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An Optical Tweezers–Based Immunosensor for Detection of Femtomoles-per-Liter Concentrations of Antigens

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Abstract

We used optical tweezers—optical trapping with focused laser beams—to pull microspheres coated with antigens off of an antibody-coated surface. Using this technique, we could quantify the force required to separate antigen to antibody bonds. At very low surface density of antigen, we were able to detect the single antigen to antibody binding. The force required to break the antigen–antibody bonds and pull the microsphere off the surface was shown to increase monotonically with increasing surface density of antigens. Using the force determination as a transducer, we were able to detect concentrations of free antigens in solution as small as 10^{-15} mol/L in a competitive binding assay.

Index Entries: Optical tweezers; laser trap; immunosensor; assay; antigen; antibody; BSA.

Introduction

The detection and quantitative measurement of ultralow concentrations of analytes is becoming increasingly important *(1)*. Methods that do not require amplification techniques for the detection of infectious organisms, viruses, and nucleic acid targets could find wide application in the clinical environment. To this end, we have carried out a series of experiments leading to the detection of femtomoles-per-liter concentrations of a protein antigen by a method we believe could have wide application and would be capable of automation. Our approach is to use an optical trapping

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technology capable of sensing single antigen-antibody bonds. With a competitive binding or displacement-type assay, we can detect extremely small quantities of a soluble antigen added to the system.

Materials and Methods

Principle

We have constructed a sensor using optical trapping technology or optical tweezers. Optical tweezers use focused laser beams to trap and remotely manipulate dielectric particles, including cells and other biological objects *(2–4)*. The change in momentum of the light transmitted by the dielectric object results in a force that traps objects, with an index of refraction greater than the surrounding medium, at the local maximum of the intensity of the electromagnetic field, i.e., the focus of the laser beam. A more complete discussion of optical tweezers can be found in ref. *4*.

Figure 1 illustrates the basic principle of our device. We use optical tweezers to trap a microsphere coated with an antigen and pull it away from a surface coated with the corresponding antibody. The force applied by the optical tweezers to break the antigen-antibody bonds and pull the microsphere away from the surface is measured. We detect the presence of small quantities of the antigen in solution by a competitive binding, displacement assay: the binding of the free antigens in solution to the antibodies on the surface is detected as a reduction in the average force required to pull the antigen-coated microsphere away from the surface.

Apparatus

Our optical tweezers apparatus consists of a Zeiss inverted microscope equipped with a ×100 (NA of 1.4) oil immersion objective lens, a video camera and monitor, a computer-driven translation stage capable of motion in three orthogonal directions, and a continuous-wave Nd:YAG laser emitting at 1.06 μm. The laser light is coupled into the back of the objective lens with a dichroic mirror enabling us to simultaneously view and trap the microspheres. Transparent, polarizable objects with an index of refraction higher than the surrounding medium, such as our microspheres in buffer, are trapped at the local maximum of the intensity of the laser light, tightly focused by the objective lens. Typically, the size of the focal spot is kept fixed so that the strength of the trapping force is proportional to the power of the laser. For a review of the principles of optical forces as well as the various configurations and applications of optical tweezers, see ref. *4*.

The microspheres, suspended in a buffer solution, were contained in a chamber constructed from a glass microscope slide with a 1.0-cm hole drilled through it and two glass cover slips on each side. The cover slip on the objective-lens side of the chamber contained the silane-coupled antibodies. Both cover slips were sealed to the microscope slide with silicone vacuum grease. The total volume of the chamber was approx 100 μL.

