In situ observation of streptavidin-biotin binding on an immunoassay well surface using an atomic force microscope

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Abstract Polystyrene microtitre wells are commonly used as supports for the enzyme-linked immunosorbent assay (ELISA) method of biomolecular detection, which is employed in the routine diagnosis of a variety of medical conditions. We have used an atomic force microscope (AFM) to directly monitor specific molecular interactions between individual streptavidin and biotin molecules on such wells. This was achieved by functionalising an AFM probe with biotin and monitoring the adhesive forces between the probe and a streptavidin coated immunoassay well. The results demonstrate that the AFM may be employed as an analytical tool to study the interactions between biomolecules involved in immunoassay systems.

Key words: Atomic force microscopy; ELISA; Force-distance curve; Molecular interaction; Streptavidin-biotin

1. Introduction

The enzyme-linked immunosorbent assay (ELISA) method of biomolecular detection is an established technique used for the diagnosis of medical conditions [1]. Several types of polystyrene microtitre well plates are now available as solid-phase supports for antibody bound assay systems [2]. Typically, biological molecules are deposited onto immunoassay well surfaces by passive adsorption. However, it is well documented that proteins often undergo conformational changes when passively adsorbed onto a surface [3], possibly leading to a loss in functionality of the antibody on the microtitre well surface. Previously the imaging abilities of the atomic force microscope (AFM) have been used to study immunoassay well functionality [4]. The AFM can also be used as a tool to measure recognition processes such as those between antigen and antibody [5]. Here we present data in which a model receptor-ligand interaction has been measured on the surface of a commercial immunoassay well.

The ability of the AFM [6] to measure forces of 10 pN or less resulting from discrete intermolecular interactions has recently been highlighted [7]. By attaching complementary biomolecules to the AFM probe and the opposing surface this ability has been exploited to measure the forces required to separate specific biomolecular interactions [8–15]. The streptavidin-biotin complex as a model receptor-ligand interaction has generated much interest by virtue of its high specificity and affinity ($K_d = 10^{-15}$ mol l^{-1}) and general applicability as an immobilisation method [8,13,16,17]. The streptavidin-biotin system also has wide applications in many areas of biomedical science [18]. For example, the ability of streptavidin to bind four biotin molecules has been exploited to increase the sensitivity of immunoassays [19].

Streptavidin, a 66–75 kDa protein, consists of four identical subunits each containing a single biotin binding site [20]. The long and short range forces controlling this specific interaction have been well characterised [16,17] and this combined with the availability of structural [21] and thermodynamic [22,23] data has made it an ideal system to study receptor-ligand interactions. Lee et al. [8] used biotin fuctionalised glass beads attached to AFM cantilevers and streptavidin coated mica surfaces to estimate the strength of a single streptavidin-biotin bond. A similar approach was adopted by Gaub et al. [9,10,12,13], who measured quantised forces of interaction between a biotinylated agarose bead and an AFM probe functionalised with avidin, a 67 kDa protein closely related to streptavidin.

In the past we have used streptavidin coated polystyrene microtitre wells and biotinylated antibodies to improve the functionality of immunoassay wells, and showed that scanning probe microscopy (SPM) could be used as a tool to study this phenomenon [4]. The AFM has also been used to differentiate between classes of antibody deposited onto the microtitre well surface [24]. In this paper, we extend that work and show that the AFM can be used to directly monitor biomolecular interactions on these industrially significant immunoassay well surfaces. We use biotin functionalised probes and monitor their interaction with streptavidin molecules immobilised on the microtitre well surface.

2. Materials and methods

2.1. Sample preparation

Polystyrene microtitre wells were supplied by Johnson and Johnson Clinical Diagnostics Ltd. (Chalfont St Giles, Bucks.). Wells were coated with streptavidin or bovine serum albumin (BSA) as previously described [4]. Before SPM analysis the wells were thoroughly washed with high purity deionised water (resistivity 15 MΩ cm^{-1}) to remove any loosely bound biological material. The base of each well was then excised using a heated scalpel blade to provide access for AFM analysis. For the streptavidin-biotin binding experiments the BSA was biotinylated using standard procedures [25].

