DETECTION AND LOCALIZATION OF ANTIBODY-ANTIGEN INTERACTIONS WITH HIGH SPATIAL RESOLUTION ON COLLAGEN TENDONS.

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ABSTRACT

In theory atomic force microscopy allows the detection of a single antibody-antigen interaction with nanometer resolution. However the application of this technique has been limited to 'ideal' situations and not been applied as a general laboratory technique. Previous studies on more realistic samples have been hampered by non-specific interactions. We have modified the technique with different spacer molecules between tip and antibody in order to reduce the number of non-specific events seen. This technique has been applied to an identical sample used for immoflurencse and electron microcopy investigations and antibody – antigen interactions were readily detected on the sample with a resolution of 50-100 nm. The presence of specific interactions was confirmed by blocking using excess antibodies.

Keywords: *AFM, force measurement, antibody-antigen, imaging*

1. INTRODUCTION

The ability of antibody to bind specifically with a particular molecule (called an antigen) only and the ease of producing specific antibodies has led to interest in commercial employment of antibodies in ultra senstiveimmunoassesys and biosensors.[1] One method of employing antibodies is to use the atomic force microscope (AFM).

The AFM, invented in 1986 by Binning et al,[2] consists of a ultrasharp tip (typically 10-20nm diameter) connected to a lightly sprung cantilever. In order to obtain an image of the surface the tip is rastered across the surface and the deflection of the cantilever is measured. This allows imaging of a non-conductive surface under ambient or fluid conditions down to sub-nanometer resolution without the need for any additional surface preparation.[3]

The AFM can also be used as a force sensing instrument where the deflection of the AFM tip is measured as it moves towards and away from the surface, used in this way the interaction forces between the surface and the tip can be detected and measured.[4] This method has been used to measure the local mechanical properties of biological systems, including living cells[5], vesicles[6] and human platelets[7]. The attachment of specific particles has been used to measure the adhesive force of fungi spores[8] and attaching ligands (such as antibodies) to the AFM tip allows the measurement of ligand receptor forces. Successful measurement of which normally requires the presence of a flexible tether molecule between the tip and the ligand.[1;9;10] This allows the ligand to move relative to the tip and to orient itself with the receptors on the surface and by moving the ligand away from the tip leds to a characteristic saw tooted shaped force distance curve which is easy to distinguish from a non-specific adhesion which produces a straight line force curve.

This technique has been used extensively to detect and measure antibody-antigen interaction on ideal systems, for example indiviual antigens adsorbed onto mica.[9-19] Studies on samples similar to those used in other immulogical studies have been hampered by the presence of interactions due to the pulling of organic molecules.[20] Here we have investigated the ability of AFM to detect antibody –antigen interactions on thin sections of collagen prepared identically to those used in traditional immological studies and in particular the affect of changing the structure of the spacer molecule.

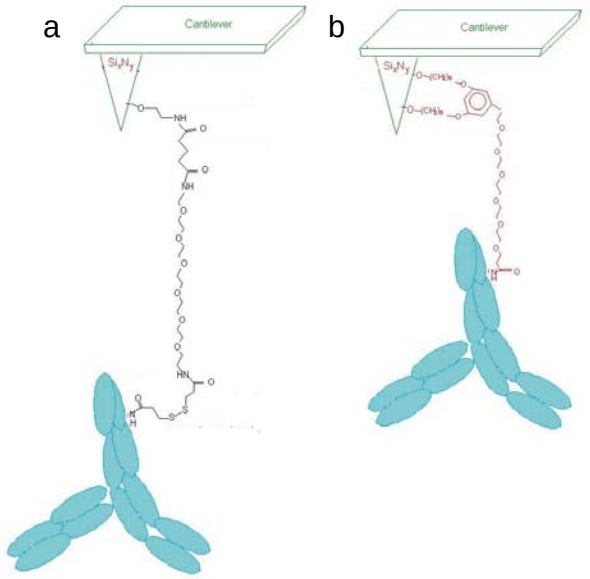

Figure 1: Showing the structure of the spacer molecules used. (a) single attachment and (b) double attachment.

2 EXPERIMENTAL

2.1 Preparation of collagen.

Tendons were dissected from fresh chicken legs and fixed in 2.5% paraformaldehyde prepared in 0.2 M Sodium Cacodylate buffer ($C_2H_6AsO_2Na$, Sigma) at 4°C for 24 hours. Individual collagen strands were teased apart, rinsed in a phosphate buffer solution (PBS) and dehydrated by immersion in aqueous ethanol solutions of 30%, 50%, 75% and 100%, then allowed to air dry. The dried strands were infiltrated with LR White Hard Grade embedding resin (London Resin Company Ltd, Berkshire England) at 4°C with 10 changes. To achieve total infiltration of resin, samples were placed under vacuum for 24 hours, then polymerized at 56 °C for an additional 24 hours. Sections (250 nm thick) were collected on gelatin-coated 8 mm circular glass disks.

