Anal. Chem. **2009,** *81,* 7510–7514

Correspondence

Antibody−Antigen Exchange Equilibria in a Field of an External Force: Design of Reagentless Biosensors

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This correspondence presents a new strategy for detecting biological molecules that relies on competitive exchange interactions of an analyte with two-component molecular tethers attaching superparamagnetic microspheres (4 μm in diameter) to a sensor surface. The individual tethers consist of an antibody−antigen complex and are designed to selectively detect antigenic proteins in a sensitive reagentless fashion. In order to impart a driving force to the otherwise free energy neutral antibody−antigen exchange equilibrium, a small mechanical force of ~10 pN was applied to stretch the antibody−antigen tethers using a massively parallel magnetic tweezers device. The experimental work was carried out with human cardiac troponin I. This serum heart attack marker was used as an example of analytes of credible relevance to biomedical diagnostics. The initial results illustrate the functioning of a cardiotroponin sensor and offer a preliminary estimate of its sensitivity of 16 pM.

Detection of biological molecules in a fast, simple, sensitive, and inexpensive manner is currently one of the most actively pursued topics of scientific inquiry in the analytical sciences. The qualitative and quantitative identification of such molecules is important in medicine, biology, forensics, and many other areas. Proteins are of particular interest as disease markers. In the majority of cases, an immunoassay is the method of choice. Modern immunoassays are generally capable of quantifying a protein of interest in less than an hour.[1] Most currently used immunoassays fall into one of two broad categories: competitive and immunometric assays.[1,2] In competitive assays, a small quantity of antibody is used in order to limit the number of binding sites. The analyte antigen competes with trace quantities of labeled antigen, and the ratio of bound to free labeled antigen is measured and calibrated to the analyte antigen concentration. The principal advantage of competitive immunoassays is that only a single antibody binding site is required and so smaller proteins can be used as the antigen.[1] Indeed, competitive assays are now principally used with analytes that are too small for immunometric assays, as competitive assays generally have lower sensitivities and more problems with nonspecific protein binding interferences than immunometric assays.[1]

In an immunometric assay, two antibodies are used and antigen is the limiting factor. Generally, one antibody is bound to a solid support to enable separation. The second is labeled to enable and often to enhance detection of the antibody−antigen−antibody "sandwich" complex. This is done by the use of enzymes (in ELISA), although fluorescence, isotope decay, and even visual inspection can also be used. Immunometric assays have some substantial advantages over competitive assays, most importantly, a greater sensitivity. Also, since two antibody−antigen binding events are required to generate a signal, reduced interference from nonspecific protein binding is another advantage.[3−5] There are also some drawbacks to immunometric assays. In particular, finding two antibodies that bind with good specificity and selectivity at different epitopes can be a challenge or even impossible in cases of smaller analytes.[3−6]

Whether a competitive or immunometric assay is employed, most immunoassays require a dedicated lab, with large instruments and often with substantial sample workup and reagent requirements.[1,2] In many cases, only a single analyte can be measured at a time and each run can take up to an hour, making the time and cost required to run a large volume of immunoassays significant. Despite difficulties related to antibody instability and other constraints of immunoassays, some progress has been made in developing simpler to operate, disposable test kits.[6−9] Home

