Atomic force microscopy study of immunosensor surface to scale down the size of ELISA-type sensors

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Abstract

Here we describe the use of atomic force microscopy (AFM) to study the nanoscale mechanics of the molecular layers of a popular immunosensor, ELISA (enzyme-linked immunosorbent assay) type. We characterize the sensor surface in terms of brush length and grafting density of the molecular layers. The obtained data demonstrated that a reliable reading of the immunosignal (a suggested dimensionless combination of brush length and grafting density) can be attained from an area as small as $\sim 3 \mu m^2$. This is approximately 4 million times smaller compared to typical ELISA sensors. The immunosensor described is composed of a molecular mix of two different antigens. Intriguingly, we find that AFM can reliably distinguish between having the immunosignal from either antibody and from both antibodies together. This was impossible to get by using standard optical detection methods.

(Some figures in this article are in colour only in the electronic version)

1. Introduction

Immunosensors, a broad class of biosensors based on the antigen–antibody specific affinity [1–6], have proven their effectiveness in a broad variety of applications, ranging from the detection of explosives to the identification of different diseases. One of the most popular immunosensors used for medical diagnostics and research, ELISA (enzyme-linked immunosorbent assay) is used in every hospital. Since being invented in 1971, the approach to ELISA design has being through various further developments. An ELISA plate has typically 96 wells allowing for the detection of 96 immunosignals simultaneously. Each well is a separate sensor 6 mm in diameter. Modern technology, however, makes it feasible to read signals from an area of submicron sizes (for example, when using a fluorescent-based immunosensor), while keeping a reliable signal/noise ratio. Thus, there is a clear potential for improvement. Miniaturization of such sensors is typically justified by the increase in the speed of immunosignal processing, as well as the decrease in cost and improved durability of the sensors. The increase in sensitivity and speed is of obvious great interest [1–9] for both medical and non-medical applications. There have been several attempts to make micro-immunosensors [10–20] which would have the above advantages. Most of the detection schemes used are based on electrochemical detection [1, 16, 18], mass detection by means of a quartz microbalance [1, 21–23], surface plasmon resonance (SPR) spectroscopy [24, 25] and fluorescence detection [26–28].

It should be noted that there is a popular sensor design called micro-ELISA, which typically involves the use of polystyrene beads of several tens of microns in diameter [26, 27, 29] instead of the flat bottom of ‘macro’ ELISA wells. Micro-ELISA is an order-of-magnitude faster in processing immunosignals than the regular ELISA method and requires smaller amounts of analyte. However, it is typically not as quantitative as the macro-ELISA method. Additionally, the sensing surface of ‘micro-ELISA’ is not necessarily small.
Despite the described developments, the in situ study and control of the molecular assembly of each molecular layer of the sensors at the nanoscale is only beginning [28, 30–32]. Atomic force microscopy (AFM) has proven its ability to provide information about the nanomechanics of molecular layers [31, 33, 34] with essentially molecular resolution [31, 33, 35–45]. By studying AFM force dependences, it is possible to extract information about grafting density (or rigidity) and thickness of the molecular layers attached to a sensor surface [32, 33, 46–48]. The sensitivity of the AFM method is so high that it is possible, for example, to detect the change of the force curves, and consequently requires neither primary one). In contrast to such detection, AFM records the change, lateral resolution is restricted by the radius of curvature of the AFM probe [49], which can be as small as several nanometers.

In this work, we demonstrate the use of AFM to study the nanomechanics of molecular surfaces used in ELISA sensors, which are relatively flat polystyrene surfaces functionalized with two different antigens. In a typical ELISA sensor the signal is collected optically by using a special dye which changes its absorbance as a result of the activity of an enzyme attached to the secondary antibody (complementary to the primary one). In contrast to such detection, AFM records the change of the force curves, and consequently requires neither the enzyme markers nor secondary antibodies. Furthermore, AFM can collect the force data from an area as small as the AFM probe contact, which can be of the order of several square nanometers. Here we use the AFM technique to evaluate the minimum area from which one can obtain a reliable immunosignal.