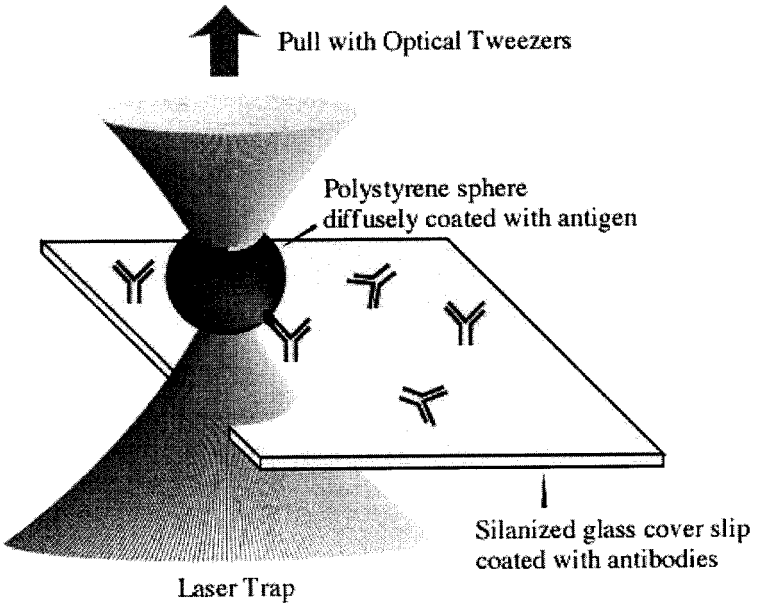

Fig. 1. Basic scheme of our optical tweezer–based immunosensor. An antigen-coated microsphere is trapped and pulled away from an antibody-coated surface using optical tweezers. The minimum amount of force applied by the optical tweezers to break the microsphere-coupled antigen to antibody bonds is measured. Detection of free antigens in solution is manifested as a reduction in this applied force owing to the displacement of microsphere-coupled antigen by free antigen in the binding to the antibody.

Sample Preparation

The antigen used in these experiments was bovine serum albumin (BSA). The BSA was covalently coupled to 4.5-µm-diameter latex microspheres with carboxyl groups (Bangs, Carmel, IN). The covalent coupling was accomplished by first removing a 0.1-mL aliquot of the original 5% suspension of microspheres and washing with a buffer at pH 6.6. containing 0.05 mol/L of potassium phosphate containing 0.1 mol/L of sodium chloride, 0.2% gelatin, and 0.01% thimerosal solution. Next, 1.0 mL of 2-(*N*-morpholino)ethane sulfonic acid buffer (Sigma, St. Louis, MO) (0.05 mol/L, pH 5.5) was added to the desired quantity of BSA plus the microspheres and vortexed. A freshly prepared 1% solution of the water-soluble carbodiimide (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimde (Sigma) was added to the microspheres and stirred for 4 h. The unreacted carbodiimide was removed by centrifuging and washing the microspheres three times at 15°C with phosphate-buffered saline (PBS) consisting of 0.05 mol/L of potassium phosphate, 0.1 mol/L of sodium chloride, and 0.01% thimerosal solution at pH 7.5. The microspheres were then stored in PBS at 4°C. The BSA-coupled microspheres were diluted to a concentration of approx 10^{-7} mol/L for the experiments, which was about 1000 microspheres in the sample chamber.

The corresponding antibodies (mouse monoclonal, anti-BSA) were covalently attached to glass cover slips through silane coupling. Silane-coated cover slips were prepared by first boiling them in 10% nitric acid for 1 h followed by washing with distilled water until the water had a neutral pH. Silane solution was prepared by adding 5 mL of 3-glycidoxypropyl-trimethoxysilane (Aldrich, Miliwaukee, WI) and 5 mL of tetramethyl-orthosilicate (Aldrich) to 100 mL of deionized water. The pH of the silane solution was adjusted to 4.0 with 10% acetic acid solution. The cover slips were dipped in the silane solution and dried at room temperature followed by heating in an oven for 90 min at 110°C. The cover slips were then mounted on glass slides and 100 µL of 0.05 mol/L potassium phosphate at pH 8.0 was added to the cover slip surface. To this was added 10 µL of 2.8 mg/mL monoclonal anti-BSA (Sigma). The slides were incubated at 5°C for 72 h. The cover slips were then rinsed with the PBS solution.

Measurement Procedure

We detected the binding of the microsphere to the cover slip surface according to the following procedure. Microspheres resting on the surface of the cover slip were located optically with the microscope. The microscope objective was focused on the surface of the cover slip and then positioned in the center of the microsphere in the plane of the cover slip. The objective lens was then displaced 5.0 µm toward the cover slip surface by the computer-driven stage. This corresponds to placing the focus of the laser beam about one microsphere radius above the middle of the microsphere, in the chamber, away from the surface. We then slowly increased the laser power from zero until the microsphere was visually observed to jump away from the surface into the focus of the laser beam. The minimum power at which the microsphere would be pulled into the optical trap was recorded. The laser power was typically increased on a timescale of approx 5 s. We observed that the minimum laser power required to lift the microsphere off the surface and into the trap was unchanged when we increased or decreased this time by a factor of 2.