2.2. SPM analysis

Force-distance curves were recorded using a Topometrix Explorer AFM (Topometrix Corporation, Saffron Walden, Essex). All force experiments were carried out in a glass liquid cell, built and developed in our laboratory, which was thoroughly cleaned prior to use. Freshly prepared potassium phosphate buffer (100 mM, pH 7) filtered using a 0.2 μm filter (Sartorius A.G., Göttingen) was used for each experiment. Cantilevers (Park Scientific Instruments, Mountain View, CA)

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with silicon nitride, Si_3N_4, probes and spring constant (k)$\approx$0.05 Nm^{-1}, were used.

During a force measurement cycle (see schematic Fig. 1) the probe is moved towards the surface at constant velocity until it is brought into contact with the sample (B). As the forward motion continues the probe is pressed into the sample surface until a point of maximum load is reached (C). The direction of motion is then reversed and the probe is withdrawn from the sample surface. A plot of cantilever deflection against distance moved by the fixed end of the cantilever is obtained as raw data. During the retract portion of the force measurement, i.e. when the probe is withdrawn from the sample surface, the probe 'sticks' to the surface due to interactions between the probe and sample. The magnitude of this adhesive force is calculated from the difference between the maximum cantilever deflection (D) during the retract portion of the curve and the point of zero cantilever deflection (A). In these experiments the cantilever deflection signal, measured in nAmp, was converted to a deflection distance (nm) using the gradient of the retract trace in the contact region of the force curve (see Fig. 1) [8]. This distance, d, was converted to the force acting on the AFM probe (nN) using the cantilever spring constant (k), and Hooke's law ($F=-kd$). The spring constants used in these experiments are those quoted by the manufacturer and therefore all force values in this paper are subject to the errors associated with this constant [26].

2.3. AFM probe functionalisation

AFM probes were functionalised with biotin by overnight incubation at room temperature in a solution of biotinylated bovine serum albumin (BBSA) (600 μg/ml in 100 mM potassium phosphate buffer, pH 7). BSA has previously been shown to irreversibly bind to the

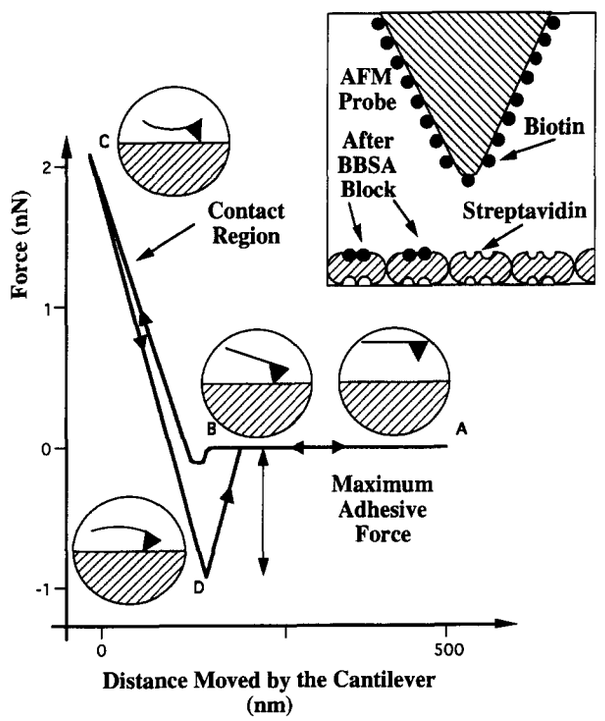

Fig. 1. (Main diagram) A schematic diagram of a typical force measurement cycle. The relative position of the AFM probe to the sample surface is indicated in the circles and the direction of motion is indicated by the arrowheads; (A) zero cantilever deflection, (B) the probe is brought into contact with the sample surface, (C) the point of maximum load, (D) the point of maximum adhesive force. The cantilever deflection then returns to its original equilibrium position as the tip-sample separation is increased. (Inset) A schematic diagram of the streptavidin coated well surface and the biotinylated probe. After blocking biotin binding sites (left corner) the biotinylated probe cannot specifically bind with streptavidin molecules on the well surface.

