

Single Molecule Force Spectroscopy to Compare Natural versus Artificial Antibody–Antigen Interaction

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Biorecognition is central to various biological processes and finds numerous applications in virtually all areas of chemistry, biology, and medicine. Artificial antibodies, produced by imprinting synthetic polymers, are designed to mimic the biological recognition capability of natural antibodies, while exhibiting superior thermal, chemical, and environmental stability compared to their natural counterparts. The binding affinity of the artificial antibodies to their antigens characterizes the biorecognition ability of these synthetic nanoconstructs and their ability to replace natural recognition elements. However, a quantitative study of the binding affinity of an artificial antibody to an antigen, especially at the molecular level, is still lacking. In this study, using atomic force microscopy-based force spectroscopy, the authors show that the binding affinity of an artificial antibody to an antigen (hemoglobin) is weaker than that of natural antibody. The fine difference in the molecular interactions manifests into a significant difference in the bioanalytical parameters of biosensors based on these recognition elements.

Artificial antibodies or synthetic biorecognition elements based on molecular imprinting have gained significant attention, owing to their superior stability,^[1] reusability, and cost-efficiency,^[2] as well as enormous potential applications in bio/chemical sensing,^[3] separation science,^[4] and catalysis.^[5] The molecular imprinting process typically involves the polymerization of functional monomers around template species

followed by the removal of the templates, which results in cavities that are complementary in size, shape and chemical functionality to the template species.^[6] A wide variety of chemical and biological species, such as low molar mass compounds, proteins, viruses, and even bacteria have been employed as templates.^[7] The cavities, often referred to as artificial antibodies, recognize the target antigens through the

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complementarity in size, shape, and concerted noncovalent interactions (e.g., electrostatic, hydrogen-bonding, aromatic acceptor–donor) emanating from the rationally chosen functional monomers. Recent efforts have focused on the rational selection and incorporation of appropriate functional monomers to improve recognition abilities, for example, by incorporating supramolecular or aromatic acceptor–donor interactions.^[8] In fact, one of the long-standing goals in the design of artificial antibody is to achieve highest binding affinity and selectivity to target antigens that rival the biorecognition ability of natural antibodies. However, so far, there have been no reports on direct comparison of the binding affinities of artificial and natural antibodies to target antigen at the molecular level. Moving forward, experimental techniques that provide a detailed insight into the biorecognition ability of the artificial antibodies at the molecular scale is critical.

Atomic force microscopy-based force spectroscopy (AFM-FS) is a powerful tool for investigating molecular scale processes. By covalently immobilizing the biomolecules exhibiting specific interaction (e.g., antibody–antigen) on an AFM tip and a substrate, interaction forces between them can be measured with pico-newton resolution.^[9] A better understanding of the interaction forces between antibodies and antigens at the molecular scale is important to rationalize the observed differences in recognition abilities between artificial and natural antibodies at an ensemble level (e.g., sensitivity and selectivity of biosensors based on these biorecognition elements^[10]). Ultimately, this fundamental knowledge can provide design rules for realizing artificial recognition elements that rival their natural counterparts. Here, using plasmonic nanotransducers, which offer a fine control over the imprinting process and bioconjugation at the nanometer scale,^[11] and hemoglobin as a prototypic protein target, we compare the pair-wise interaction forces of two systems, artificial antibody–hemoglobin and natural antibody–hemoglobin, at the molecular scale. By varying the loading rates of the AFM tip (known as dynamic force spectroscopy, DFS), we determine the kinetic and energetic parameters (including dissociation rates (k_{off}) and heights of energy barrier during dissociation (ΔG)) of the two systems. In the end, we investigate the biosensing capabilities of the two recognition elements using a plasmonic nanobiosensor based on refractive index sensitivity of localized surface plasmon resonance (LSPR) wavelength and correlate with their corresponding k_{off} and ΔG .

