, T.; Galli, S. J. IgE and Mast Cells in Host Defense Against Parasites and Venoms. *Semin. Immunopathol.* **2016**, 38 (5), 581–603.
- (12) Li, J. L.; Liu, J.; Ye, Y. H.; Yang, P.; Tu, Z. C. Reduced IgE/IgG Binding Capacities of Bovine Alpha-Lactalbumin by Glycation after Dynamic High-Pressure Microfluidization Pretreatment Evaluated by High Resolution Mass Spectrometry. *Food Chem.* **2019**, 299, 125166–125173.
- (13) Roux, H. K. Immunoglobulin Structure and Function as Revealed by Electron Microscopy. *Int. Arch. Allergy Immunol.* **1999**, 120 (2), 85–99.
- (14) Hook, F.; Voros, J.; Rodahl, M.; Kurrat, R.; Boni, P.; Ramsden, J.J.; Textor, M.; Spencer, N.D.; Tengvall, P.; Gold, J.; Kasemo, B. A Comparative Study of Protein Adsorption on Titanium Oxide Surfaces using in situ Ellipsometry, Optical Waveguide Lightmode Spectroscopy, and quartz crystal microbalance/dissipation. *Colloids Surf., B* **2002**, 24 (2), 155–170.
- (15) Wiseman, M. E.; Frank, C. W. Antibody Adsorption and Orientation on Hydrophobic Surfaces. *Langmuir* **2012**, 28 (3), 1765–1774.
- (16) Funari, R.; Della Ventura, B.; Altucci, C.; Offenhauer, A.; Mayer, D.; Velotta, R. Single Molecule Characterization of UV-Activated Antibodies on Gold by Atomic Force Microscopy. *Langmuir* **2016**, 32 (32), 8084–8091.
- (17) Alsteens, D.; Gaub, H. E.; Newton, R.; Pfreundschuh, M.; Gerber, C.; Müller, D. J. Atomic Force Microscopy-Based Characterization and Design of Biointerfaces. *Nat. Rev. Mater.* **2017**, 2 (5), 17008.

- (18) Krieg, M.; Fläschner, G.; Alsteens, D.; Gaub, B. M.; Roos, W. H.; Wuite, G. J. L.; Gaub, H. E.; Gerber, C.; Dufrêne, Y. F.; Müller, D. J. Atomic Force Microscopy-Based Mechanobiology. *Nat. Rev. Phys.* **2019**, *1* (1), 41–57.
- (19) Thomson, N. H. The Substructure of Immunoglobulin G Resolved to 25 kDa using Amplitude Modulation AFM in Air. *Ultramicroscopy* **2005**, *105* (1–4), 103–10.
- (20) Martinez-Martin, D.; Herruzo, E. T.; Dietz, C.; Gomez-Herrero, J.; Garcia, R. Noninvasive Protein Structural Flexibility Mapping by Bimodal Dynamic Force Microscopy. *Phys. Rev. Lett.* **2011**, *106* (19), 198101.
- (21) Muller, D. J.; Engel, A. Atomic Force Microscopy and Spectroscopy of Native Membrane Proteins. *Nat. Protoc.* **2007**, *2* (9), 2191–2197.
- (22) Sofinska, K.; Wojtkiewicz, A. M.; Wojcik, P.; Zastawny, O.; Guzik, M.; Winiarska, A.; Waligorski, P.; Ciesla, M.; Barbasz, J.; Szaleniec, M. Investigation of Quaternary Structure of Aggregating 3-Ketosteroid Dehydrogenase from *Sterolibacterium Denitrificans*: In the Pursuit of Consensus of Various Biophysical Techniques. *Biochim. Biophys. Acta, Gen. Subj.* **2019**, *1863* (6), 1027–1039.
- (23) Pfreundschuh, M.; Alsteens, D.; Hilbert, M.; Steinmetz, M. O.; Müller, D. J. Localizing Chemical Groups While Imaging Single Native Proteins by High-Resolution Atomic Force Microscopy. *Nano Lett.* **2014**, *14* (5), 2957–2964.