Results and Discussion

Specific vs Nonspecific Binding Experiments

We performed an initial series of experiments to study the specific vs nonspecific binding of antigens to the silanized surfaces with and without antibodies, respectively. Two series of measurements were made, according to the procedure described in the previous section, using microspheres coated with BSA and silane-coated cover slips with and without anti-BSA coupled to them. For each series of measurements, we varied the surface coverage of the microspheres by changing the amount of BSA offered during the coupling procedure. Concentrations of BSA ranging from 1.45×10^{-7} to 1.45×10^{-15} mol/L were used for preparing the coated microspheres. Figure 2 presents the titer data for the BSA-coated microspheres. Each value

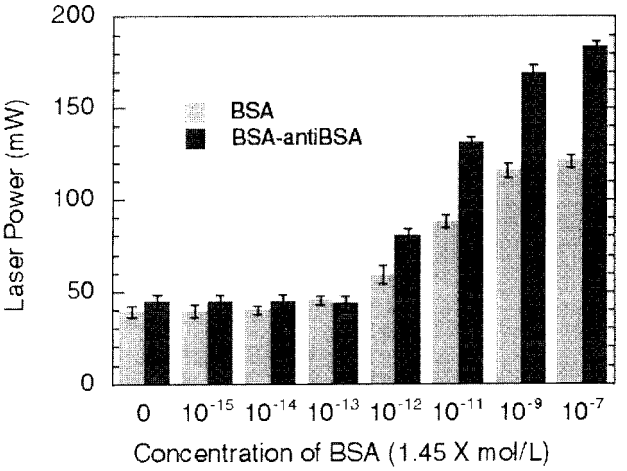

Fig. 2. Power required to pull microspheres coated with BSA off a silane-coated glass cover slip (light shaded bars) and a silane-coupled anti-BSA-coated cover slip (dark shaded bars). The increase in the measured laser power corresponds to increasing binding force of the BSA-coated microspheres with the silanized surface. The increase in the binding force with increasing BSA on the surface of the microspheres is interpreted as arising from the increasing number of BSA to anti-BSA bonds and BSA to methyl-terminated silane bonds for the anti-BSA-coupled and -noncoupled silane-coated surfaces, respectively. The BSA concentrations indicated on the bar graph are concentrations used in the coupling reactions and not the quantities bond; therefore, they represent the maximum quantity of protein that could be coupled to the microspheres. The mean and SD of 10 measurements made with independent microspheres are shown.

of the laser power required to pull a microsphere, coated at a particular concentration of BSA, off the surface represents the average of 10 measurements with 10 different microspheres. The uncertainties shown are the standard deviation (SDs) of the 10 measurements.

The data presented in Fig. 2 show that for microspheres treated with BSA concentrations of 1.45×10^{-13} mol/L or less, the binding force of the microsphere to the surface was essentially the same as if there were no BSA on the microsphere. For microspheres coupled at BSA concentrations of 1.45×10^{-12} mol/L and higher, the binding force of the microsphere to the silanized surface increased with increasing BSA concentrations; however, the measured binding force of BSA to anti-BSA was significantly larger than the measured binding force of BSA to only the silane-coated cover slip. We interpret this as arising from both the specific and nonspecific binding of the BSA to the silanized surfaces with and without anti-BSA, respectively.

Based on the number of microspheres used in the coupling reaction, and assuming 100% coupling of available BSA molecules to the microspheres, we calculate that the average number of BSA molecules coupled to the microspheres at the offered BSA concentration of 1.45×10^{-13} mol/L is one. (This estimate of the number of BSA molecules coupled to the

microsphere is probably good to an order of magnitude. In practice, there will be <100% coupling of the BSA to the microspheres; however, loss of microspheres during washing will tend to compensate for the reduced coupling.) Thus, we expect that for microspheres treated at BSA concentrations $<1.45 \times 10^{-13}$ mol/L, there should be essentially no BSA on the microsphere available to bind to the cover slip surface. As the number of BSA molecules on the microsphere increases, there should be an increase in the number of BSA-to-surface bonds for both specific and nonspecific binding, and a corresponding increase in the measured binding force.