We have previously demonstrated the feasibility of molecular imprinting on gold nanostructures, which serve as nanotransducers for plasmonic biosensors.^[11–13] In the case of artificial antibodies, the molecular imprinting process on gold nanorods (AuNRs) involves multiple steps as schematically illustrated in **Figure 1A**. Refractive index sensitivity of the plasmonic nanostructures can also be employed to monitor each step along imprinting process, which involves a change in the refractive index of the medium surrounding the nanostructures.^[11] The imprinting process first involves the synthesis of AuNRs and their immobilization onto a glass substrate. We synthesized AuNRs with a length of 49.3 ± 1.9 nm and a diameter of 19.2 ± 1.3 nm using a

seed-mediated approach (Figure 1C, inset).^[14] These AuNRs are then immobilized onto the 3-mercaptopropyl-trimethoxysilane (MPTES)-modified glass substrates. AFM image reveals the uniform distribution of AuNRs with built-in artificial antibodies on the glass substrate (Figure 1C). Each step in the molecular imprinting process is monitored by following the LSPR shift of AuNRs (Figure 1D,E). The accumulated red shift following various steps of imprinting process is found to be ≈ 15 nm followed by a ≈ 8 nm blue shift upon template protein removal. In the case of natural antibodies, anti-hemoglobin IgG is conjugated onto the AuNR surfaces using polyethylene glycol (PEG) as the linker. The successful bioconjugation is confirmed by a 9.6 nm increase in the hydrodynamic size of AuNRs (Figure 1F). Also, the longitudinal LSPR wavelength of the AuNRs exhibits a red shift of 6.8 nm due to the increase in the refractive index of the medium surrounding the AuNRs (Figure 1G). These AuNR-IgG conjugates are then adsorbed onto the MPTES-modified glass substrates (Figure S1, Supporting Information).

Next, we set out to investigate the pair-wise interaction forces of the two systems: artificial antibody–hemoglobin and natural antibody–hemoglobin (schematic illustrations shown in Figure 1B,H). In order to measure the interaction forces using AFM-FS, the hemoglobin is immobilized onto the AFM tip. The PEG linker strategy mentioned above is applied for tip biofunctionalization as well, owing to several advantages of the PEG, including (i) serves as a flexible spacer, increasing the accessibility of hemoglobin molecules to antibodies;^[15] (ii) resists nonspecific protein adhesion and tip–surface interaction;^[16] and (iii) provides an internal standard (i.e., well-defined rupture length range) to screen AFM force–displacement curves. For example, PEG chain rupture length in the range of 15–70 nm (for PEG with M_w of 5000 g mol^{−1}, contour length 47.9 nm^[17]) is employed as a criterion to choose force curves exhibiting specific binding events.^[18]

Force measurements are conducted using AFM tips, functionalized with hemoglobin, on the artificial antibodies at a loading rate of ≈ 350 nN s^{−1} in buffer. We have collected a total of 1000 force curves for each condition. The typical force curves representing specific binding, nonspecific interaction and no interaction are shown in **Figure 2C**. Of these, $10.4 \pm 2.0\%$ force curves exhibiting a specific binding event are analyzed to construct the force distribution histogram (Figure S2, Supporting Information). This binding percentage is reasonable considering the total coverage area of AuNRs on the substrate, which can be estimated from footprint of a single AuNR and the number density of AuNRs in a given region. For example, AFM image shows ≈ 280 AuNRs in a $2 \times 2 \mu\text{m}^2$ area (Figure 1C). Based on the dimensions of single AuNR ($\approx 50 \times 20$ nm²), we estimate the surface coverage percentage of AuNRs in this area to be $\approx 7\%$, which is very close to the binding probability. A too low or a too high binding probability compared to the surface coverage % is indicative of the loss of tip functionality or increase of nonspecific binding. The distribution of the rupture force of artificial antibody–hemoglobin shows two peaks (most probable rupture force) including the primary peak at 188.4 pN and a secondary peak at 361.3 pN (Figure 2A). It should be noted that the rupture force associated with the secondary peak is almost 100%

