The influence of epitope availability on atomic-force microscope studies of antigen–antibody interactions

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The ability of the atomic-force microscope (AFM) to detect interaction forces between individual biological molecules has recently been demonstrated. In this study, force measurements have been obtained between AFM probes functionalized with the β -subunit of human chorionic gonadotrophin (β hCG) and surfaces functionalized with anti- β hCG antibody. A comparison of the obtained results with previous anti-ferritin antibody-binding data identifies differences when the antigen molecule expresses only a single epitope (β hCG), rather than multiple epitopes (ferritin), for the monoclonal antibodies employed. Specifically, the probability of observing probe–sample adhesion is found to be higher when the antigen expresses multiple epitopes.

However, the periodic force observed in the adhesive-force distribution, due to the rupture of single antigen–antibody interactions, is found to be larger and more clearly observed for the mono-epitopic system. Hence, these findings indicate the potential of the AFM to distinguish between multivalent and monovalent antibody–antigen interactions, and demonstrate the influence of the number of expressed epitopes upon such binding studies.

Key word: ferritin, force measurements, human chorionic gonadotrophin β -subunit, protein interaction, scanning-probe microscopy.

INTRODUCTION

The interaction between an antibody and its antigen is of fundamental importance to many techniques, including immunoassays [1], immunocytochemistry [2] and affinity chromatography [3]. The ability of the atomic-force microscope (AFM) [4] to measure unbinding forces between complementary biomolecular pairs, such as those between an antigen and an antibody, has been highlighted by various groups [5–8]. Previously, we have demonstrated that such an approach can be applied to the measurement of biomolecular interactions on immunoassay substrates [9], and also to monitor interactions between individual molecules employed in such systems [7]. Here we extend these studies and measure adhesive forces between AFM probes functionalized with the free β -subunit of human chorionic gonadotrophin (β hCG) and surfaces functionalized with anti- β hCG antibody.

Human chorionic gonadotrophin (hCG) belongs to the family of glycoprotein hormones, which also contains luteinizing hormone, follicle-stimulating hormone and thyrotrophin-stimulating hormone [10,11]. The hormones have related structures, each consisting of two subunits, namely an α -subunit and a β -subunit. The α -subunit (92 amino acids, 22 kDa) is virtually identical in each of the proteins, whereas the β -subunit is characteristic for the particular hormone. The β -subunit of hCG is a single chain of 145 amino acids (34 kDa). Whereas it is the β -subunit of the hormone that defines its functional characteristics, both subunits are required for biological activity. The structure and function of the related glycoprotein hormones have been reviewed [10]. To date, all pregnancy tests are based around the qualitative detection of hCG, in urine or serum, using

immunoassay techniques [11]. As it is the β -subunit of the hormone that defines its biological activity, most pregnancy tests utilize antibodies that are specific for the β -subunit alone, but which do not discriminate between the subunit and the intact molecule of hCG.

In previous experiments, we measured adhesive forces between human spleen ferritin and anti-ferritin antibody molecules employed in a clinical immunoassay [7]. Quantized forces of approx. 50 pN were observed in the adhesive-force distribution, which we interpreted as single unbinding events between individual antigen and antibody molecules. It was also noted that some force measurements displayed multiple adhesions in their retract traces, and we postulated that such force curves could arise from multiple interactions between the ferritin molecules on the probe and surface-immobilized antibodies. However, as ferritin is a protein that is comprised of 24 homologous subunits, it contains many repeats of the same binding site or epitope for the monoclonal antibody employed. Thus the surface-bound antibodies could bind in numerous ways with the ferritin molecules on the AFM probe. The further interpretation of the force data was therefore hindered by the complexity of the ferritin–anti-ferritin interaction.

The present studies employed the protein β hCG as an antigen that, in contrast to ferritin, expresses only a single epitope for the antibodies employed. A comparison of the results obtained with previous anti-ferritin–ferritin binding data allows an investigation of the influence of the number of expressed epitopes upon such binding studies. In addition, they demonstrate that, by choosing a relatively simple receptor–ligand system, the interpretation of obtained force data can be more readily facilitated. These findings indicate the potential of the AFM to distinguish

Abbreviations used: AFM, atomic-force microscope; hCG, human chorionic gonadotrophin; β hCG, β -subunit of hCG.