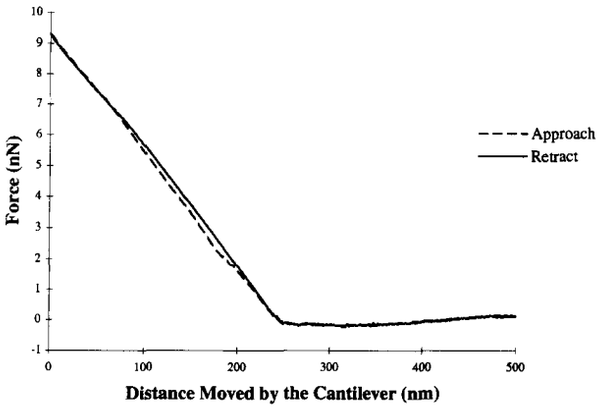

Fig. 2. A force measurement between a BSA coated AFM probe and BSA coated immunoassay well displaying no discrete adhesion points.

Si_3N_4 probe surface [9]. Before use, probes were rinsed in deionised water to remove loosely bound biological material.

2.4. Streptavidin-biotin binding experiments

AFM probes were derivatised using the method outlined in Section 2.3. Force measurements between the derivatised probes and streptavidin coated microtitre wells were obtained in freshly prepared potassium phosphate buffer (100 mM, pH 7). To confirm the presence of specific binding, biotin binding sites on the well surface were subsequently blocked by flooding the system with BBSA (600 μg/ml in 100 mM potassium phosphate buffer, pH 7). Potassium phosphate buffer was flowed through the AFM liquid cell after 1 h to remove excess BBSA. Force measurements were then obtained between the derivatised probe and the blocked streptavidin coated well. After these measurements, force curves were obtained between the same probe and a BSA coated well, as a second experimental control.

3. Results and discussion

To measure the small forces required to separate complementary biomolecular pairs it is first necessary to shield the non-specific interaction forces which may occur when surfaces interact. Lee et al. [8] achieved this by coating surfaces with BSA monolayers and established that such surfaces had no specific or non-specific interactions. Fig. 2 displays a force curve between a BSA coated AFM probe and BSA coated well in which adhesive forces are not observed. In these experiments we have employed BBSA molecules to shield the surface chemistry of the probe and also to effectively biotinylate the probe surface.

Fig. 1 displays a schematic diagram of the interaction between a biotin functionalised probe and a streptavidin coated well, including the expected appearance of the force curve. The maximal cantilever deflection during the retraction phase of the force measurement is related directly to the magnitude of the force required to break the biotin-streptavidin bond(s) formed on contact, as discussed in Section 2.2. Fig. 3A displays a typical force curve between a probe fuctionalised with BBSA and a streptavidin coated well. Repeated force curves yielded an adhesive force of 409 ± 166 pN. A force of 340 ± 120 pN has been reported corresponding to the rupture of a single streptavidin-biotin bond [8]. Force curves within this set of data display a sharp adhesion 'pull off' as can be seen in Fig. 3A. To confirm that streptavidin-biotin binding was causing the observed force discontinuity, the experimental system was flooded with BBSA to effectively block the biotin