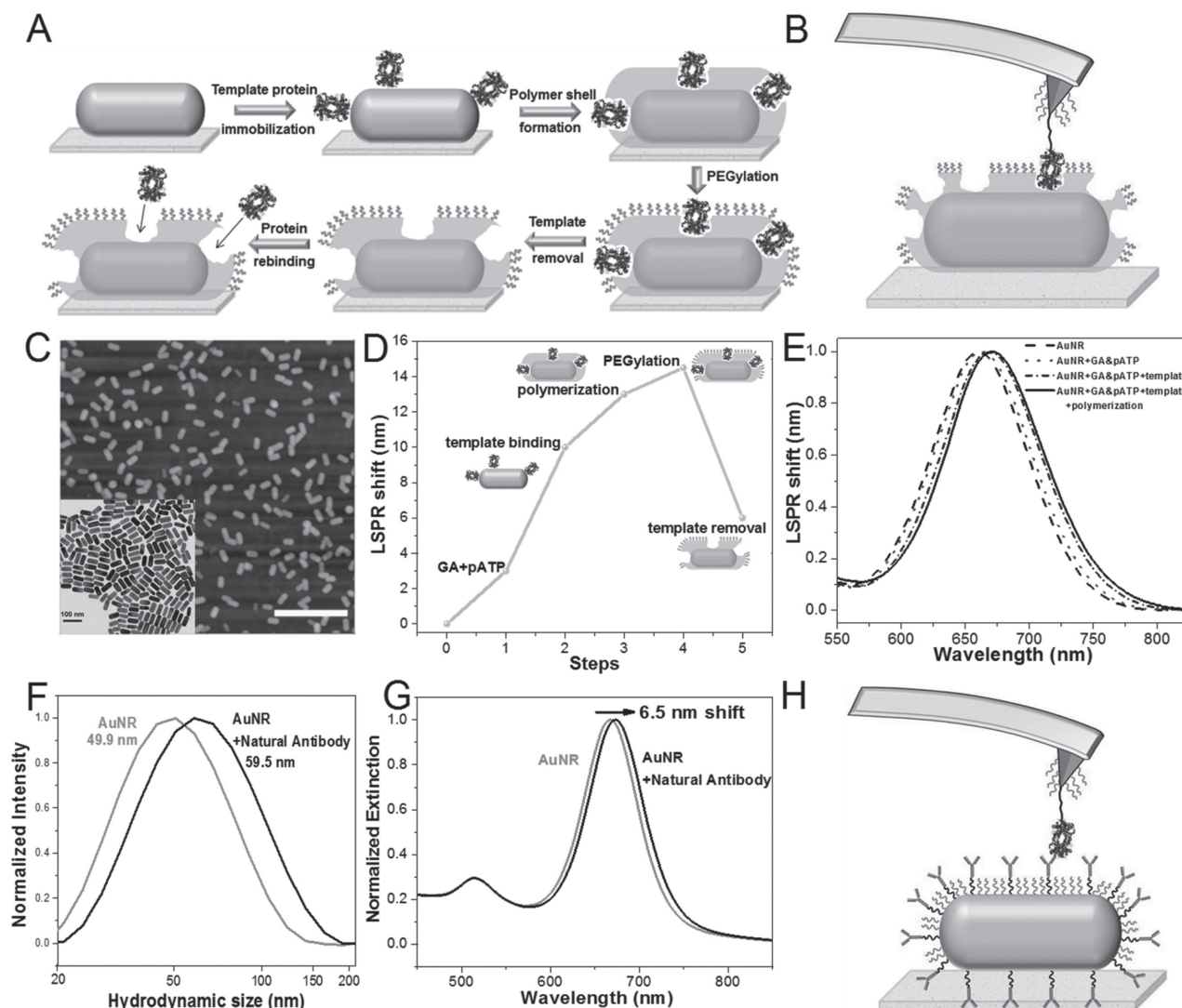


Figure 1. A) Schematic illustration of molecular imprinting of hemoglobin on AuNR surface. First, template proteins (hemoglobin) are conjugated to AuNR through the formation of reversible imine bonds using *p*-aminothiophenol and glutaraldehyde as the linker. Trimethoxysilane, (3-aminopropyl) trimethoxysilane, and trimethoxyphenylsilane are then copolymerized around the AuNR-hemoglobin conjugates. The methoxy group of the three monomers undergoes a rapid hydrolysis and subsequent condensation to form an amorphous polymer matrix, with amine, hydroxyl, methyl, and phenyl groups interacting with the template hemoglobin through electrostatic, hydrogen bonding, hydrophobic, and aromatic donor–acceptor interactions, respectively. To minimize the nonspecific protein binding during the recognition and detection, bifunctional polyethylene glycol (methoxy-PEG-silane) is then covalently grafted onto the noncavity regions (i.e., regions not interacting with the template) of the siloxane copolymer (Figure S3, Supporting Information). Subsequently, the template hemoglobin is then removed by treating the molecular imprints with copolymer acid and sodium dodecyl sulfate to break the imine bonds and overcome noncovalent interactions, respectively. Following the template protein removal, the AuNRs are left with siloxane copolymer layer with cavities complementary in shape, size, and chemical functionality to the template protein. B) Schematic representation of AFM-based force spectroscopy set-up for measuring interaction force of artificial antibody–hemoglobin. For the tip biofunctionalization, hemoglobin molecules were conjugated to bifunctional polyethylene