The high selectivity provided by the molecular recognition of antibodies for antigens is observed in the data of Fig. 2. In each case in which binding of a microsphere coupled with BSA was observed, the binding force of the BSA to a silane-coupled anti-BSA surface was larger than the binding force of the BSA to the silane surface without anti-BSA. Despite the strong tendency of BSA to bind to surfaces terminated with methyl groups such as our nonfunctionalized silane surface *(5)*, the measured binding force of BSA to the anti-BSA was 30–50% larger for microspheres coupled at BSA concentrations from 1.45×10^{-12} to 1.45×10^{-7} mol/L, respectively. The data in Fig. 2 suggest that the increase in the specific binding of a microsphere coupled with BSA to the anti-BSA-coated silane surface occurs for microspheres coupled at BSA concentrations between 1.45×10^{-13} and 1.45×10^{-12} mol/L. In this range, the measured increase in the binding force would arise from a single or, at most, a few antigen-to-antibody binding pairs.

We performed an additional experiment to test our interpretation that the increased force observed with the BSA-coupled microspheres on the anti-BSA coated cover slip was owing to specific binding and not to the presence of any nonspecific immunoglobulin (IgG) to BSA interaction. We coupled a nonspecific mouse IgG (Sigma; mouse IgG recognizes mouse serum albumin and does not crossreact with BSA) to a silanized cover slip and repeated the binding force measurement experiments with microspheres prepared at BSA concentrations ranging from 1.45×10^{-7} to 1.45×10^{-15} mol/L. The results of these experiments showed that the force required to break the microsphere-to-surface bonds remained constant across the entire range of BSA concentrations used to couple to the microspheres. This finding is consistent with the interpretation that the increased binding force observed in the presence of the specific antibodies is owing to the specific antigen-to-antibody interaction. In contrast to the data of Fig. 2, the absence of any increase in the nonspecific binding force of the BSA-coated microsphere with the silanized cover slip as the coverage of the microsphere increased suggests that the cover slip was, in effect, fully coated with the nonspecific mouse IgG. This would correspond to an average spacing between nonspecific IgG molecules of, at most, the radius of the microsphere. Hence, the minimum average surface density of nonspecific IgG is 2×10^{7} mol/cm^2.

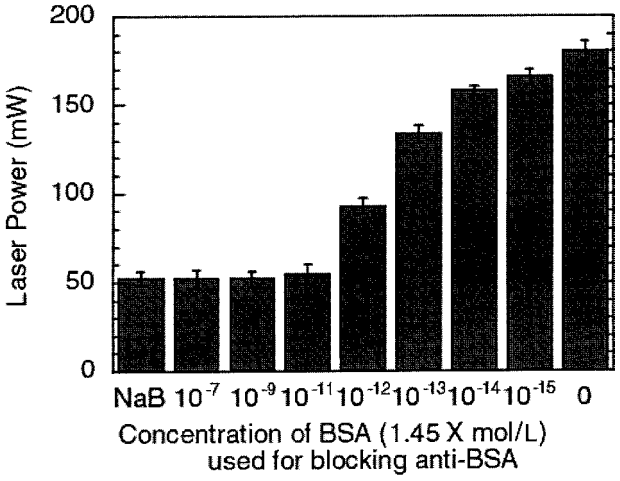

Fig. 3. Power required to pull microspheres coated with BSA (coupled at a BSA concentration of 1.45×10^{-7} mol/L) off a silane-coupled anti-BSA-coated glass cover slip in the presence of free BSA in solution. The increase in the measured laser power corresponds to increasing binding force of the BSA-coated microspheres with the silanized surface. The decrease in the binding force with increasing free BSA concentrations is interpreted as arising from the decreasing number of anti-BSA-binding sites available to the BSA-coated microsphere owing to displacement by the free BSA in solution. The BSA concentrations indicated on the bar graph are concentrations of free BSA in the chamber. Shown are the mean and SD of 10 measurements made with independent microspheres. NaB, measurements made with BSA-coated microspheres and a surface only coated with silane (no anti-BSA).

