Lab on a Chip

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Efficiency Optimisation of Protein on a Chip

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Abstract

This study elucidates the protein reorientation on a chip can be changed by an external electric field (EEF) and optimised for achieving strong effective binding between proteins. Protein A and its binding protein immunoglobulin G (IgG) were used as an example, in addition to an anticancer peptide (CB1a) and its antibody (anti-CB1a). The binding forces (BF) were measured by atomic force microscopy (AFM) with EEFs applied at different angles (EEF°). The optimal angle (OA) of the EEF ($_{OA}$EEF°) corresponding to the maximum binding force (BF_{max}) was obtained. The results showed that the BF_{max}s between IgG/Protein A and anti-CB1a/B1a were 6424.2±195.3pN ($_{OA}$EEF°=45°) and 729.1±33.2pN ($_{OA}$EEF°=22.5°), respectively. Without an EEF, the BF was only 730.0±113.9pN and 337.3±35.0pN, respectively. Based on these observations, we concluded that the efficient optimisation of protein–protein interaction on a chip is essential. This finding is applicable to the industrial fabrication of all protein chips.

Introduction

Biochip developments (DNA chips in particular) have progressed steadily in recent years[1-3]. The main detection techniques used are fluorescence (inner and outer reflections) and fluorescence-freemethods (atomic force microscopy, optical spectroscopy, surface plasma resonance, dark-field light scattering, and electrochemistry)[4-6]. One of the main applications of biochips is diagnostics, because of their ease of use and capability to provide quick results, especially for their development as 'portable' chips in the future. However, for using such chips for proteins (protein chip)[7], certain problems must be overcome, including the fragility of the three-dimensional (3D) structure and the temperature/pH-sensitive characteristics of proteins. For example, protein unfolds at high temperatures or in acidic/alkaline pHs. Furthermore, fragility of the protein structure implies that the biological functions of proteins are performed only because of the presence of network connections and through weak forces (non-covalent) such as hydrogen bond, the van der Waal force, and charge–charge interaction[8,9]. These weak forces supporting protein functions can be easily eliminated by changing the pH, temperature, or ionic strength. To overcome these problems, therefore, we present requirements for fabricating efficient protein chips: The first requirement is to ensure that the protein functions are effectively performed with the 3D conformation (folded state) when the protein is either in a solution[10,11] or on a chip[12]. For protein immobilised on a chip, this is essential; otherwise, the protein chip could be rendered useless. The second requirement involves using a method that ensures 'optimising' the performance of the protein on the chip. The reason is that a protein such as an antibody has only one unique binding site that can be relocated on a chip at any location referring to its binding partner, the antigen. In this situation, the 'angle' of protein insertion into the chip is logically a crucial factor influencing the binding efficiency of the protein. An apt example entails the binding site of an antibody protein on a chip being exactly opposite to the binding direction to its antigen; therefore, the efficiency of the protein chip can be predicted to be 'zero'. In other words, the antibody and antigen cannot feasibly bind together perfectly to interact. Accordingly, the term 'optimisation angle' (OA) is coined in this study as the second requirement for denoting the angle of protein insertion for optimal protein-antibody interaction. Both the first and second requirements should be considered for producing highly efficient protein chips in the future.

In this study, we developed and demonstrated a method for determining the OA to immobilise a protein on a chip. We considered that most proteins have their own polarities and can be reoriented by applying an external electric field (EEF). Thus, protein reorientation (PRO) can be conducted to optimise the performance of a protein

on a chip. To prove the feasibility of this concept, Protein A, which is frequently used in biochemical research, with the binding protein immunoglobulin G (IgG)[13] and a small peptide, CB1a (≈4kD, an anticancer peptide),which we used in our previous study[14], with its antibody anti-CB1a[15] were used as examples. Both proteins, IgG and anti-CB1a,[15] were immobilised on a chip to prove the feasibility of the concept of PRO under the influence of EEFs at different angles (EEF°). Atomic force microscopy (AFM) was used as the binding force measuring system[16-18]. Quantitative analysis of the binding force for different extents of PRO under the influence of EEFs yields the OA of the EEF corresponding to the maximum binding force (BF_{max}). The BF_{max} between an antigen and its antibody indicates the optimal achievable efficiency of the protein chip. Consequently, we proved that the relationship between PRO and the EEF exists. Investigating all influencing angles (0° to 360°) can facilitate identifying the OA. According to the OA, the most efficient protein chip can be produced. The OA defined in this study may therefore be applied to optimise protein–protein interactions and the fabrication of all protein chips.

Experimental

Silicon tips used for AFM (NanoWizard, JPK Instruments, Berlin, Germany) imaging and force measurements were ordered from Nanosensor (Neuchatel, Switzerland). Silicon wafers (6′) were purchased from Summit-Tech Resource Corp (Hsinchu, Taiwan). [3-(2-Aminoethylamino)propyl]tri-methoxysilane (3-APTMS) and glutaraldehyde (GTA) were obtained from Sigma–Aldrich (St. Louis, MO, USA). The peptide CB1a (33 amino acids; molecular weight ≈ 4kD) was prepared according to a previous study[14]. The antibody anti-CB1a was produced from mice according to a previous study[15]. Protein A (molecular weight ≈ 42kD) and its binding protein (IgG) were purchased from R&D System (Minneapolis, MN, USA) and used without further purification. Protein solutions were prepared with a phosphate-buffered saline buffer (pH 7.6), and deionised distilled water was used in this study.

Preparation of antibody on chip without EEF. The chip used without the addition of an EEF was prepared as follows: **(a)** Hydroxylation: To create a hydroxyl (-OH) group on the chip surface, the chip was incubated in piranha solution for 10 min and cleaned with ethanol/H_2O to remove organic compounds and other impurities. The chip was then treated with oxygen plasma for 3 min.**(b)** Self-assembled monolayer: 3-APTMS was used to form a self-assembled monolayer (SAM), which was the first layeron the chip[19]. The procedure used for it was as follows: The chip treated in Step (a) was incubated for 1 h in a solution containing 97% 3-APTMS diluted with 99%

ethanol. The chip was then removed from the solution and cleaned with ethanol in an ultrasonic cleaner. **(c)** Cross-linking[20,21]: The chip with the SAM was incubated for 1 h in 25% GTA(the second layer) diluted 10 times with pure water for 1 h. The aldehyde group of GTA formed covalent bonds with the amide group of the SAM on the chip. The chip was then cleaned with water in the ultrasonic cleaner to remove free GTA. The cleaned chip then contained two layers: SAM and GTA layers. **(d)**Target protein (the third layer)[22]: A protein antibody (IgG or anti-CB1a)with a concentration of 20mg/mL of 1xPBS buffer was incubated for 30 min on the chip containing SAM+GTA layers. During incubation, the amino group (-NH_2) of IgG (or anti-CB1a) was coupled (covalent bonding) with the aldehyde group of GTA. Subsequently, 0.05M NaOH was used to remove the free antibody protein from the chip. The main chemical bonding processis shown in Fig.1 (A). The three layers (SAM+ GTA+ IgG) of the constructed chip were proved layer by layer for their covalent bonding by using FTIR (Fig. 1(B). Finally, the treated chip contained three layers (SAM+GTA+antibody protein) and was ready for use in the experiments. Antigens such as CB1a and Protein A immobilized on the tips of AFM were similarly prepared as for the antibodies immobilized on the chip, but without EEF.