glycol (COOH–PEG–SH), yielding hemoglobin–PEG–SH. Then the gold-coated AFM tip is biofunctionalized by immersing the tip into the mixture solution (molar ratio 1:10 000) of hemoglobin–PEG–SH and methoxy–PEG–SH for the formation of Au–S linkage. This biofunctionalization strategy limits the density and number of proteins on the tip surface and ensures that most of interaction events are from single-molecular binding. C) AFM image showing uniformly adsorbed AuNR-molecular imprints on glass substrate after template removal (Scale bar: 0.5 μ m). Inset: TEM image of AuNRs used as plasmonic nanotransducers (Scale bar: 100 nm). D) LSPR shift corresponding to each step of hemoglobin imprinting on AuNRs. E) Representative extinction spectra of hemoglobin imprinting including bare AuNRs on glass, attaching cross-linker on AuNRs, hemoglobin (template) conjugation on AuNRs, and polymerization around template proteins. F) Hydrodynamic size obtained from DLS showing an increase of $\approx$ 10 nm after natural antibody conjugation on AuNRs. G) Extinction spectra showing the LSPR shift after conjugation of AuNR with anti-hemoglobin IgG in solution. H) Schematic representation of AFM-based force spectroscopy set-up for measuring interaction force of natural antibody–hemoglobin. AFM tip biofunctionalization strategy described above is used.

higher than that of the primary peak, implying that multiple molecules (two hemoglobin molecules in this case) may be interacting with artificial antibodies (two adjacent cavities)

simultaneously. In contrast, the force histogram of natural antibody–hemoglobin shows single peak at 225.3 pN (Figure 2B). The lack of multibinding for natural antibody may be due to