2.2 Preparation of functionized tips.

Rabbit polyclonal antibodies, raised against extracted and purified Collagen type I from chicken skin, were obtained commercially (Chemicon International, Temecula, CA). These antibodies are specific for collagen I. A silicon nitride AFM tip was first functionalized with amine groups by treatment with ethylamine, as described by Gruber et al. Either a hetero-bifunctionalized PEG-400 molecule with amine and PDP reactive ends or a di-carboxylate PEG200 molcule (Sensopath, Bozeman, MT) was then attached to the AFM tip via amino groups or hydroxyl groups respectively. Finally, derivatized antibodies were attached to the PDP end of the PEG molecule.[4]

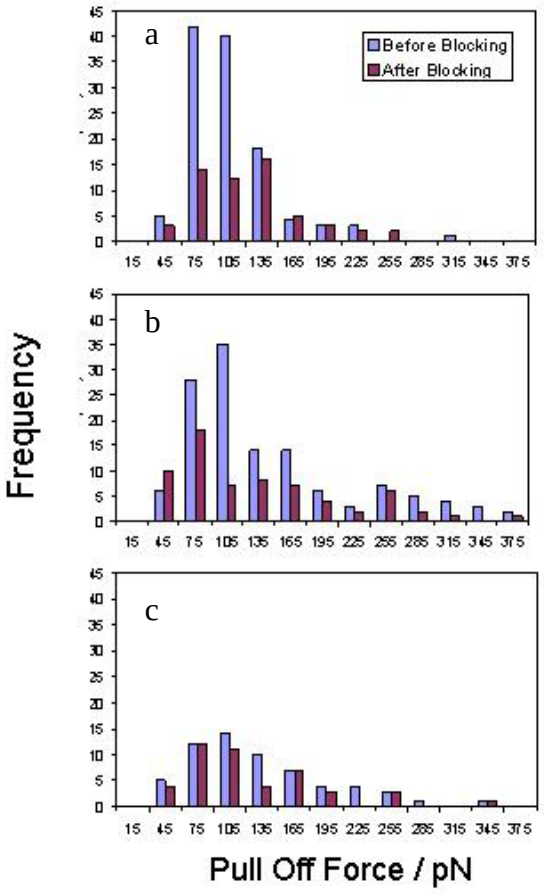

Figure 2. Distribution of unbinding events. Only those unbinding events that fell between 0 to 40 nm range are used in the counting statistics. Blue (light) band indicates forces measured with a functionalized tip on uninhibited sample, while red (dark) band correspond to interactions between tip and sample after antigenic sites on fibers have been blocked with excess antibodies. (a) double attachment, (b) single attachment and (c) control using unmodified tip.

2.3 AFM imaging and force measurements.

Force measures were taken in PBS solution and collected over a 4 x 4 μm^2 area with 32 x 32 resolution (1024 total force curves). Each force distance curve was obtained at 1Hz frequency, with a z-scan size of 200 nm and a relative deflection offset of 20 nm. After obtaining this data, 50 µl of 200 µg/ml anti-collagen primary antibodies were introduced into the AFM sample chamber and were incubated with the fibers for one hour. The excess antibodies in solution bound to the antigens and prevented the AFM-coupled antibody from interacting with the sample. Finally, after force-curve data were collected, the same functionalized tip was used to obtain a liquid tapping mode image to verify that data was collected from the correct area on the sample. as was identified at the beginning of the experiments