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between multivalent and monovalent antibody–antigen interactions, and allow a discussion of the relative advantages/disadvantages of each of the employed antibody–antigen systems.

MATERIALS AND METHODS

Sample and probe preparation

β hCG (Zymed Laboratories, San Francisco, CA, U.S.A.) was reconstituted and diluted to a concentration of 0.1 mg/ml using high-purity deionized water (resistivity 17 M Ω ·cm, purified on an ELGA-Maxima system, USF Elga, High Wycombe, Bucks, U.K.), and stored at -20°C prior to use. Using the immobilization protocol described previously [7], the surfaces of silicon nitride probes (Digital Instruments, Santa Barbara, CA, U.S.A.) were functionalized with β hCG (3 $\mu\text{g}/\text{ml}$) in potassium phosphate buffer (100 mM, pH 7). Mouse IgG1 anti- β hCG antibody (1 mg/ml; Ortho-Clinical Diagnostics, Chalfont St. Giles, Bucks, U.K.) was immobilized on to silicon substrates using the immobilization protocol described previously [7].

AFM analysis

Force measurements were recorded at a speed of 1 $\mu\text{m}/\text{s}$, using a Topometrix Explorer AFM (Topometrix Corporation, Saffron Walden, Essex, U.K.), in a glass liquid cell built and developed in our laboratory. Freshly prepared potassium phosphate buffer (100 mM, pH 7) was used in all experiments. V-shaped cantilevers (Digital Instruments) with silicon nitride probes and spring constants $k \cong 0.12$ N/m, were employed. To confirm specific probe–sample binding, antibody-functionalized surfaces were flooded with an excess of free β hCG (3 $\mu\text{g}/\text{ml}$), and force measurements were then re-recorded.

Raw data were obtained as cantilever deflection (nA) versus displacement of the z-piezo (nm). Cantilever deflection was first converted to a deflection distance (nm) using the gradient of the linear portion of the retract trace [12]. Using the cantilever spring constant (k), and Hooke's Law [$F = kd$, where F is force (pN) and d is cantilever-deflection distance (nm)], the cantilever deflection was converted to force (as described previously [7]). The spring constants of individual cantilevers were determined experimentally using the resonant frequency method of Cleveland et al. [13], and were found to be within the range of 0.043–0.14 N/m. The horizontal distance axis (nm) was converted from z-piezo displacement to probe–sample separation by subtracting the cantilever deflection distance (nm) from the z-piezo displacement for every data point in each force curve.

To confirm that experimental observations were due to an increase in epitope availability, and not to differences in the surface densities of immobilized antigen molecules on the AFM probe surface, planar silicon substrates were functionalized with β hCG or ferritin utilizing conditions identical to those employed for probe functionalization. The prepared surfaces were then imaged in air using a multimode AFM with Nanoscope IIIa Controller (Digital Instruments). Images were obtained in tapping mode using single-beam silicon cantilever probes (Digital Instruments), with resonant frequencies of 274–280 kHz and drive amplitude voltages of 42–140 mV. Topography and phase images are displayed as greyscale representations, i.e. with higher features lighter in colour in the topography images, and increased phase shifts lighter in colour in the corresponding phase images.

RESULTS AND DISCUSSION

To perform measurements between complementary biomolecular pairs, the probe and surface are functionalized with the biomole-

Figure 1 Typical force measurements recorded between a β hCG-functionalized AFM probe and an anti- β hCG-antibody-coated surface

(a) A typical force measurement obtained between a β hCG-functionalized AFM probe and a silicon substrate coated with the monoclonal antibody to that protein. (b) Adhesive forces were not observed in force measurements recorded after the addition of excess ligand, confirming the specificity of the probe–sample interaction.

Figure 2 The distribution of adhesive forces obtained between β hCG-functionalized AFM probes and silicon substrates coated with anti- β hCG antibody

The data are normalized to indicate the probability of observing an adhesive force of a particular magnitude. The forces appear to be quantized with a period of 132 ± 16 pN ($n = 132$). See reference [7] for further details of the data analysis.

cules of interest, and forces measured as they are brought into and out of contact [5–9,12,14–24]. The maximum cantilever deflection during the retract phase of the force measurement is related directly to the magnitude of the force required to break the receptor–ligand bond(s) formed on probe–sample contact.