Re-orientation of antibody on chip by applying EEF. Because proteins are large polar molecules (especially antibody proteins), their reorientation may be affected by the presence of an EEF. Accordingly, we expect that high binding efficiency can be achieved by reorienting the protein (such as IgG) on the chip to the optimal angle for binding with its binding protein, Protein A. To confirm this special case, we designed a rotatable and adjustable parallel electrode device for reorienting IgG on the chip before it approached GTA for binding. As shown in Fig.2 (A), this specific design consists of a centre vessel that holds the protein chip and a 360° rotatable frame attached with adjustable parallel copper electrodes. A real photograph of the EEF rotating device set-up was shown in Fig. 2(B). An EEF can reorient IgG immediately before its immobilisation. Similar procedures were conducted for CB1a and its antibody (anti-CB1a). The extent of PRO on the chip varies with the EEF angle (EEF°). Optimisation can therefore be achieved by using the most appropriate OA among the EEF° values ($_{OA}$EEF°) on the basis of BF measurements. The basic principle by using EEF to re-orient IgG or anti-CB1a on a chip is due to a torque produced along the direction of protein polarity.

Measurement of BF between antibody and antigen. According to Hooke's law ($F=k\Delta x$, where F denotes the BF in this experiment, k is the spring constant of the

atomic force microscope tip, and Δx is the displacement of the tip from its original position), BF, which is proportional to the compression distance Δx, can be calculated from k and Δx (Fig. 3)[16,23]. In this experiment, the EEF applied to the IgG solution for 15 min was applied at different angles before IgG was immobilised on the chip (chip_IgG). Protein A was stabilised on the atomic force microscope tip (afm_Protein A). After the application of EEFs at different angles, the BFs were measured using AFM as afm_Protein A approached chip_IgG. Similar procedures were performed for the CB1a antibody (chip_anti-CB1a) and CB1a (afm_CB1a).

Results

Optimisation of the BF between IgGand Protein A under an EEF. The EEF angle (θ) was varied by 22.5°in each test, and the angle ranged from 0° to 360°. IgG was reoriented under the influence of an EEF applied at different angles (EEF°) before it approached and was immobilised on the chip (chip_IgG). The BFwas measured as afm_Protein A approached to interact with chip_IgG. Figs. 4 (A) & (B) showed typical examples of AFM spectra without and with the influence of an EEF, respectively, for chip_IgG and afm_Protein A. Figs. 4 (C) & (D) were the examples of AFM spectra for chip_anti-CB1a and afm_CB1a without and with EEF, respectively (see below section). The results of the relationship between the BF and the EEF° are shown in Fig.5. The BF_{max} between two protein interactions was 6424.2±195.3pN at an EEF° of 45°. Compared with the BF (730.0±113.9pN) without an EEF, the interaction between IgG and Protein A was highly optimised at a particular angle (OA=45°) of the EEF ($_{OA}EEF^{45}$). Fig. 6 shows the relationship between the EEF strength and the BF for IgG/Protein A (Fig. 6(A)) and for anti-CB1a/CB1a (Fig. 6 (B) (the distance between the two electrodes was 3 cm). The results showed that BF increased with the EEF strength. The optimal EEF strength that could be produced in this study was 8×10^5 V/m; therefore, this EEF strength was used in the experiments of this study. Fig. 7(A) showed that smaller angles were used around the OA (ranging from 22.5° to 67.5° with an increment of 7.5° in each experiment). For IgG and Protein A, only one outstanding peak was observed at an OA of 45°. Fig.7 (B) shows the images of IgG chip surface measured by AFM. Results show that the proteins were better ordered on the chip surface (more dense; see Fig. 7(B)-(b)) when EEF at its OA (45^o) was applied as compared with (a) without EEF and (c) EEF at 90^o (less dense). These proteins are reoriented at their best position in the solution under the influence of OA-EEF before they are bounded to the chip (SAM+GTA). Functionalization of IgG remained was proved by its best binding with antigen,

Protein A (see Fig. 5).

Optimisation of the BF between anti-CB1a and CB1a under an EEF. Another set of examples considered was the anti-CB1a antibody and CB1a antigen. The preparation procedures were similar to those for IgG and Protein A. The chip was incubated in an anti-CB1a antibody solution for 15 min under the influence of EEFs at different angles (EEF°). Anti-CB1a on the chip was then induced to interact with afm_CB1a on the atomic force microscope tip, and the BF was measured. Fig. 8 shows the relationship between the BF and the EEF° over a cycle (0°–360°). A peak (first peak) was observed at an EEF angle of 22.5°, and the BF_{max} was approximately 729.09pN. Compared with the BF (337.3pN) without an EEF, the interaction between the anti-CB1a antibody and the CB1a antigen was highly optimised at a specific angle (OA=22.5°) of the EEF ($_{OA}EEF^{22.5}$). Another peak (second peak) was observed at 135° with a BF of approximately 567.9pN.

In nature, there has only a unique binding site between antibody and antigen. If this unique binding site is deformed due to the conformational change, the efficiency of binding would jump to very low situation (or even to zero). Reflecting to this fact, in this work, we observed that all binding efficiencies were purely dependent on the angles of EEF (see Figs. 5 & 8) where $8x10^5$ V/m was applied. The low binding efficiencies for all angles of EEF applied were not observed. Therefore, the strength of EEF ($8x10^5$ V/m) used in this experiment was strong enough only for rotating the protein. Consequently, a significant optimum angle of EEF (OA) can be obtained.

Discussion

The main objective of this study was to examine whether a protein chip can be optimised and whether the optimisation can be included as an essential step as a standard operating procedure (SOP) for fabrication to be provided subsequently. We considered how the protein could be efficiently optimised when it is used on a chip as a protein chip for investigations in the future. If the protein can be efficiently optimised, the application of biochips (especially protein chips) would increase to a useful stage in the market. Optimisation implies that the investigated proteins can perform their functions with the highest efficiency in each pilot production they are used. Currently, improving the efficiency of proteins used in chip applications (protein chips) is not considered. In other words, manufacturers may not have a reliable method for quantifying their protein chip products. In this study, we present a concept and method for elucidating these concerns and offer a direction for establishing an SOP for producing protein chips in the future.

The main difficulty in applying biotechnology to diagnostic investigations is that the immobilisation of proteins cannot be confirmed. The efficiency of a protein chip cannot be manipulated if proteins are simply immobilised on the chip without further treatment. For example, as shown in Fig. 9(A), any antibody (such as IgG) can be immobilised on a chip by different reorientations. If the reorientation of IgG is completely 'wrong' (e.g., exactly opposite to the direction of binding with its antigen, Protein A, or the EEF angle being 90°), the efficiency of this protein chip could be extremely low (or even '0%'; Figs. 9(B) and 5). However, if IgG can be reoriented to the optimal position (e.g., perfectly matching the binding direction or the EEF angle being 45°), the efficiency of the protein chip could be extremely high (or even show '100%'optimisation; Figs. 9(B) and 5). These two situations (0% and 100% optimisation) differ considerably. Similarly, anti-CB1a can interact with CB1a at its optimal position for an EEF angle of 22.5° (Figs. 9(C) and 8). Otherwise (EEF angle of 90°, for example), the efficiency would be less than 10% (Figs. 9(C) and 8). Therefore, the efficiency optimisation of a protein chip is extremely crucial.