2. Materials and methods

2.1. Chemicals and reagents

Anti-nitrotyrosine IgG from rabbit (anti-NT), 2,4-dinitrophenyl-modified human serum albumin (DNP–HSA; ∼35 DNP species per protein), bovine serum albumin (BSA), 3,3′,5,5′-tetramethylbenzidine (TMB), 3-nitro-L-tyrosine ethyl ester piperazineethanesulfonic acid (HEPES) were purchased from Sigma-Aldrich. Antidinitrophenyl IgG polyclonal (anti-DNP), 3',5'-dinitro-L-tyrosine ethyl ester hydrochloride, N-(3-dimethylaminopropyl)-N'-ethycarbodiimide hydrochloride (EDC) and (4-(2-hydroxyethyl)-1)-piperazinethanesulfonic acid (HEPES) were purchased from Sigma-Aldrich. Antidinitrophenyl IgG polyclonal (anti-DNP) from goat was purchased from Oxford Biomedical. Mouse origin IgG against goat immunoglobulin and IgG specific against rabbit IgG, both labeled with HRP (horseradish peroxidase) (antigoat-IgG-HRP and antirabbit-IgG-HRP, respectively), were purchased from Jackson Immuno. All commercial chemicals were used as supplied without further purification. The nitrotyrosine–BSA conjugate (NT–BSA; ∼60 NT species per protein) was prepared via standard carbodiimide coupling [40]. Ultrapure water (18 MΩ cm) from a NANOpure Diamond (Barnstead) source was used in all of the experiments.

2.2. Preparation of immunosensing surfaces

Standard polystyrene ELISA microtiter plates (VWR) were used as the substrate of the immunosensing surface. The bottoms of the 6 mm wells were removed and cleaned by ultrasonication in water for 15 min. The water was replaced and the sonication was repeated two more times.

The antigen conjugate mixtures (DNP–HSA and NT–BSA), in a protein ratio of 1:1 (10 μg ml⁻¹ each conjugate), were prepared in 50 mM carbonate buffer, pH 9.6, deposited on the substrate (100 μl per well) and incubated overnight at 4 ± 2 °C to adsorb on the surface of the well. The excess antigen was removed by washing each substrate four times with 300 μl of phosphate-buffered saline (PBS) with Tween-20 (PBST) (137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄ and 1.76 mM KH₂PO₄, pH = 7.4 with 0.05% (v/v) Tween-20). The blocking step to prevent nonspecific adsorption of antibodies was accomplished via the addition of 300 μl of blocking solution (10 mg ml⁻¹ BSA dissolved in PBS) to each antigen-coated surface. The excess BSA was removed after 1 h of incubation at 25 ± 2 °C by washing the surface four times with PBST, yielding the antigen-functionalized surface. The primary antibodies, anti-DNP, 2.0 μg ml⁻¹, and anti-NT, 0.1 μg ml⁻¹, were applied to the antigen-functionalized surface. For further protection against the nonspecific binding of the antibodies to the well surface, 1 mg ml⁻¹ BSA in PBST was added to the antibody solutions. The antibody solutions, 100 μl, were applied in all four combinations to the antigen-functionalized surfaces: (a) no antibodies; (b) only anti-NT; (c) only anti-DNP and (d) both anti-DNP and anti-NT. These solutions were reacted with the modified surface for 1 h at 25 ± 2 °C. Afterwards, the sensing surfaces were washed again with the PBST solution.

2.3. Optical detection of immunosignals

As a benchmark for immunosignals, a standard enzyme-color-generated signal was used. After reacting the antigen-functionalized surfaces with the antibody combinations as described above, the surface was treated with a mixture of secondary antibodies, antigoat-IgG-HRP and antirabbit-IgG-HRP (0.05 μg ml⁻¹ each antibody), with 1 mg ml⁻¹ BSA in PBST buffer (100 μl) for 1 h at 25 ± 2 °C. After incubation, the plate was washed four times with the PBST solution to remove the excess secondary antibody. The generated surface was used to analyze optical output signals in the presence of TMB, 416 μM, in 50 mM citrate buffer, 1 mM H₂O₂, 300 μl, pH 5.0. Absorbance of the biocatalytically oxidized TMB was measured at 655 nm after reacting for 1 min using a BIO RAD Model 680 ELISA microplate reader.

2.4. Atomic force microscopy

A Nanoscope IIIa MultiMode (Digital Instruments/Veco, Inc., Santa Barbara, CA) atomic force microscopy (AFM) was used in the present study. Standard silicon nitride integrated pyramidal tips (Veco/DI probes) fixed on a triangular cantilever were used. The spring constant k ∼ 0.14 N m⁻¹, which was measured by using the resonance method (a built-in option in the AFM software). The sensitivity of the AFM photodetector was determined assuming no deformations of the tip and sample in the compliance (contact) region of the force curve. The AFM software used was version 5.12,
release 4. A JV Veeco scanner was used. A standard cantilever holder for operation in liquids was employed. All force measurements were performed in PBS buffer at ambient temperature (∼22°C).