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Published on Web 07/29/2009

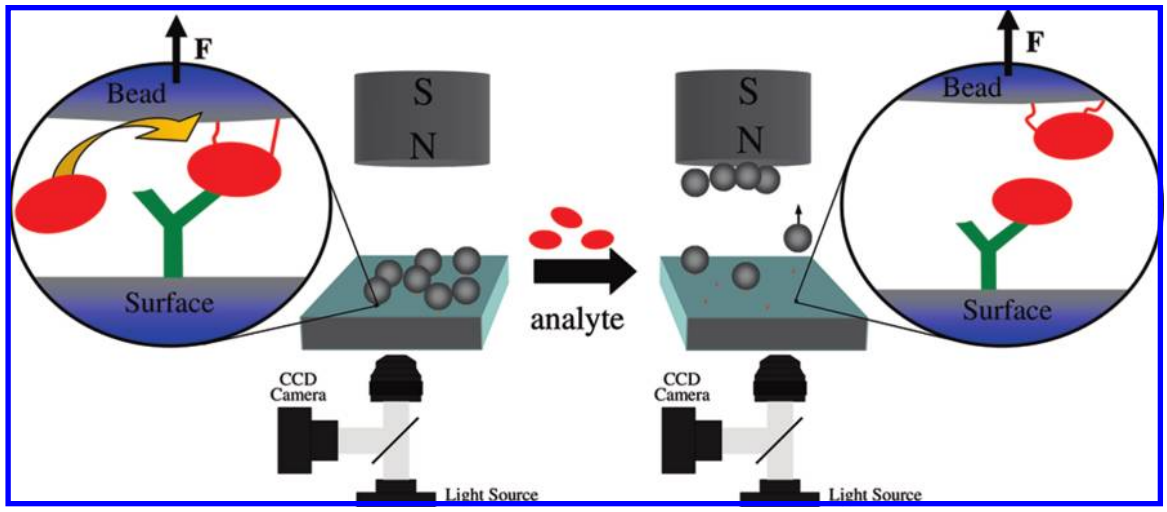

Figure 1. A schematic illustration of the design and functioning of an immunogenic protein sensor. The two circled cartoons depict the structure of a single antibody (y shaped element)−antigen (red, oval shaped element) tether linking a microbead to the sensor surface (left circle). A force, *F*, is acting on the superparamagnetic bead. This decreases the binding constant of the Ab−Ag complex and promotes the competitive exchange involving free antigen. Following the exchange and the breaking of the tether, the bead is released (right circle). The analyte antigen is engaged in a stronger Ab−Ag complex unaffected by the external force.

pregnancy tests are a well-known example of simple, portable sandwich immunoassays easily performed outside of a lab. More generally, a diagnostic system developed by Abbott Point of Care Inc. (formerly I-STAT Corp.) allows an untrained user to perform several immunoassays and other tests on whole blood, including cardiotroponin I, BNP, and clinically relevant electrolytes. Currently, clinically relevant concentrations of the heart attack markers cardiotroponin I and BNP can be measured in less than 20 min.[10]

Design of Immunogenic Protein Sensors. This section introduces the basic idea underlying the design of our sensor. Our approach relies on competitive exchange equilibria involving antibody−antigen complexes. Figure 1 illustrates the key elements of the sensor design. The left-hand side of the figure shows several microbeads attached to the device surface with a number of antibody−antigen tethers. Each tether consists of an antibody attached to the device surface and an antigen molecule bound to the microbead surface. The number of tethers per microbead can be controlled by diluting the antibody solution used in antibody surface attachment with BSA. The antibody−antigen interaction established during the initial sedimentation of microbeads results in microbead attachment to the sensor surface. A sensor glass slide carrying microbeads is positioned on the stage of an inverted microscope allowing us to continuously monitor the number of microbeads bound to the surface. When a sensor constructed this way is exposed to a small (microliter) volume of analyte solution containing the target antigen (identical to the bead-bound antigen), the latter engages in a competitive exchange with the antibody−antigen complex constituting a tether:

$$\mathrm{Ab{-}Ag_{bead}} + \mathrm{Ag_{analyte}} \leftrightarrow \mathrm{Ab{-}Ag_{analyte}} + \mathrm{Ag_{bead}} \qquad (1)$$

It is apparent that since the protein forming antibody−antigen tethers and the analyte are identical, this reaction lacks any driving force and thus its equilibrium constant, *K*, is 1. This is not a desirable situation. Our goal is to create conditions under which the equilibrium in eq 1 is shifted to the right so that every analyte antigen that diffuses into the binding region breaks one antibody−antigen tether. Ultimately, this would result in breaking of all tethers and a release of microbeads as shown on the right-hand side of Figure 1. To accomplish this, we apply a small force (*F*) directed to stretch the antibody−antigen tethers using a massively parallel magnetic tweezers apparatus (see Experimental Section). According to Bell's theory,[11] this exponentially increases the dissociation rate constant $k_{off}(F)$ relative to its value in the absence of the force:

$$k_{off}(\mathrm{F}) = k_{off}(0)\exp(Fx/kT) \qquad (2)$$

where *x* is the distance over which the force acts on the Ab−Ag complex as it is gradually stretched. This, in turn, decreases the binding constant of the affected antigen by its antibody, $K_{binding}$:

$$K_{binding} = k_{on}/k_{off} \qquad (3)$$

Since the equilibrium constant of the reaction in eq 1 can be expressed by the ratio of the binding constants, it is apparent that $K > 1$ in the presence of a small magnetic force. In fact it is easy to show that in order to increase *K* from 1 to ~100 and thus to render the reaction in eq 1 to be nearly irreversible, we need to apply a force of ~10−50 pN.

In addition to the selectivity intrinsic to the antibody−antigen association and the competitive exchange processes, we expect this sensor design to embody three additional important features worth mentioning. (1) The beads are superparamagnetic, thus their release in a small external magnetic field of the magnetic tweezers can be easily followed with the aid of an optical microscope and constitutes the sensor's signal transduction mechanism. (2) This signal transduction also offers a significant

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signal amplification and high sensitivity. In principle, if each microbead were attached with just a few antibody−antigen tethers, a small number of single-molecule events of the reaction in eq 1 would result in the release of a microbead, an easily detectable event. (3) Since, as postulated above, the antigen exchange equilibria can be rendered essentially irreversible by an external magnetic force, each target antigen molecule is captured as it diffuses into the active region of the sensor and exchanges with the antigen of the tethers. In other words, the sensor acts as an integrating device. In the limit of low analyte concentrations, the sensor response (number of released microbeads) becomes time dependent. As the concentration of target antigen in the analyte sample decreases, the time delay between the introduction of an analyte and microbead release is expected to increase, reflecting diffusion of antigen molecules to the sensor's reaction centers of the individual microbeads.