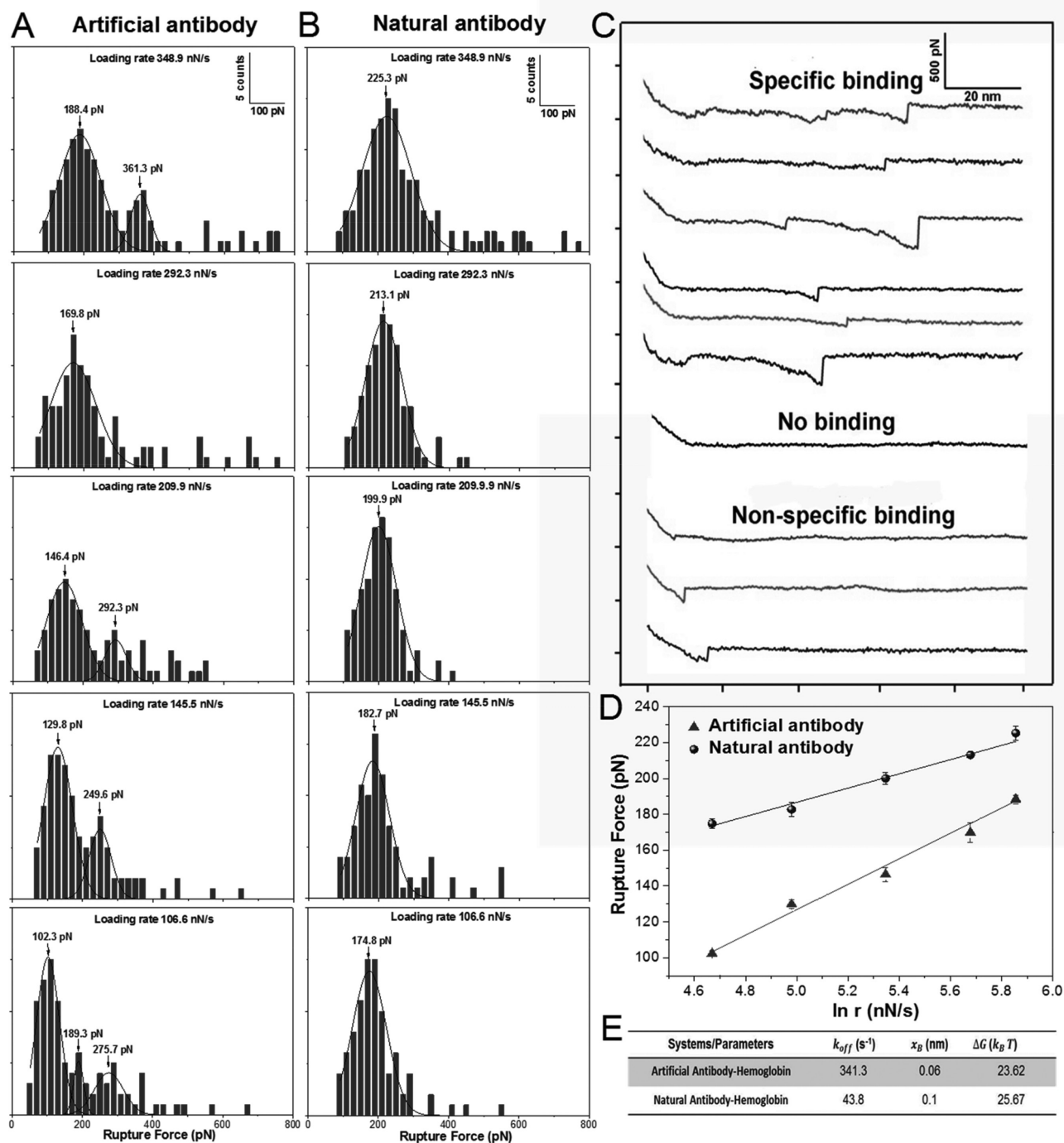


Figure 2. A) Force distribution histograms of artificial antibody–hemoglobin at different loading rates with Gaussian fits of the data. The fitted peaks represent the most probable rupture forces at different loading rates. B) Force distribution histograms of natural antibody–hemoglobin at different loading rates with Gaussian fits of the data. C) Typical AFM force–distance curves obtained in the experiments: The curves classified as specific binding events are manifested in a cantilever deflection observed as a nonlinear delayed retraction curve with a different slope as that of the contact region. The rupture lengths of the three curves (at the bottom) fall out of the range of 15–70 nm (less than 15 nm) and were considered as nonspecific binding events. D) Dynamic force spectra of both systems at different loading rates. Fitting the data with the Bell–Evans model yields linear fits for both systems. E) Dissociation kinetic parameters of both systems.

the steric hindrance originating from the larger size of anti-hemoglobin IgG (150 kDa) compared to the artificial antibody (64 kDa, equivalent to the size of the template protein). Interestingly, similar force distributions are observed at other loading rates for both artificial and natural antibodies, further supporting the mechanism suggested above. Furthermore, two

control experiments confirm that the measured forces are indeed those of the artificial antibody–hemoglobin interaction (Figure S2, Supporting Information). Overall, the force spectroscopy clearly indicates that the single molecular rupture force of artificial antibody–hemoglobin is $\approx 20\%$ lower than that of natural antibody–hemoglobin.

To further compare the molecular interactions of the two systems, DFS is employed to determine the dissociation dynamics and energy barriers in the dissociation event, by evaluating the most probable rupture forces at different loading rates. We probed the interaction forces over a loading rate range of ≈ 100 to ≈ 350 nN s^{-1} . According to classical Bell–Evans model,^[19] many interaction systems exhibited “double energy barriers” including an outer barrier representing near-equilibrium unbinding regime at slow loading rates (usually less than 50 nN s^{-1}), and an inner barrier representing kinetic unbinding regime at fast loading rates. However, a recent study pointed out that the common existence of two energy barriers could be a misconception based on a false interpretation of these nonlinear force value trends across the slow and fast loading rate regimes.^[20] The force at the near-equilibrium loading regime was affected by single bond reforming or asynchronized rupture of multiple individual bonds. By fitting these nonlinear force value trends using the model proposed by Friddle et al.,^[20] the dissociation kinetic parameters are found to be very similar to the data fitted by Bell–Evans model at high loading rate regime. Therefore, in this study, to avoid the discrepancy of two different models and also to mimic flow condition during recognition and detection, we probe the interaction forces at the high loading rate regime (from ≈ 100 to ≈ 350 nN s^{-1}).