In this study, adhesion-force measurements were obtained between β hCG-coated AFM probes and silicon substrates coated with anti- β hCG antibody. A typical force measurement displaying specific probe–sample adhesion is displayed in Figure 1(a). Sets of at least ten measurements could be repeated at one

Table 1 Summary of the number of adhesion points observed in the retract traces of force measurements

The measurements correspond to those recorded in this study, and previous ferritin–anti-ferritin-binding experiments. They are displayed as percentages of the total number of force measurements. The ferritin data include those already presented in [7].

Antibody–antigen system	No adhesion (%)	One adhesion point (%)	Two points of adhesion (%)	More than two adhesion points (%)
β hCG ($n = 185$)	29	41	18	12
Ferritin ($n = 201$)	9	40	15	36

Figure 3 AFM images of ferritin- and β hCG-coated silicon substrates

(a) A typical AFM image (size 500 nm $\times$ 500 nm; z-scale 20 nm) of the surface topography of a ferritin-coated silicon substrate. (b) The simultaneously recorded phase image of the same area (phase z-scale 100 $^{\circ}$). Globular features of 12–25 nm are observed. (c) Topography (size 500 nm $\times$ 500 nm; z-scale 10 nm) and (d) phase AFM images (phase z-scale 100 $^{\circ}$) of a silicon-substrate coated with β hCG. Globular features with diameters of 6–24 nm were observed.

particular point on the sample surface, illustrating that molecules were not removed from the probe or sample surface during serial force measurements. To confirm the specificity of the probe–sample interaction, the surface was flooded with an excess of free antigen. Force measurements recorded after this time displayed no probe–sample adhesion, demonstrating that the specific probe–sample interactions had been blocked (see Figure 1b).

The adhesive forces, recorded in these β hCG-binding experiments, ranged from 45 to 1500 pN. The distribution of the adhesive forces ($n = 132$ force measurements) was then analysed, as in previous studies [7], to determine the rupture force for the interaction between an individual antibody and antigen molecule (Figure 2). The data displayed in Figure 2 are normalized to

indicate the probability of observing an adhesive force with a particular magnitude. Within the distribution, the forces appear to be quantized with a periodicity of 132 ± 16 pN. During the acquisition of these data, the background noise level was approx. 30 pN. The small peak in the force distribution centred around this value is, therefore, most probably representative of non-specific binding and was not included in the estimation of the periodic force.

In our previous ferritin–anti-ferritin-binding studies, it was noted that many of the obtained force curves displayed multi-adhesion points in their retract traces. We proposed that such curves could arise from multiple interactions between the ferritin-coated AFM probe and surface-immobilized antibodies. The

immobilization strategy employed in these, and previous, experiments attaches proteins to surfaces by the ϵ -amino groups of lysine residues, and hence the antigen molecules are immobilized on to the probe surfaces in a variety of orientations. Due to the distribution of epitopes on the surface of the ferritin molecule, their availability for antibody binding is not highly dependent on the molecular conformation/orientation. In contrast, for proteins expressing only single epitopes for the antibodies employed, e.g. β hCG, the availability will be much more influenced by molecular conformation and orientation. It would be expected, therefore, that ferritin-coated probes would be able to form more antigen–antibody pairs than a corresponding β hCG-coated probe of the same size. In addition, if force curves displaying multi-adhesion points do indeed correspond to the sequential rupture of a number of antigen–antibody bonds, it would also be expected that ferritin–anti-ferritin force curves would be more likely to display multi-adhesion points than β hCG–anti- β hCG-binding curves.

Table 1 displays the number of adhesion points observed in the retract traces of force measurements recorded in this study, together with those recorded in previous ferritin-binding studies. It can be seen that for the ferritin experiments, from a total of 201 curves, over half of the force measurements displayed more than one adhesion minimum in their retract trace, with 36% of the measurements displaying more than two points of adhesion. It should be noted that these figures differ from those stated previously [7], resulting from a more thorough analysis of a larger data set. In contrast, for the β hCG studies, out of a total of 185 measurements only 30% of force curves displayed more than one adhesion point, with 12% of them displaying more than two. Thus it can be seen that multi-adhesion points were indeed observed more frequently in the ferritin-binding studies, and sharp single adhesion points were more commonly observed in the β hCG experiments. In addition, only 9% of the ferritin–anti-ferritin force curves displayed no adhesion, in comparison with 29% of the measurements obtained in the β hCG studies. This indicates that the probability of observing probe–sample adhesion was also greater in the ferritin experiments, which is again as would be expected if the availability of the epitopes were higher for the ferritin-coated probes.