The effect of the EEF on the interaction between IgG and Protein A was considerably stronger than that on the interaction between anti-CB1a and CB1a because only one peak was observed for the interaction between IgG and protein A, and the BF_{max} was greater (6424.2±195.3and 729.1±33.2pN for IgG/protein A and anti-CB1a/CB1a, respectively). Furthermore, the results show two peaks (EEF angles of 22.5° and 135°) for the optimal binding between anti-CB1a and CB1a (Fig.8). This may be due to the smaller size of CB1a (4 kD); therefore, it can bind with either the Fa or the Fb section of anti-CB1a (Fig.9(C)). However, the section corresponding to the optimal binding (Fa or Fb section) could not be ascertained from the results of this study. Similarly, as shown in Fig. 9 (B), IgG can be reoriented to any angle and Protein A can select the optimal angle for binding with IgG (e.g., optimisation). In contrast to the results for the interaction between anti-CB1a and CB1a, only one outstanding peak was found for the interaction between Protein A and IgG. The remainder of the peaks were quite small. Our explanation is that because Protein A is larger (42 kD) than CB1a (4 kD), the optimal binding corresponds to the Protein A binding and covers the two sections (Fa/Fb) of IgG simultaneously (Fig. 9(B)). Hence, only one peak that is uniquely 'outstanding' compared with those obtained at other EEF angles was obtained at the EEF angle of 45°. These observations indicate that EEF° values of 45° and 22.5° can optimise IgG and anti-CB1a, respectively, when they are immobilised on a chip. Therefore, at these optimised EEF angles, the efficient optimisation of a protein chip and SOP fabrication can be achieved. This method may be used for optimising any protein on a chip.

Conclusion

The efficiency optimisation of IgG and anti-CB1a chips can be achieved using the method proposed in this study. Thus, this study developed an innovative method involving an EEF for enabling the effective use of protein chips. Furthermore, this study proved that an EEF can be used for PRO and that the optimised EEF angle can be determined.

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Notes and references

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Figure Legends

Fig, 1 Preparation of a protein chip without an EEF. (A) A silicon oxide chip was hydroxylated by treating it with oxygen plasma. 3-APTMS was added to form a SAM layer. The COH group of GTA was cross-linked with the NH_2 group of 3-APTMS (releasing H_2O). The target protein (side chain or terminal NH_2 group) was then bound tothe COH group of GTA that was coupled with 3-APTMS on the chip. (B) FTIR spectra of the three layers (SAM+GTA+IgG) on chip. (a) The first layer was treated by SAM (3-APTMS as a material). Two main peaks (circled ones) were obtained: (i) Deformed amine peak was shown between 1495 and 1650 cm^{-1}; (ii) Si–O–Si bond peak was shown at 1099 cm^{-1}. (b)&(c) The second layer was constructed by bonding NH_2 of 3-APTMS with COH of GTA. This bonding causes to reduce the absorbance of NH_2/COH between 3200 and 3400 cm^{-1}. (d) The third layer was constructed by adding IgG into the chip. The functional groups of protein were shown between 3600 and 3800 cm^{-1} for OH and between 3420 and 3550 cm^{-1} for NH_2.

Fig. 2 Determination of the optimisation angle (OA). (A) An antibody protein was placed in the centre vessel (diameter *r*) containing two parallel electrodes (length *L*).The inter electrode distance is adjustable (diameter *R*). The variables *w* and *d* indicate the length and width of the frame holder, respectively, and *h* is the distance between the two electrodes. An EEF can be produced by adjusting either the power supply or *R*. Binding forces for different EEF angles (EEF°; θ) can be measured using AFM. The OA corresponding to BF_{max} was determined according to the relationship between the BF and the EEF°. (B) A real photograph of the EEF rotating device set-up. A vacuum pump (red line connected to pump) was added to hold the protein chip well during the EEF experiments. The angles (0^o, 90^o, 180^o, 270^o) of EEF were shown by labels in yellow.

Fig. 3 AFM measurements. **(A)**The processes of 'extension' and 'retraction' between an antigen (blue dot) and its antibody (blue 'Y' type). Extension occurs as the atomic force microscope probe tip (containing the antigen) approaches the antibody protein immobilised on the chip surface, before establishing contact with the antibody protein. Retraction occurs as the antigen makes contact with the antibody. When contact is established, the tip is displaced from the original position. The displacement (Δx) can be converted into the binding force (BF). The variable *h* indicates the distance between the probe tip and the chip surface. **(B)** Plots of binding force vs. extension and retraction steps. The displacement was converted into the binding force (pN), which is plotted on the *y*-axis. The BF is obtained after the release of retraction.

Fig. 4 Typical AFM binding force distributions. The BFs of chip_IgG and afm_Protein A were shown in (A) without an EEF and in (B) for an EEF angle of 45°. The average BFs are 1070.0±174.4 and 6424.2±195.3pN for (A) and (B), respectively. The BFs of chip_anti-CB1a and afm_CB1a were shown in (C) without EEF and in (D) for an EEF angle of 22.5°. The average BFs are 337.35±35.02 and 729.1±33.2pN for (C) and (D), respectively.

Fig. 5 Plot of the BF between IgG and Protein A vs.the EEF°. (A) A sharp curve with only one peak isobserved. The OAwas determined to be 45°. The BF_{max} is approximtely 6424.2±195.3pN. (B) Radar chart showing a clear relationship between the BF and the EEF°. A peak at 45°is evident in the cycle.

Fig. 6 Binding force measurements as a function of the EEF strength. The BFs between IgG and protein A (A) and between anti-CB1a and CB1a (B) were measured as a function of the EEF strength. The EEF strength ranged from 0 to 8×10^5 V/m. The

distance between the two electrodes was fixed at 3 cm. The EEF anglesused were 45° and 22.5^{o} for IgG and anti-CB1a, respectively. An EEF of 8×10^5 V/m was applied in the experiments.

Fig. 7 Detailed EEF experiments within the OA range. The angles considered were 22.5°, 30°, 37.5°, 45°, 52.5°, 60°, and 67.5°. The OA is still 45°,and the BF is approximately 6424.2pN. The BFs for the other two points at 22.5° and 67.5° are 456.9 and 515.4pN, respectively. The outstanding curve with one peak can still be observed. (B) images of IgG chip surface measured by AFM: (a) without EEF, (b) EEF at 45^{o}(OA) and (c) EEF at 90^{o}.