The force distribution histograms at different loading rates of the two systems are shown in Figure 2A,B. In both systems we note a linear dependence of the rupture force on the $\ln(r)$, where r is the loading rate (Figure 2D). Therefore, the Bell–Evans model can be used to extract the kinetic parameters using the following equation (Figure 2E):^[19]

$$F = \frac{k_B T}{x_B} \ln\left(\frac{r x_B}{k_B T k_{\text{off}}}\right) \quad (1)$$

where F is the most probable rupture force, r is the loading rate, k_{off} is the dissociation rate of bond at zero applied force, x_B is the distance between bound state and unbound state for the transition state, k_B is the Boltzmann constant, T is the absolute temperature. Both k_{off} and x_B are important kinetic parameters for evaluating the susceptibility of the bond dissociation under applied force or flow conditions.^[21] Once k_{off} is determined, the height of the energy barrier, ΔG can be deduced using the following equation:^[22]

$$\Delta G = -k_B T \ln \frac{k_{\text{off}} h}{k_B T} \quad (2)$$

where h is Planck’s constant and $k_B T$ is the thermal energy. The linear relation between rupture force and $\ln(r)$ in Figure 2D indicates that both artificial antibody–hemoglobin and natural antibody–hemoglobin complexes overcome single energy barrier during their dissociation under applied force with a height of 23.62 and 25.67 $k_B T$, respectively. The corresponding k_{off} for artificial antibody–hemoglobin and natural antibody–hemoglobin are found to be 341.3 and 43.8 s^{-1} , respectively. The higher k_{off} and lower energy barrier height indicate the formation of a less stable complex

between artificial antibody and hemoglobin compared to the natural antibody and hemoglobin.

Finally, we turn our attention to the biosensing capabilities of artificial and natural antibody based plasmonic nanobiosensors. Different concentrations of hemoglobin are used to evaluate sensitivity and detection limit of the two biosensors. **Figure 3** shows a monotonic increase in the longitudinal LSPR shift with an increasing concentration of the hemoglobin in both cases. At different concentrations of hemoglobin, the natural antibody-based biosensor exhibits significantly higher LSPR shift compared to the artificial antibody. For instance, for a hemoglobin concentration of 40 $\mu\text{g mL}^{-1}$, the plasmonic biosensors based on artificial and natural antibodies exhibit a red shift of ≈ 10 and ≈ 16 nm, respectively. The limit of detection of the plasmonic biosensors is defined as the concentration corresponding to an LSPR shift of 1 nm, which represents 3σ noise level. The detection limit of natural antibody-based biosensor is found to be ≈ 10 ng mL^{-1} , which is two orders magnitude lower than that of the artificial antibody-based biosensor (≈ 1000 ng mL^{-1}). It is widely acknowledged that the LSPR shift exhibits a characteristic decay with the increasing distance from the surface of the nanotransducer due to the decay of the evanescent electromagnetic field.^[23] Here, considering the similar thickness of imprinted polymer layer and natural antibody, which can be estimated from LSPR shift (≈ 7 nm for both cases), the difference in sensitivity and detection limit cannot be attributed to the distance-dependent sensitivity from the surface of the nanotransducer. Therefore, the disparity in the sensitivity and detection limit mainly originates from the difference in the pair-wise biomolecular interactions. Specifically, the higher molecular binding force and binding stability to antigen offers the natural antibody better biosensing capabilities (higher sensitivity and lower detection limit) over artificial antibody. Although the artificial antibodies achieved here are inferior to natural antibodies in terms of their binding affinity, the single molecular interaction information will provide additional insight for the rational design (e.g., choice of functional monomers, imprinting conditions including the thickness of the polymer layer, mechanical properties controlled by the degree of cross-linking) of desired artificial antibodies that approach and even rival their natural counterparts. While the molecular affinity of natural antibodies is certainly higher than the artificial antibodies, the thermal and environmental stability of the latter is certainly attractive for applications in point-of-care and resource-limited settings, where biomolecular-friendly environments are not often guaranteed or even available. It is important to note that the limit of detection and sensitivity depend on the nanotransducers employed and the target analyte. Gold nanocages with significantly higher sensitivity compared to AuNRs have been employed to improve the sensitivity and lower the limit of detection of biosensors based on artificial antibodies.^[13]