To find the radius of curvature of the apex of the AFM probe, the probe was imaged by using an inverse grid (TG01, by NT-MDT/MicroMash Inc.) which comprised of vertical sharp silicon tips. This technique produces an inverted image of the AFM probe. It should be noted that this method is less invasive compared to electron microscopy, which can alter the surface by the electron beam and may require some conductive coating. The probes utilized in this study have curvature radii in the range of 50–60 nm.

To collect sufficient statistics, the force–volume mode was utilized. The force curves were collected over at least five areas of 1 μm² each. Averaging of collected force data was done with the help of MMAnalysis v. 2.01 software (by Dr Gorbunov, Vladimir Tsukruk’s Laboratory, Iowa State University). The AFM probe moved up and down during the force collection with a frequency of 2 Hz.

3. Results and discussion

We analyzed four different combinations of antibodies in their specific affinity to the antigen-functionalized surfaces: (a) no antibodies; (b) only anti-NT; (c) only anti-DNP and (d) both anti-DNP and anti-NT. Figure 1 shows the expected molecular layers obtained in these cases.

The prepared sensing surfaces of figure 1 were first studied with the help of the standard ELISA optical detection method. The enzyme-labeled secondary antibodies were specifically bound to the primary ones. The presence of the enzymes was detected by the change of color due to biocatalytically oxidized TMB as described in section 2. Representative force curves are shown in figure 3. Each force curve represents each 1 μm² region.

Atomic force measurements of the molecular layers shown in figure 1 were done with the help of the force–volume mode on several randomly chosen surface regions of 1 × 1 μm² each. To find the radius of curvature of the AFM probe, we used [50]:

\[
F_{\text{brush}} \approx 50kT R^{3/2} N \exp \left(-\frac{2\pi h}{L}\right) L.
\]

Here \( L \) is the equilibrium thickness of the brush, \( N \) is an effective surface density of the brush molecules (grafting density), \( R \) is the radius of curvature of the AFM probe and \( T \) is the temperature. Equation (1) is a valid description of a brush for 0.2 < \( h/L \) < 0.8. Thus, analyzing these force curves, one can get the information about the grafting density (or rigidity) and thickness of the molecular layers.

Being plotted in a logarithmic scale, the force curves of figure 3 demonstrate straight regions, which can be described by equation (1) within the range 0.2 < \( h/L \) < 0.8. It should be noted that equation (1) is written for the approximation of a spherical probe. In reality, the AFM probe used here was pyramidal. If the probe is non-spherical, the Derjaguin approximation used for derivation of equation (1) should be replaced with the approach described in detail in [45, 51–53]. Nevertheless, the apex of the probe used in this work can be approximated by a sphere with a radius of curvature ∼50–60 nm, which was defined by imaging the inverse grid.
Figure 3. Representative force curves collected over the surfaces corresponding to the cases shown in figure 1: (a) no antibodies; (b) only anti-NT; (c) only anti-DNP and (d) both anti-DNP and anti-NT antibodies attached. Each force curve represents an average of approximately 200 force curves collected in each of the $1\,\mu m^2$ regions.

In principle, exponential force dependences, similar to equation (1), can also originate in the presence of surface charges, i.e. electrical double-layer forces. Such forces are typically trivial to identify because their range (the Debye length) is unambiguously defined by the ionic strength of the buffer [31, 36, 40–42, 50, 55], which is known. In the case of the buffer used, the range of this force is within a nanometer [55].

Fitting the collected force data with equation (1), one can get the distribution of parameters $N$ and $L$. The results are presented in table 1. Comparing these data with the expected molecular arrangements shown in figure 1, one can conclude that presumably several layers (rather than one) of BSA and HSA functionalized with antigens are physisorbed on the polystyrene substrate for case (a). The derived size of the attached primary antibodies in cases (b) and (c) are slightly smaller than expected (10–12 nm) [31]. It is clear that we are dealing with just a single layer of probably tilted antibodies. It is intriguing to note that case (d) is substantially different from the expected schematic shown in figure 1(d). The nature of this phenomenon is not unambiguously clear, and will be the subject of future work.

Let us now address the question of the detection of immunosignals, showing antibody affinity. As one can see from figure 3, there are a number of 'abnormal' force curves in cases (b)–(d) which are similar to the force curves observed in case (a). This implies the lack of antibodies in the corresponding areas. Taking into account the fact that each force curve represents the average force data collected over $1\,\mu m^2$, one can conclude that such small areas are not suitable for reliable sensing. To obtain a signal statistically different from case (a), it is necessary to average the force data collected over a larger area.