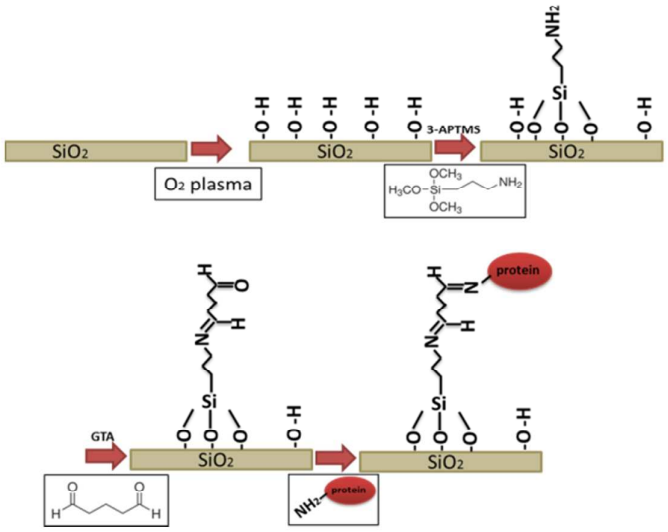
Fig. 8 Plot of the BF between anti-CB1a and CB1a vs. the EEF°. (A) A two-peak curve can be observed. The OA was determined to be 22.5°. The BF_{max} is approximately 729.1±33.2pN. (B) Radar chart showing a clear relationship between BF and EEF°. A peak at 22.5° is evident in the cycle.

Fig. 9 Reorientation of an antibody to interact with its antigen for different EEF° values. (A) A schematic illustration of protein reorientation. The antibody may have a random orientation on the chip (left diagram); therefore, the binding interaction between the antibody and the antigen maybe less efficient. If an EEF is applied at 90° (middle diagram) or 45° (right diagram), the binding between the two proteins after the reorientation of antibody may occur as shown on the right side. (B) Reorientation of IgG at different EEF° values (0°-360°). The OA is the EEF° at which Protein A perfectly interacts with IgG by binding to the Fa and Fb sections ($EEF^{45°}$ is shown by the red cycle). (C) Reorientation of anti-CB1a at different EEF° values (0°− 360°). The OA is the EEF° at which CB1a efficiently interacts with anti-CB1a by binding with the Fa or Fb section ($EEF^{22.5°}$ is indicated by the red cycle).

Figures

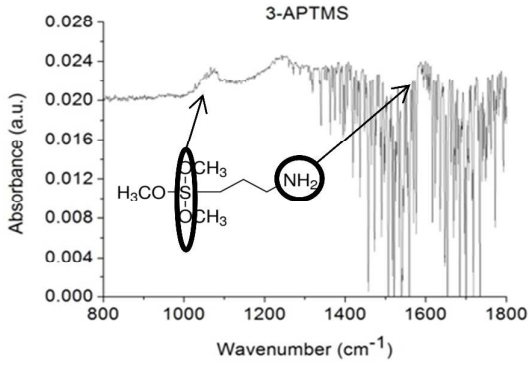
Fig. 1

(A)



(B)

(a)



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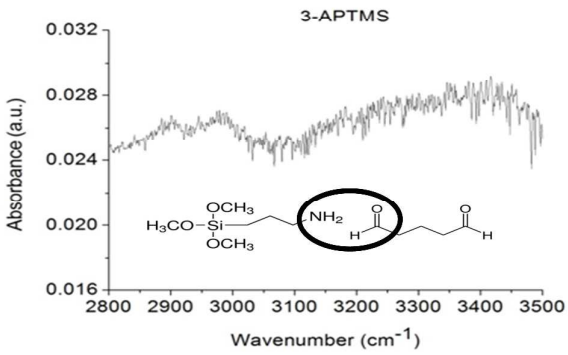

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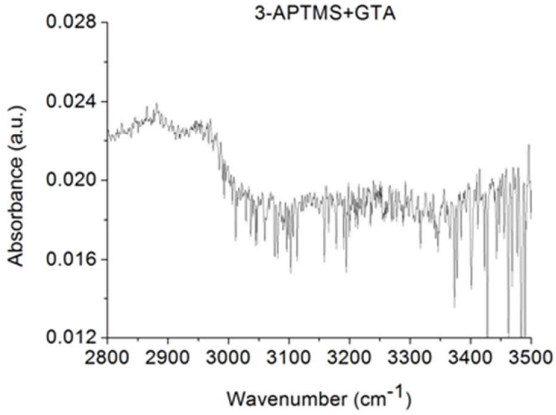


(d)

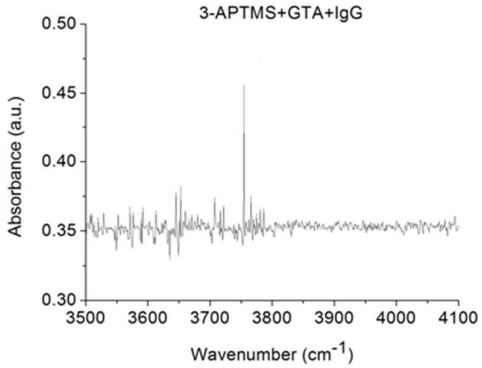
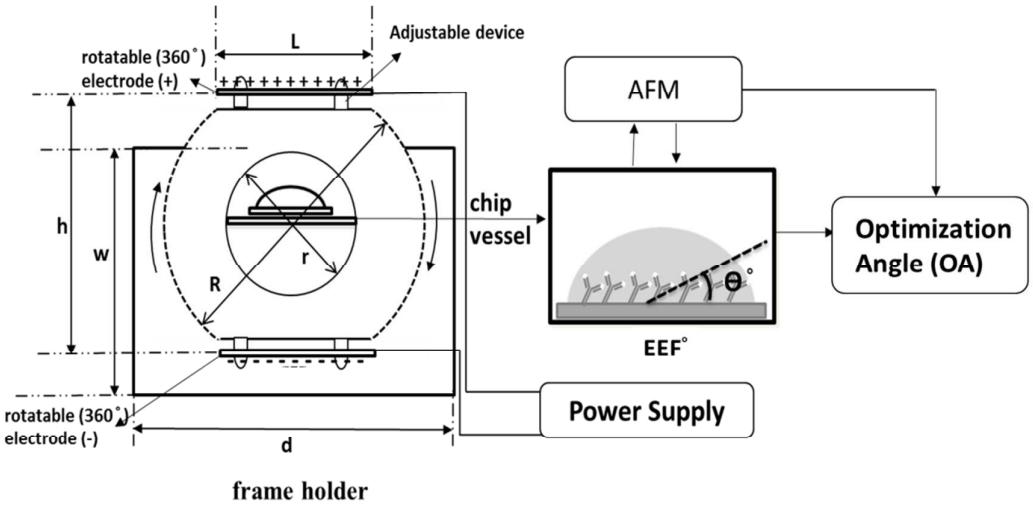


Fig. 2

(A)



(B)