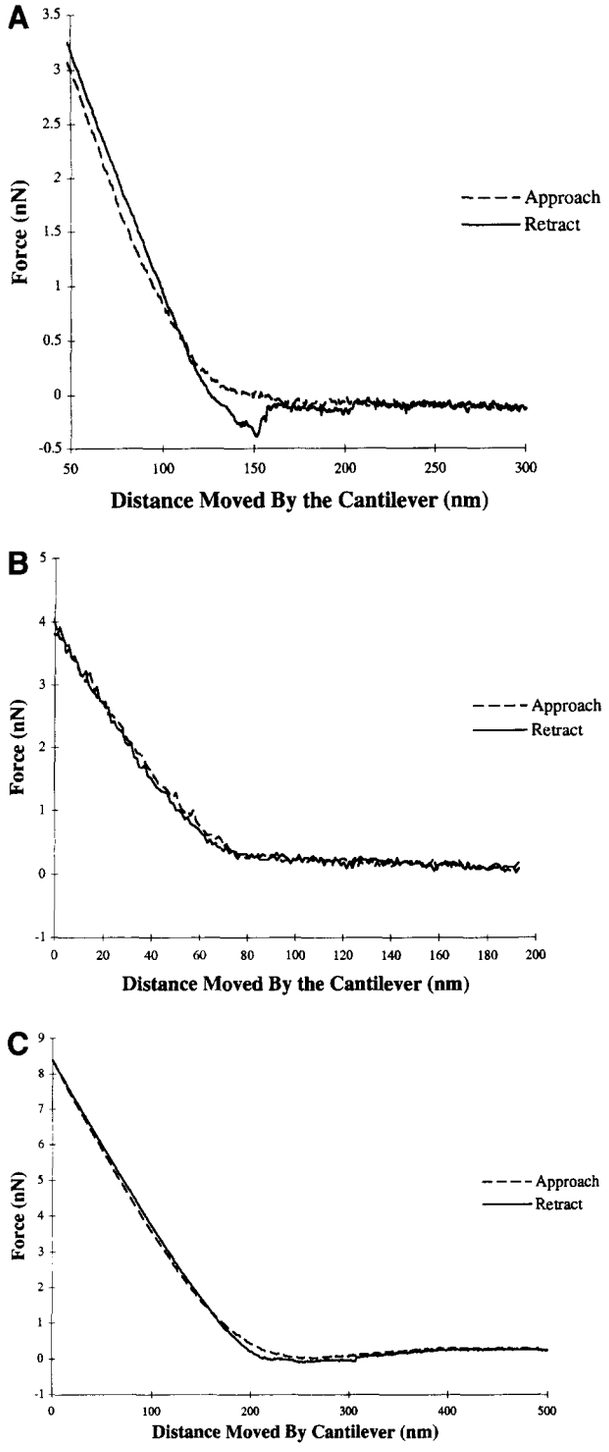

Fig. 3. (A) A force measurement between a biotinylated AFM probe and a streptavidin coated immunoassay well. A sharp adhesive 'pull off' of approx. 300 pN, typical of a specific tip-sample interaction, is observed in the retract trace. (B) A typical force measurement obtained between a streptavidin coated well and a biotinylated AFM probe after flooding the experimental system with BBSA. Adhesion points were not observed in such force curves. (C) A representative force measurement between the probe used to acquire the data in Fig. 2 and a BSA coated well surface. The curve is of similar appearance to those in B and in Fig. 2 with no adhesion points.

binding sites on the well surface. Force curves recorded after this point displayed no discrete adhesion points (see Fig. 3B).

The probe used to acquire the data in Fig. 3A was also used in further control experiments on BSA coated wells, after the above measurements had been obtained. Fig. 3C displays a representative force curve taken from these data. The appearance of this curve is similar to that of the curve in Fig. 2, and a sharp adhesion 'pull off' is not observed. The lack of discrete adhesion points in the force curves obtained in the control experiments highlights the specificity of the interaction between the biotinylated probe and the streptavidin coated well.

The force measurements between the biotinylated probe and the streptavidin surface were repeatable when measured at the same point on the sample surface, indicating that the BBSA molecules were not being pulled off the probe surface during the process. If the force measured was the force required to pull streptavidin molecules away from the well surface, rather than the streptavidin-biotin interaction, streptavidin molecules would have remained attached to the probe surface and no repeatability would have been observed. In addition, subsequent control experiments would have produced curves typical of streptavidin-biotin binding if this had been the case. Such a phenomenon was not observed indicating that the measured force was indeed the force required to separate the specific molecular interactions.

Previously similar work has centred around the measurement of biomolecular interactions on specialised flat substrates or agarose beads [8–10,12,13]. The immunoassay well surface represents a real sensor surface on which to perform measurements of biomolecular interaction, and we have shown that using fuctionalised probes the AFM can be used to measure these processes within a controlled aqueous environment on such a surface. Recent work within our group has also shown that this method can be extended to study interactions between antibodies and antigens (unpublished results). This technique could be potentially employed to measure interactions between the antibodies and antigens involved in the ELISA system to provide information regarding the functionality of the well surface. It could also be used to discriminate between antibodies with different binding affinities or to map the distribution of functional binding sites on the immunoassay surface. The AFM, therefore, has the potential to act as an analytical tool to determine the optimal binding conditions of proteins immobilised in surface assemblies employed in immunoassay systems.

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