The next step in the detection of immunosignals is to define the value of the signal based on the force curve parameters. Obviously, it is not convenient to compare just force dependences. Here we propose to use a dimensionless ‘signaling’ parameter, a combination $L^2N$. Using this parameter, one can easily consider the difference in the immunosignals. The analysis shows that, in order to distinguish cases (b)–(d) and (a), the proposed dimensionless signaling parameter should be collected over areas of at least $3\,\mu m^2$ each. The average values (with one standard deviation) of such a signal are shown in figure 4 for all cases (a)–(d).

**Table 1.** The brush length $L$ and grafting density $N$ obtained by processing of collected force data with the help of equation (1). The average and standard deviation values are presented.

<table>
<thead>
<tr>
<th>Applied antibodies</th>
<th>$L$ (nm)</th>
<th>$N$ (nm$^{-2}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No antibodies, case (a)</td>
<td>$16 \pm 8$</td>
<td>$0.19 \pm 0.10$</td>
</tr>
<tr>
<td>Anti-DNP only, case (b)</td>
<td>$23 \pm 9$</td>
<td>$0.16 \pm 0.12$</td>
</tr>
<tr>
<td>Anti-NT only, case (c)</td>
<td>$24 \pm 6$</td>
<td>$0.11 \pm 0.06$</td>
</tr>
<tr>
<td>Both anti-DNP and anti-NT, case (d)</td>
<td>$79 \pm 47$</td>
<td>$0.07 \pm 0.09$</td>
</tr>
</tbody>
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antibodies; (b) only anti-NT; (c) only anti-DNP and (d) both anti-DNP and anti-NT antibodies attached. The horizontal line can be used as a separator between the presence and absence of antibodies.

Ten values of the signal are used for each case. One can clearly see the presence of antibodies in cases (b)–(d). Moreover, the proposed signaling parameter is capable of unambiguously distinguishing the presence of both types simultaneously (case (d)) and only either one of each type (cases (b) and (c)), which was impossible to distinguish by using the standard optical detection shown in figure 2.

4. Conclusion

Here we demonstrated the use of the AFM technique to study the mechanics of the molecular layers of an ELISA immunosensor at the nanoscale. In the investigated type of ELISA sensor, the immunosignal was recorded when complementary (primary) antibodies were captured by the corresponding antigens attached to the sensor surface. The study was done using an ELISA sensor surface, which is composed of a molecular mix of two different antigens. The force data were processed through the entropy brush model. By combining the derived brush parameters, grafting density and length of the ‘immunobrush’, we proposed the dimensionless parameter as a signal for immunodetection. Using this parameter, we demonstrated unambiguous immunodetection collected from an area as small as \(3 \mu \text{m}^2\), which is \(\sim 4\) million times smaller than the area used in ELISA sensors today. Using the dimensionless parameter introduced, we found that AFM can reliably distinguish between the presence of either antibody and from both antibodies together. This differentiation was impossible to detect with the standard optical detection methods. In addition, AFM records the change of the force curves, and consequently requires neither the enzyme markers nor secondary antibodies.

Reducing the size of the sensing area will reduce the quantity of the sample, reagents used and the time required to perform the immunodetection. Moreover, one should expect an increase in sensitivity due to the smaller amount of signaling molecules required to produce a detectable signal. The exact increase of sensitivity will be a function of time, dissociation and association constants of the analyte. It will be defined in future work.

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References

individual commercial immunoassays using clinical samples

Biotechniques 42 30–3

[31] Pita M, Cui L, Gaikwad R M, Katz E and Sokolov I 2008 High sensitivity molecular detection with ELISA-type immunosensing Nanotechnology 19 375502


[37] Ong Q K and Sokolov I 2007 Attachment of nanoparticles to the AFM tips for direct measurements of interaction between a single nanoparticle and surfaces J. Colloid Interface Sci. 310 385–90

[38] Sokolov I, Subba-Rao V and Luck L A 2006 Change in rigidity in the activated form of the glucose/galactose receptor from E-coli: a phenomenon that will be key to the development of biosensors Biophys. J. 90 1055–63


[54] Sokolov I, Ong Q K, Shodiev H, Chechik N, James D and Oliver M 2006 AFM study of forces between silica, silicon nitride and polyurethane pads J. Colloid Interface Sci. 300 475–81