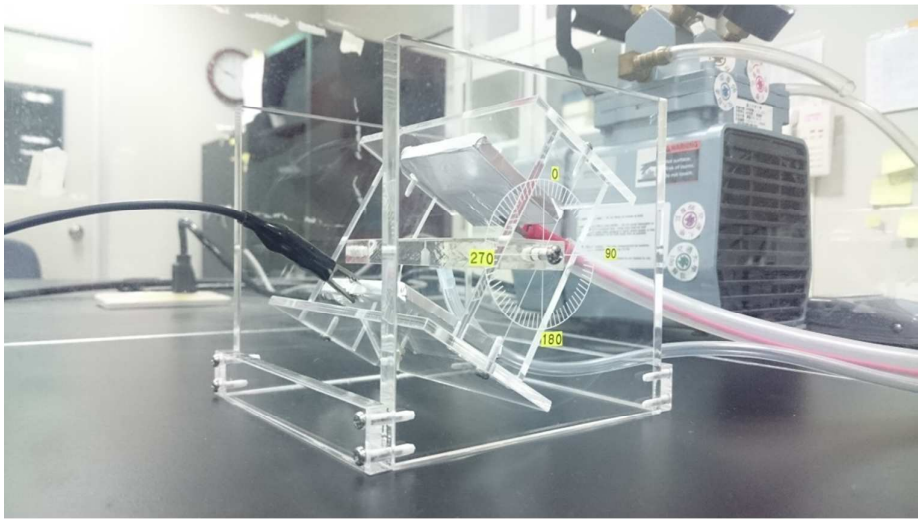


Fig. 3

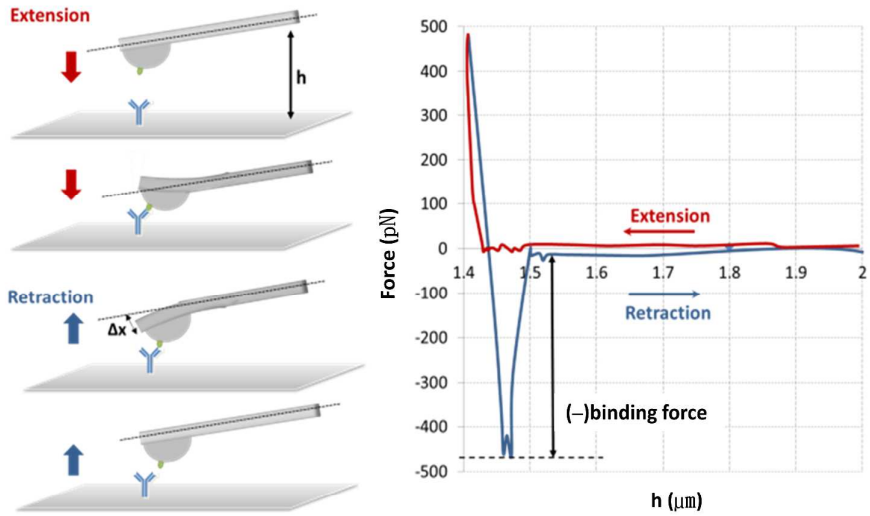
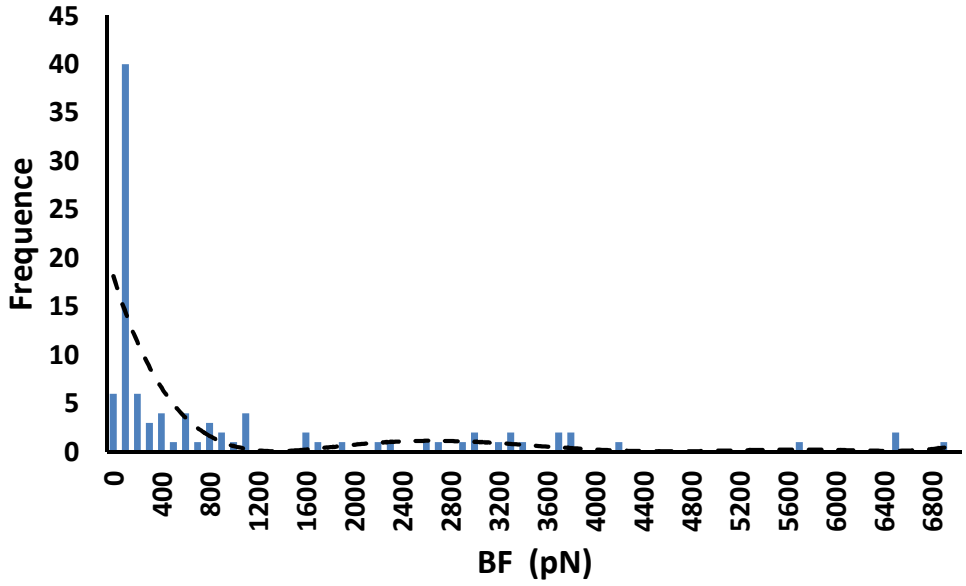


Fig. 4

(A)



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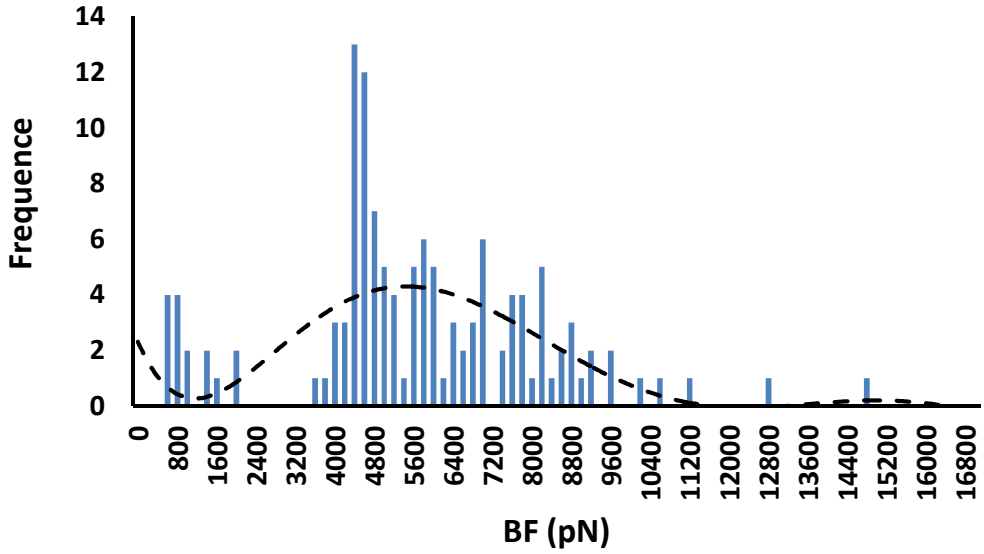
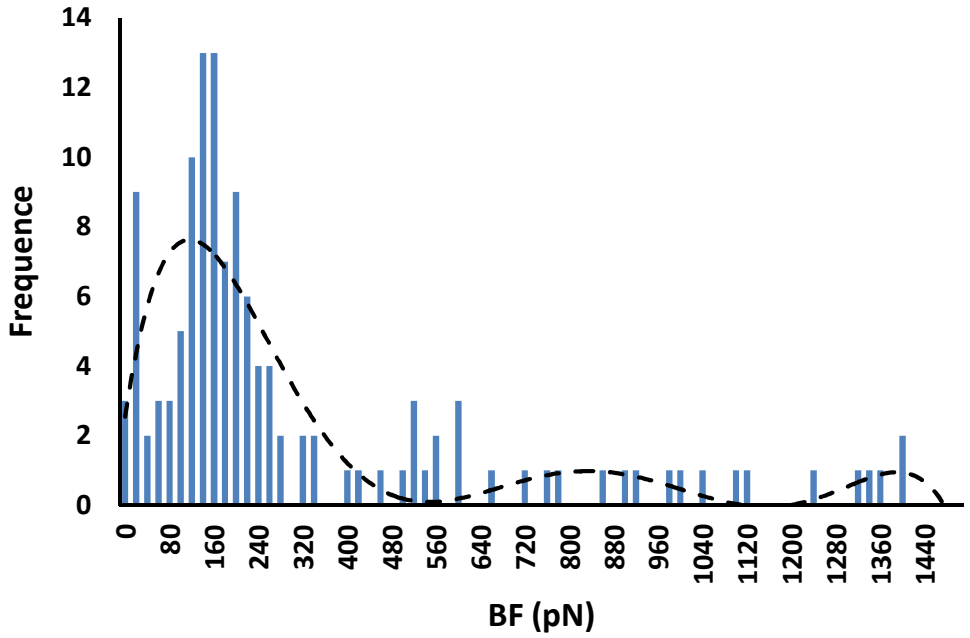