Such observations may also be explained by differences in the densities of antigen molecules on the probe surfaces in the ferritin and β hCG experiments. To eliminate this possibility from our studies, planar silicon substrates were functionalized with each antigen, using conditions identical to those employed for probe functionalization. Figure 3 displays typical topographic and phase AFM images of substrates coated with either ferritin or β hCG. Phase images (e.g. Figures 3b and 3d) are simultaneously acquired with topographic images (e.g. Figures 3a and 3c), and can contain additional information about the probe–sample interactions during imaging [25]. Here phase images are included as they display enhanced surface detail.

From Figure 3 it can be seen that the silicon substrates are covered with a layer of globular features. From X-ray crystallographic structures, the molecular diameters for ferritin and β hCG can be estimated to be 12 nm ([26]; PDB code 2FHA) and 4–6 nm ([27]; PDB code 1HCN) respectively. The globular features observed in Figure 3 fall within the size range expected for individual molecules and small molecular aggregates. If the above molecular dimensions are considered, it can also be said that a greater number of the smaller antigen molecules (e.g. β hCG) are required to achieve complete coverage of a given surface area, i.e. the surface density of antigen molecules should be greater for β hCG than for ferritin, due to its smaller molecular size. If the density of immobilized antigen molecules was an

Figure 4 Schematic representation of possible unbinding scenarios for the ferritin and β hCG experimental systems

(a) Due to the distribution of epitopes on the surface of ferritin, their availability for epitope binding is not highly dependent on molecular conformation/orientation. Thus many unbinding scenarios exist for the ferritin–anti-ferritin interaction. (b) Epitope availability will be much more influenced by molecular conformation/orientation for proteins expressing only single epitopes, e.g. β hCG. It is therefore unlikely that an antibody molecule would be able to bind to epitopes on neighbouring molecules, and hence fewer unbinding scenarios exist. Bound portions of antibodies are coloured black.

important factor in this study, an increased probability of binding would be observed for the probes with the higher antigen density, i.e. those coated with β hCG. As this is not the case, our experimental observations can only be attributed to an increase in the number of available epitopes on the ferritin-coated probes.

The small number (approx. 20) of molecular aggregates of 10–12 nm in diameter in Figures 3(c) and 3(d) may correspond to dimers or trimers of β hCG molecules. If such protein complexes were to occur on the probe surface the epitope availability may be further reduced, particularly if the epitope was situated at the complex interface. In addition, were protein complexation significant on the probe surface, the repeatability of force measurements may also be impaired. For example, on retraction of the probe from the antibody-coated substrate the β hCG complex could disrupt, rather than the antigen–antibody bond, leaving β hCG bound to the surface-attached antibody. The number of sites available for antigen binding in subsequent measurements would then be reduced. However, most of the image areas in Figures 3(c) and 3(d) are composed of smaller features with dimensions similar to single molecules, indicating that the majority of molecules are immobilized in their monomeric form. This observation, coupled with the high repeatability of the force measurements, indicates that for these experiments the dimerization or trimerization of molecules on the surface of the AFM probe does not play a significant role.

As discussed in our previous publication [7], many different unbinding scenarios exist for the interaction of ferritin-coated AFM probes with surfaces functionalized with anti-ferritin antibody (Figure 4a). Many of these are due to the high number of available epitopes on the probe surface. In contrast, the

Figure 5 The distribution of adhesive forces obtained with ferritin-functionalized AFM probes and silicon surfaces coated with anti-ferritin antibody

The data are normalized as in Figure 2 [7].

interaction of AFM probes coated with a single-epitope antigen and antibody-functionalized surfaces is less complicated (Figure 4b), i.e. due to the random orientation of the β hCG molecule, it is unlikely that an antibody molecule would be able to bind to epitopes on neighbouring molecules. Due to the small size of the β hCG molecule, a similar unbinding scenario to that depicted in Figure 4(a) (part iii) may also be possible, in which a single antibody would bind two molecules of antigen. However, again it is unlikely that this scenario would occur in reality due to the random orientation of the β hCG.