In summary, using AFM-based force spectroscopy, we have unveiled single molecular pair-wise interaction force between artificial (and natural) antibody and antigen. We found that the rupture force associated with natural antibody and antigen is higher compared to artificial antibody and antigen. Furthermore, our single molecule force

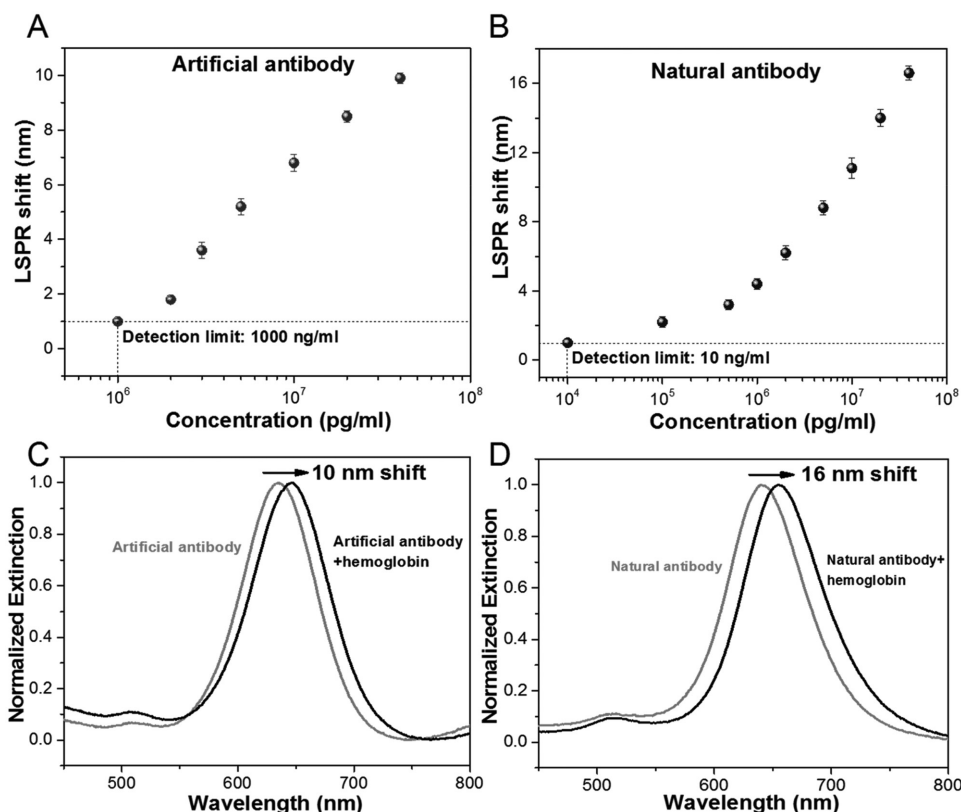


Figure 3. LSPR shift of A) artificial antibody-based and B) natural antibody-based plasmonic biosensor exposed to different concentrations of hemoglobin (mean $\pm$ standard deviation, $N = 3$). The detection limits of artificial antibody-based and natural antibody-based biosensor are 1000 and 10 ng mL⁻¹, respectively. C) Typical extinction spectra of artificial antibody-based biosensor before and after exposure to 40 μ g mL⁻¹ of hemoglobin showing a shift of $\approx$ 10 nm. D) Typical extinction spectra of natural antibody-based biosensor before and after exposure to 40 μ g mL⁻¹ of hemoglobin showing a shift of $\approx$ 16 nm.

spectroscopy study reveals that the natural antibody–antigen complex exhibits a higher binding stability compared to artificial antibody–antigen complex. These fine differences at single molecular level manifest into the significantly different biosensing characteristics of plasmonic biochips based on natural and artificial antibodies. These results highlight the importance of understanding the single molecular interaction in design of highly sensitive biosensors. The AFM-based force spectroscopy-based approach presented here is a powerful tool to rationalize and guide the design of synthetic biorecognition elements and relevant highly sensitive biosensors for resource-limited environments.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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