(C)



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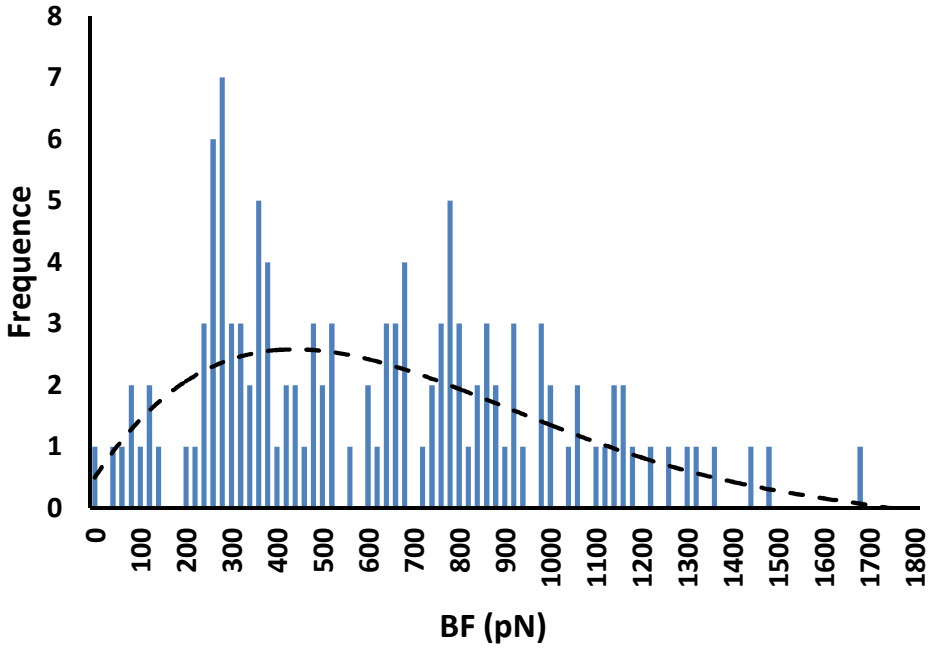
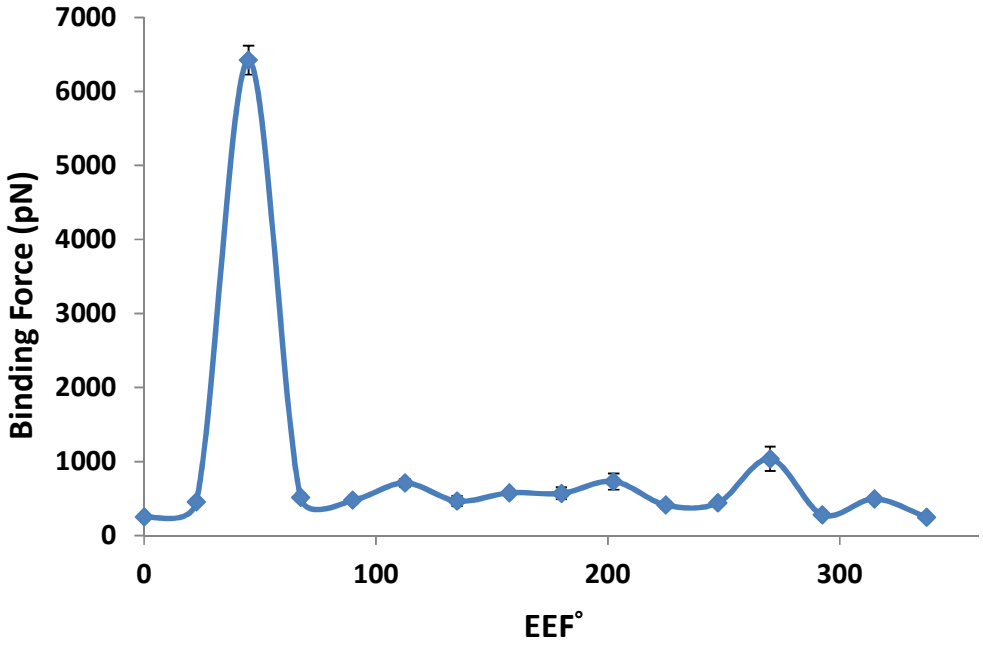


Fig. 5

(A)