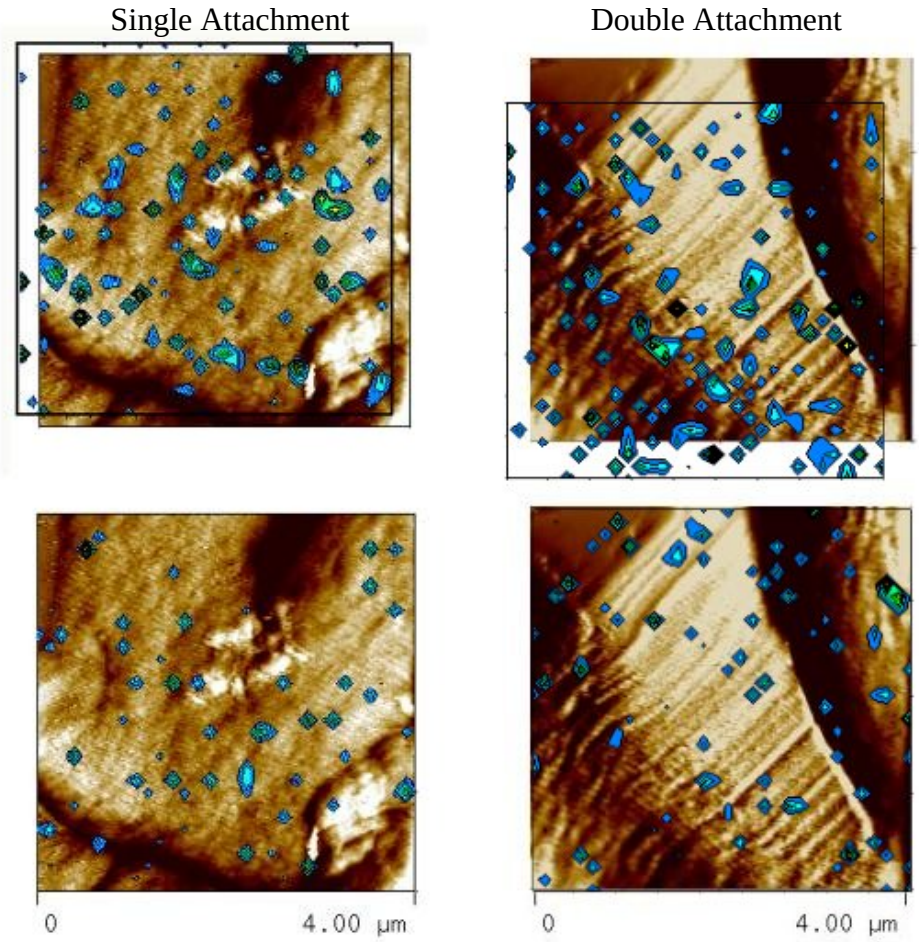

Figure 3. Liquid tapping mode AFM image of collagen fibers taken with a functionized tip after force measurements combined with the spatial distribution of unbinding events (a) before and (b) after blocking with excess antibodies. The data is offset to compensate for the drift of the piezo scanner with time. The drift was determined form the height images obtained with the force volume data.

3. RESULTS AND DISSCUSION

The majority of AFM force curves taken from the collagen tehdons using both types of modified probes show no or non-specific interactions. However a significant minority (approximately 200- 300 out of a total of 1024) do show evidence of specific interactions, some of which show a single interaction indicative of antibody-antigen interactions.[9]. This is complicated by the presence of curves caused by the pulling of the collagen fibrils which gives similar shaped force curves.[20]. Previous studies have shown ligand receptor unbinding forces are in the range 50-400 pN and with a pull off length of less than 40 nm [9]. Therefore only single event curves within these limits were chosen for analysis. In addition the analysis was repeated after the antibody-antigen interactions were blocked with excess antibodies, figure 2.

For both the single and doubly attached tether the effect of blocking is to reduce the number of events detected. In both cases this is particularly strong between 60-120 pN which aggress well with earlier analysis. The residue interactions are due to unblocked antibody-antigen interactions and short distance pulling of collagen fibrils. Data taken using unmodified probe showed no change upon blocking. The results from the doubly attached tethers, figure 2(a), show a comparably larger number of events before and a lower number after blocking compared to data taken using a singly attached tether. The action of the doubly attached tether is then two fold. The first is with the stronger bonding to the tip more antibodies are available for binding with antigens and by covering more of the tip less bare silicon nitride is available for collagen fibrils to bond to which happens just prior to collagen pulling.

An amplitude liquid tapping mode AFM image of collagen is shown in figure 3 taken with a functionized tip after the force measurements. The liquid tapping mode image shows the characteristic-banding pattern associated with collagen.[5] The spatial distribution of events before and after blocking is also shown in figure 3. Although events are seen across the majority of the sample the distribution is not uniform. In both cases a correlation between the distribution of events and the image is seen. In the case of the single attached tether the area to the bottom left hand side of the image shows relatively lower number of events, similarly for the upper central area for the doubly attached tether where the binding pattern is not apparent. This suggests either these areas are either not collagen or collagen fibers where the antigens have been damaged.

4. CONCLUSIONS

It has been shown that AFM can be used to detect antibody-antigen interactions on actual biological samples and be able to map the distribution of the interactions with tens of nanometer resolution. A doubly attached tether improves the technique by both increasing the number of events detected and reducing pulling of organic molecules which can complicate the analysis.

ACKNOWLEDGEMENTS

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