EXPERIMENTAL SECTION

A three cartridge Millipore water purification system provided nanopure water. Mono- and dibasic sodium phosphate, hydrogen peroxide (30%), sodium chloride, and glass microscope slides (precleaned) were obtained from Fisher. Tris hydrochloride (Ultra) was purchased from MP Biomedicals. Sulfuric acid (concentrated) and sodium citrate were obtained from EMD. Bovine serum albumin (BSA) was obtained from Sigma. Protein stabilization solution (PSS), a proprietary mixture consisting principally of lactitol, tris, and DEA-dextran with other chemicals in small quantities, was manufactured by Applied Enzyme Technology Ltd. and was provided by Abbott Point of Care, Inc., a division of Abbott Laboratories. Cardiac troponin ITC complex (cardiotroponin) and polyclonal mouse antibodies to cardiotroponin were received in PSS in high concentrations (1−50 μM) from Abbott Point of Care, Inc. The proteins were purified by size exclusion chromatography. In order to minimize protein loss due to interactions with container walls, an excess of BSA (50 μL of 6 mg/mL solution) was added with the protein. The protein was collected in centrifuge filter devices and concentrated in a centrifuge. Dynabeads M-450 tosylated magnetic microbeads (4.5 μm diameter) were purchased from Invitrogen. Parafilm "M" was obtained from Pechiney plastic packaging. Glass slides with surface bound aldehyde moieties (Nexterion Slide AL) were purchased from Schott North America, Inc. Amicon Ultra centrifuge filter devices with a molecular weight cutoff of 10 000 Da were supplied by Millipore. Size exclusion desalting columns (10 mL) were supplied by Bio-Rad.

To bind the antibodies to slides, aldehyde slides were treated with an antibody solution for at least 5 h (up to overnight). The latter was diluted with a BSA solution to control the surface density of antibodies. Subsequently, the slides were rinsed with PBS. A volume of 2 μL of 25% (by mass) PSS solution was then added to each 4 mm diameter circular well (made on slides by melting precut parafilm at ~100 °C). The solution was then dried, resulting in a final concentration of 2.5 mg of solids/cm^2.

Tosylated Dynabeads were treated with cardiotroponin by washing a 50 μL aliquot of beads, resuspending it in 500 μL of PBS, and then adding 50 μL of concentrated cardiotroponin solution (~300 ng/mL). The beads were rotated for 24 h, and then 50 μL of 6 mg/mL BSA and 0.1 M tris were added and the solution was mixed for another 24 h. The treated beads were washed with PBS and then resuspended in a 25% PSS solution for storage. They could be refrigerated in this solution for up to 2 weeks. When the beads were to be used, an aliquot was diluted to 1−2% PSS. To form the tethers, 3−5 μL of bead solution were added to the slides (which were coated with dried PSS) and the slides were again dried. At this point, the slides could be kept desiccated for up to 2 days. To initiate experiments, the spots were rehydrated using deionized water, as appropriate buffer was dried with the PSS.

In all runs, the tethered beads were imaged with a Nikon TE 2000-S inverted microscope using a Photometrix CoolSnap HQ2 camera and a Nikon Intensilight C-HGFI light source. The camera was controlled and the images were analyzed using NIS Elements 2.3 image acquisition and analysis software. A Newport M460A three directional translation stage was used to position a stack of five 0.2 cm thick and 0.9 cm in diameter N52 grade neodymium rare earth disk magnets obtained from K&J Magnetics, Inc. By control of the distance between the stack of magnets and the microscope stage, a magnetic force in a range of 0−110 pN can be applied to microbeads with a relative precision of ~±20%.