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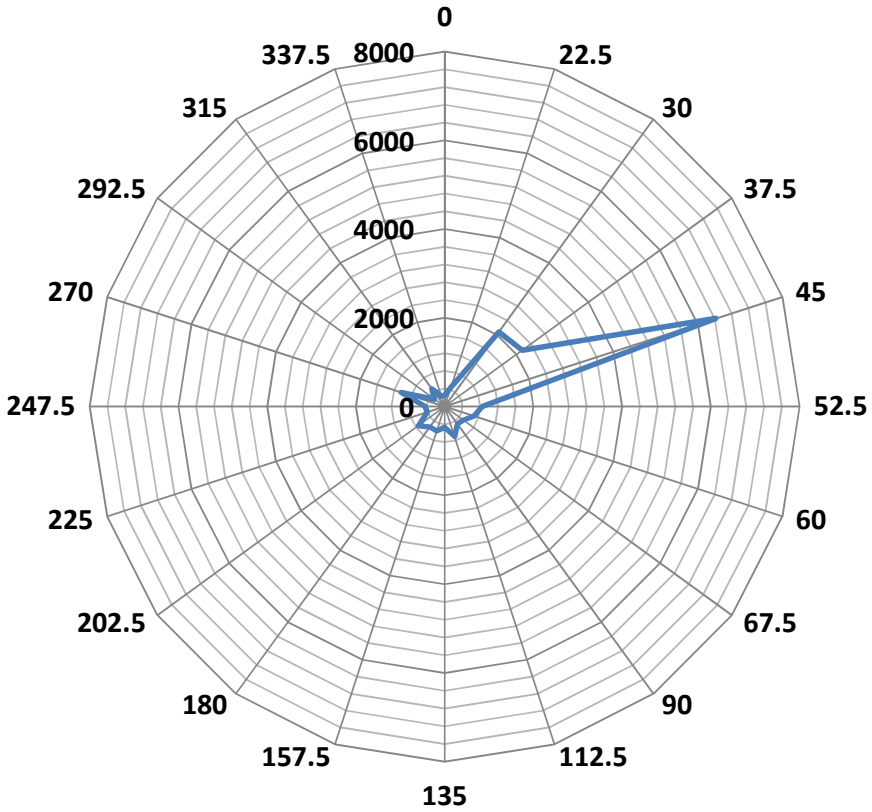
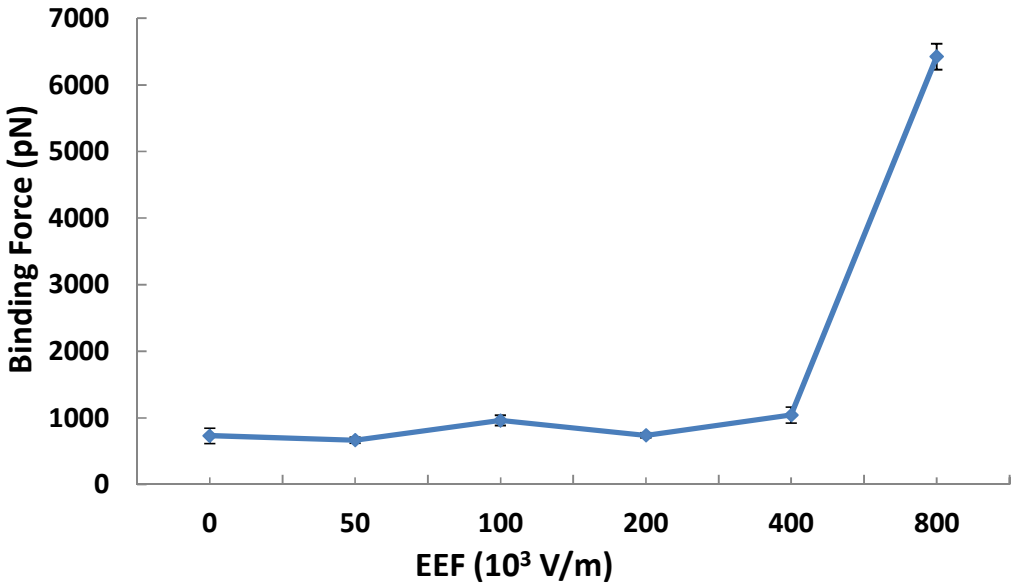


Fig. 6

(A)



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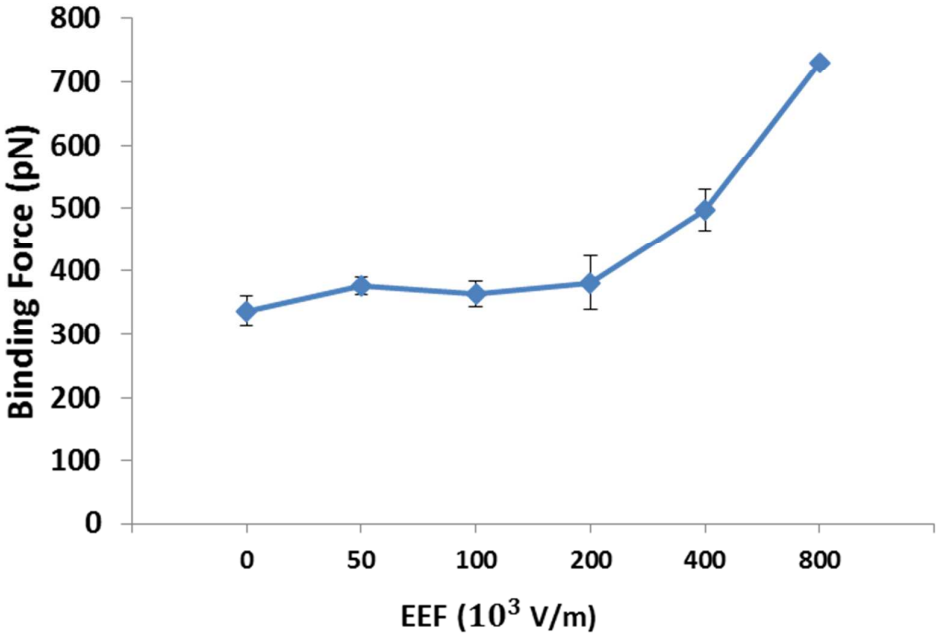
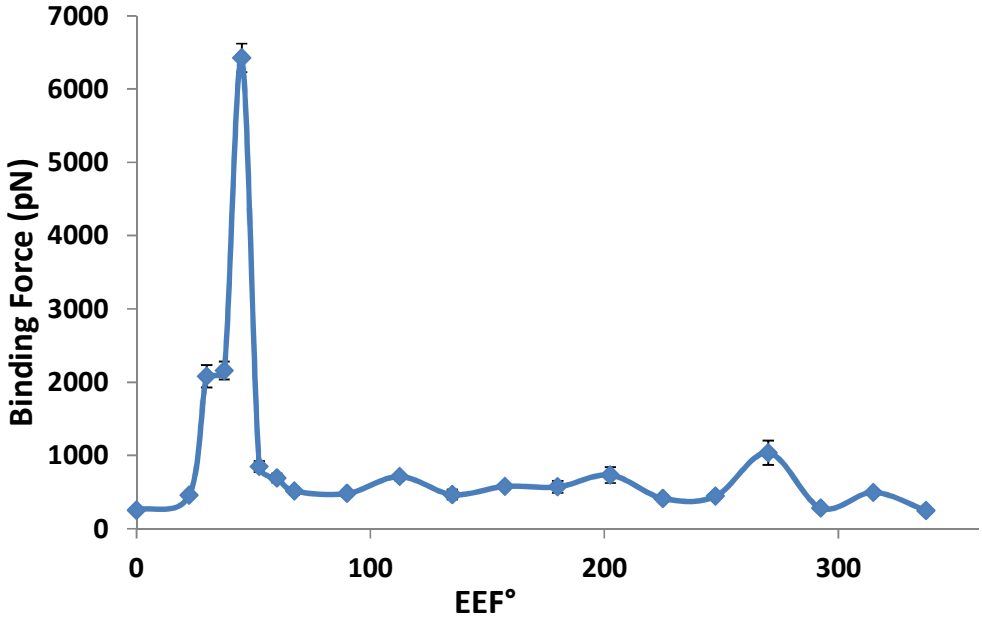


Fig. 7

(A)