Immunosensor Experiment

To demonstrate the device as an immunosensor, we performed a typical competitive or displacement-type assay using a BSA-coated microsphere, coupled at a BSA concentration of 1.45×10^{-7} mol/L, and a silane-coupled anti-BSA-coated surface. For each experiment, we added 1 µL of buffer solution with free BSA at various concentrations to the chamber containing the microspheres in 100 µL of buffer and incubated this solution for 2 h at room temperature. We then made measurements of the binding force of the microspheres to the anti-BSA-coated surface according to the procedure described earlier. Figure 3 shows the results of our experiments as a function of free BSA in solution. The BSA concentrations indicated on the bar graph are concentrations of free BSA in the chamber. The height of each bar represents the average of 10 measurements with 10 different microspheres. The uncertainties shown are the SDs of the 10 measurements. The first bar, labeled NaB (no anti-BSA), is the result of measurements made with BSA-coated microspheres and a surface coated with only silane.

The data in Fig. 3 indicate that the range of sensitivity of our assay covers at least three orders of magnitude. We observe an increase in the

binding force of the microspheres to the surface for concentrations of BSA in solution between 1.45×10^{-12} and 1.45×10^{-15} mol/L. The difference in the binding force of the microsphere to the surface for no BSA in solution and a concentration of BSA in solution of 1.45×10^{-15} mol/L shows that the assay is sensitive to femtomoles-per-liter concentrations of antigens. At concentrations of BSA in solution of 1.45×10^{-11} mol/L and higher, the binding force of the microsphere to the anti-BSA-coated surface is indistinguishable from the binding force of the microsphere to a silanized surface without anti-BA (the NaB value). We interpret this as arising from a complete displacement of the microsphere-coupled BSA to anti-BSA reaction by the free BSA. We can estimate an upper limit for the average surface density of anti-BSA based on the minimum concentration for complete displacement. A concentration of 1.45×10^{-11} mol/L of BSA in solution corresponds to about 9×10^8 mol, and, hence, the maximum average surface density of anti-BSA is 9×10^8 mol/cm^2; however, this value is an overestimate since, in equilibrium, a fraction of the BSA is not bound but in solution. According to the data in Fig. 2, the increase in the difference between specific and nonspecific binding force for BSA concentrations of 1.45×10^{-12} and 1.45×10^{-7} mol/L is only a factor of 3, even though the BSA surface coverage of the microsphere has gone up by 10^5. This implies that the number of BSA to anti-BSA bonds is limited by the surface coverage of anti-BSA on the silanized cover slip and is at least a factor of 3 higher than the lower estimate based on the results with the nonspecific mouse IgG. We conservatively estimate the average surface density of anti-BSA to be between 6×10^7 and 9×10^8 mol/cm^2.

At a concentration of 1.45×10^{-15} mol/L, there are only about 10^5 mol of BSA in solution. A possible explanation for the detection of such a low concentration of analyte is that the dielectrophoretic force *(6)* of the laser acting on the free BSA molecules can effectively concentrate the free BSA in the region of contact between the microsphere of interest and the surface. In the presence of a gradient electric field, the interaction of the induced dipole moment of a molecule with the field results in a dielectrophoretic force on the molecule; this is essentially the same force responsible for the trapping of the microspheres by the optical tweezers. A simple calculation shows that the time required for a free BSA molecule (with a diffusion constant of 0.59 cm^2/s) to diffuse, in the presence of the dielectrophoretic force of the laser field used in the experiments, a distance of 100 μm (approximately the average distance between free BSA molecules at 1.45×10^{-15} mol/L) to the focus of the laser beam is comparable to the timescale in which we make our measurements (5 s). The polarizability of the glass cover slip should also enhance the electric field where the laser beam is passing through it and increase the dielectrophoretic force at the location where the microsphere is binding to the surface, thereby further concentrating the free BSA. While our simple estimate shows that the timescale for bringing the free BSA molecules to a location where they could bind to anti-

BSA and block the microsphere from sticking is reasonable, a more detailed calculation would include the binding of the BSA to the anti-BSA. Such a calculation, however, is beyond the scope of this article, but further studies of this interesting dielectrophoretic effect are warranted.

Conclusion

We have demonstrated an optical tweezers–based immunosensor capable of detecting femtomolar concentrations of antigen in a competitive binding assay. Although we have shown that the system described is capable of these highly sensitive measurements, an investigation of the limits of sensitivity as well as the development of some form of sample processing and microfluidics will be necessary for maximum utilization of this technology in the clinical environment. We believe that it should be possible to improve the sensitivity of this technology further than demonstrated in this study. The broad potential of this approach for detection an quantification of binding pairs is under further investigation.

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