If the distribution of the obtained adhesive forces (Figure 2) is compared with that obtained in the previous anti-ferritin–ferritin binding experiments (Figure 5), it can be seen that the peaks of quantization are sharper or more obvious for the experiments in which β hCG is employed. It is possible that each of the unbinding scenarios, envisioned for the ferritin–anti-ferritin interaction, would produce different unbinding pathways and therefore a range of adhesive forces. The distributions of the adhesive forces (Figure 5) could, therefore, represent a convolution of the adhesive forces obtained for the various antigen–antibody–unbinding pathways. In comparison, due to the decreased availability of epitopes on the β hCG-coated probes, the antibodies would typically bind monovalently with antigen molecules on the probe surface. The force distribution (Figure 2) would arise only from various multiples of the individual antigen–antibody interaction. It would be expected, therefore, for the quantization of the unbinding force for such an interaction to be more pronounced than that recorded for the multi-epitopic antigen, e.g. there would be no or little convolution of data.

Also apparent, on comparison of the β hCG data with previous ferritin data, is that the periodic force is much larger for the β hCG experiments, i.e. 132 ± 16 pN compared with 49 ± 10 pN. Adhesion forces ranging from 60 to 244 pN [5–8] have been reported for similar experiments employing different antigen–antibody systems. In addition, it has been estimated that antigen–antibody complexes with affinity constants ranging from 10^2 to 10^{10} M $^{-1}$ require rupture forces of 35–135 pN [5]. The periodic force observed in these β hCG studies, and the previous ferritin experiments, fall within these ranges and thus may be attributed to the rupture forces of individual antigen–antibody molecules. In addition, the periodic force observed for the β hCG–antibody interaction lies towards the top of this range, suggesting that this antigen–antibody interaction is of higher affinity than the previous ferritin–anti-ferritin system.

The presented data therefore demonstrate the applicability of a previously employed approach [7] to a second antigen–antibody system which, like the ferritin–anti-ferritin system, is also employed in a clinical immunoassay. Many of the difficulties that arose during the interpretation of previous ferritin–anti-ferritin binding data were due to the presence of many epitopes on the ferritin-molecule surface. A comparison of the results obtained with those of previous ferritin–anti-ferritin-binding experiments revealed differences between the sets of data. Specifically, we found that multi-adhesion points were observed more commonly in the ferritin experiments than the β hCG studies, and also that the probability of observing probe–sample adhesion was greater in the ferritin studies. We suggest that this was due to the increased availability of epitopes on the probe surface in comparison with those coated with β hCG. In addition, a comparison of the adhesive-force distributions from each antigen–antibody system revealed that the quantized peaks were much sharper in the β hCG experiments, most likely due to a reduction in the number of possible unbinding scenarios.

These findings demonstrate the effect of the number of available epitopes on AFM studies of antigen–antibody interactions and indicate the potential of the AFM to distinguish between multivalent and monovalent antibody–antigen interactions. They also illustrate that when selecting a receptor–ligand system for initial AFM-binding studies both the valency and the number of available binding sites should be considered. For example, in early binding experiments it may be desirable to increase the probability of binding. Thus a multi-epitopic antigen that interacted with an antibody multivalently would be of choice.

However, to facilitate the interpretation of data and progress in the understanding of these recognition processes, there is a need to simplify the systems under study. Recent advances in probe-functionalization methods have enabled an improved control of the numbers of interacting molecules [6,8,24]. The use of such approaches together with monovalent antibody–antigen systems should in the future allow an improved understanding of the detailed nature of the force measurements and the associated force distributions that complement the already existing data for such interactions.

The authors are grateful for the funding of this programme via a BBSRC LINK Scheme, with Ortho-Clinical Diagnostics, Chalfont St. Giles, Bucks, U.K. and Digital Instruments, Santa Barbara, CA, U.S.A.

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Received 20 November 1998/25 March 1999; accepted 21 April 1999