- (24) Thomson, N. H. Imaging the Substructure of Antibodies with Tapping-Mode AFM in Air: the Importance of a Water Layer on Mica. *J. Microsc.* **2005**, *217* (3), 193–199.
- (25) Dabkowska, M.; Adamczyk, Z.; Kujda, M. Mechanism of HSA Adsorption on Mica Determined by Streaming Potential, AFM and XPS Measurements. *Colloids Surf., B* **2013**, *101* (1), 442–449.
- (26) Makky, A.; Berthelot, T.; Feraudet-Tarisse, C.; Volland, H.; Viel, P.; Polesel-Maris, J. Substructures High Resolution Imaging of Individual IgG and IgM Antibodies with Piezoelectric Tuning Fork Atomic Force Microscopy. *Sens. Actuators, B* **2012**, *162* (1), 269–277.
- (27) San Paulo, A.; Garcia, R. High-Resolution Imaging of Antibodies by Tapping-Mode Atomic Force Microscopy: Attractive and Repulsive Tip-Sample Interaction Regimes. *Biophys. J.* **2000**, *78* (3), 1599–1605.
- (28) Patil, S.; Martinez, N. F.; Lozano, J. R.; Garcia, R. Force Microscopy Imaging of Individual Protein Molecules with sub-pico Newton Force Sensitivity. *J. Mol. Recognit.* **2007**, *20* (6), 516–523.
- (29) Kienberger, F.; Mueller, H.; Pastushenko, V.; Hinterdorfer, P. Following Single Antibody Binding to Purple Membranes in Real Time. *EMBO Rep.* **2004**, *5* (6), 579–583.
- (30) Martínez, N. F.; Lozano, J. R.; Herruzo, E. T.; Garcia, F.; Richter, C.; Sulzbach, T.; Garcia, R. Bimodal Atomic Force Microscopy Imaging of Isolated Antibodies in Air and Liquids. *Nanotechnology* **2008**, *19* (38), 384011.
- (31) Ouerghi, O.; Touhami, A.; Othmane, A.; Ben Ouada, H.; Martelet, C.; Fretigny, C.; Jaffrezic-Renault, N. Investigating Antibody-Antigen Binding with Atomic Force Microscopy. *Sens. Actuators, B* **2002**, *84* (2), 167–175.
- (32) Yeow, N.; Tabor, R. F.; Garnier, G. Direct Measurement of IgM-Antigen Interaction Energy on Individual Red Blood Cells. *Colloids Surf., B* **2017**, *155*, 373–378.
- (33) Hernandez-Garcia, A.; Stuart, M. A. C.; Vries, R. D. Templated Co-Assembly into Nanorods of Polyanions and Artificial Virus Capsid Proteins. *Soft Matter* **2017**, *14* (1), 132–139.
- (34) Bergkvist, M.; Carlsson, J.; Karlsson, T.; Oscarsson, S. TM-AFM Threshold Analysis of Macromolecular Orientation: A Study of the Orientation of IgG and IgE on Mica Surfaces. *J. Colloid Interface Sci.* **1998**, *206* (2), 475–481.
- (35) Perrino, A. P.; Garcia, R. How Soft is a Single Protein? The Stress-Strain Curve of Antibody Pentamers with 5 pN and 50 pm Resolutions. *Nanoscale* **2016**, *8* (17), 9151–9158.
- (36) Gao, C.; Qu, N. Electrolysis-Assisted Recovery of Superhydrophilicity from Superhydrophobized Surfaces. *Surf. Coat. Technol.* **2019**, *372*, 343–352.
- (37) Wang, G.; Zhang, T. Y. Easy Route to the Wettability Cycling of Copper Surface between Superhydrophobicity and Superhydrophilicity. *ACS Appl. Mater. Interfaces* **2012**, *4* (1), 273–279.
- (38) Hansma, H. G.; Laney, D. E. DNA Binding to Mica Correlates with Cationic Radius: Assay by Atomic Force Microscopy. *Biophys. J.* **1996**, *70* (4), 1933–1939.