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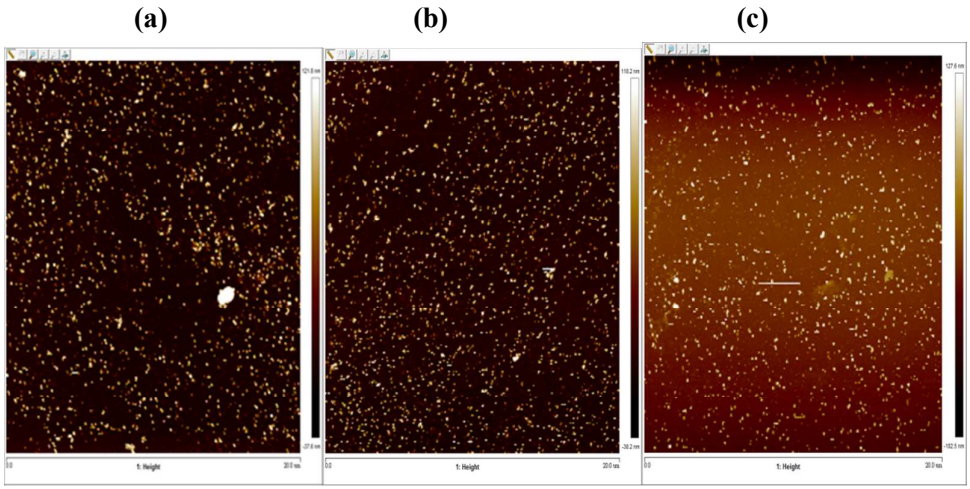
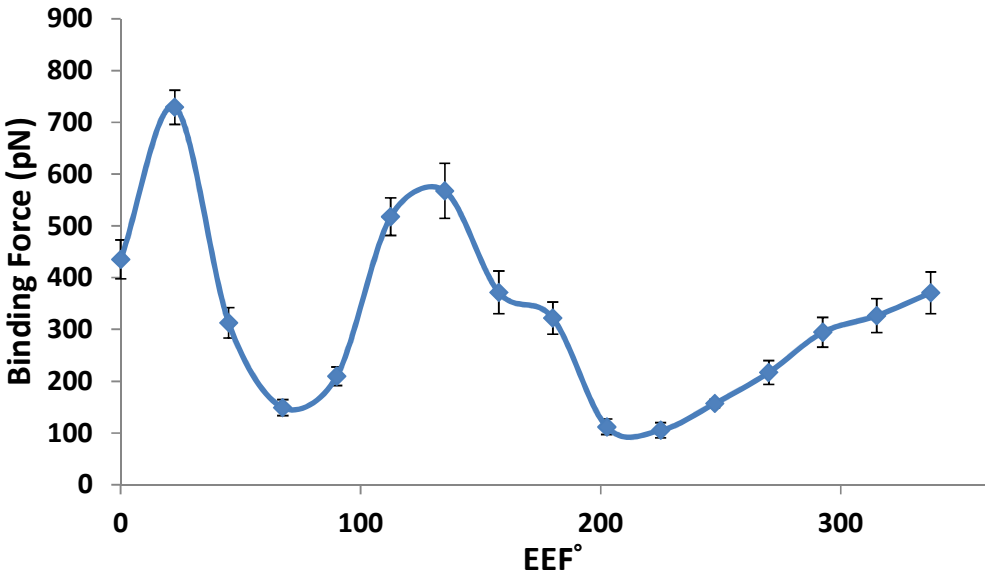


Fig. 8

(A)



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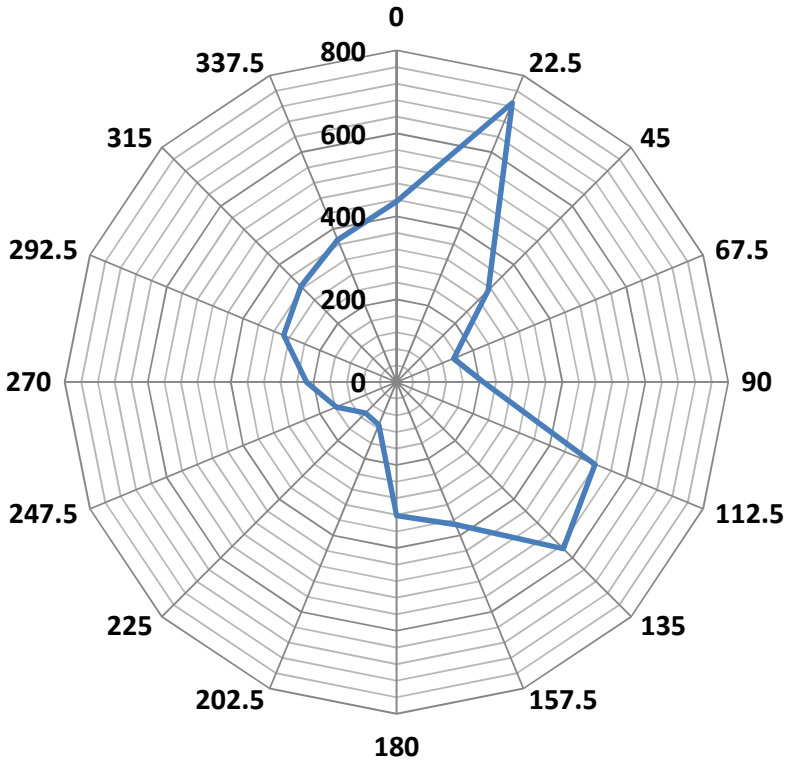
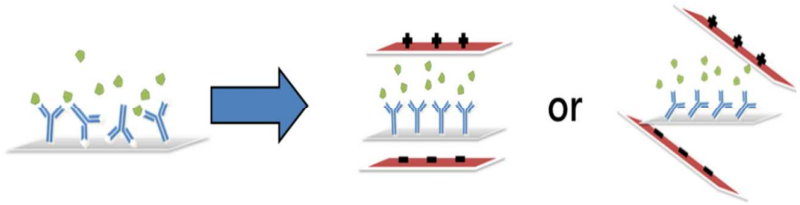


Fig. 9

(A)



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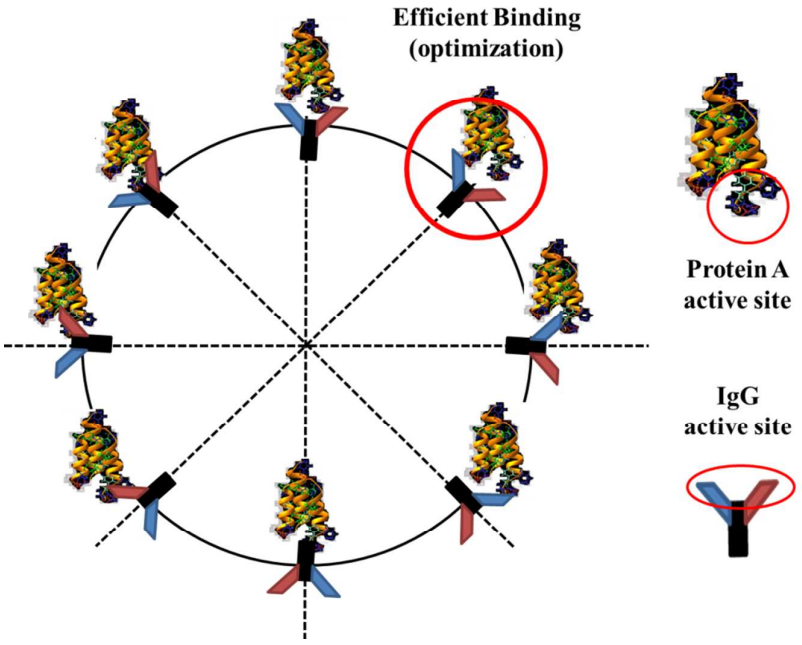

(C)