During force runs, the number of beads in the circular well (several hundred to a few thousand beads were present initially) was tracked as the stack of magnets exerted a 5, 25, or 90 pN force on the beads. The force−distance calibration curve of the stack of magnets with Dynabeads and the method used to obtain it have been discussed elsewhere.[12]

RESULTS AND DISCUSSION

In this section, we narrowly focus on the preliminary results illustrating the performance of a protein sensor designed to respond to human cardiac troponin I. Cardiotroponin is involved in the calcium ion mediated regulation of muscle contraction, along with actin and tropomyosin.[13,14] Cardiotroponin has three subunits, the calcium binding C subunit, the tropomyosin-binding T subunit, and the inhibitory I subunit, and is often abbreviated as simply ITC. While the C subunit is identical in both cardiac and skeletal muscle, the I and T subunit differ sufficiently to develop specific antibodies.[15] The presence of cardiotroponin in serum has been shown to be a marker for myocardial injury (heart attack). Therefore, the detection of the cardiotroponin I or T subunit is the current method of choice for the diagnosis of this medical condition.[10]

Attachment of superparamagnetic microbeads to glass slides relied on antibody−antigen tethers as outlined in Figure 1 and the Experimental Section. Cardiotroponin antibodies were covalently bound to aldehyde slides using dilute solutions of the antibody in BSA. The ratio of the two proteins determined the surface concentration of antibodies bound to the surface and thus the average number of antibody−antigen tethers involved in binding of individual beads.

Simple sedimentation of microbeads carrying surface bound cardiotroponin onto antibody derivatized glass slides led to antibody−antigen tethering of the beads as illustrated in Figure

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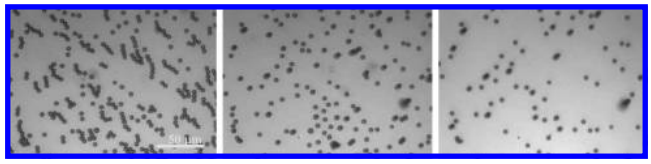

Figure 2. Details of larger micrographs that illustrate the change of microbead population as a function of time and the applied force. The image on the left shows the initial bead population before any force is applied (although the magnets are close enough to cause the beads to arrange themselves linearly). The center micrograph shows the same slide 3 s after a 5 pN force was applied. The one on the right shows the same area after 90 pN was applied for 2 min.

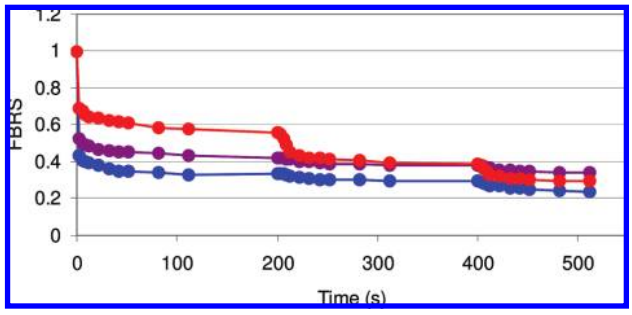

Figure 3. The fraction of the beads remaining on the surface (FBRS) as a function of time and the applied force. Cardiotroponin antibody−antigen tether system (red, top data set). BSA (surface)−cardiotroponin antigen (microbeads) system (purple, middle data set). Cardiotroponin antibody (surface)−streptavidin (microbeads) system (blue, bottom data set). The force of 5, 25, and 90 pN was applied at 0, 200, and 400 s, respectively. The initial number of the microbeads was ~500. FBRS is normalized to that initial number of the microbeads.

1. At that point, we investigated the extent to which Ab-Ag tethers formed and were capable of attaching the microbeads. We also asked whether our procedures designed to limit attachment of microbeads to the sensor surface via nonspecific interactions (see Experimental Section) were successful. To answer these questions, a typical experiment involved application of a magnetically induced force of a specific magnitude to a population of microbeads residing on the sensor surface, followed by optical monitoring the decrease of the bead population with time. Clearly, a successful Ab−Ag tether formation would be concluded if we observed a large fraction of the microbeads remaining on the surface despite application of a force of 5−90 pN. It is well-known that a force of ~5 pN is sufficient to lift a microbead such as those used in this work that are not surface bound other than through van der Waals interactions.[16] Only a small fraction of 1 pN is sufficient to overcome the gravitational force acting on microbeads. Conversely, we would like to see a large fraction of microbeads being lifted off the surface in blank experiments in which no Ab−Ag tethers could form. Unfortunately, we found the problem of nonspecific binding to be severe and difficult to control fully.[12] In typical runs, a relatively large population fraction of ~40% of the beads were attached via nonspecific binding. We do not devote much space to this problem here as this part of our research will be the subject of a separate report.

Typical results of a magnetic force induced bead detachment experiment are shown in Figures 2 and 3. The first of these figures shows microscopic images of a population of microbeads and its decrease during a force induced bead detachment experiment. Quantitative analysis of such images leads to the data such as those shown in Figure 3 where the fraction of the initial number of microbeads remaining on the sensor surface (FBRS) is plotted as a function of time in a run during which a force of 5, 25, and 90 pN was applied at $t = 0$, 200, and 400 s, respectively. The top data set (red circles) represents the antibody−cardiotroponin tether system. Approximately 60% of the beads in this run remain attached over 200 s when 5 pN was acting on them. A slow decrease of FBRS to ~42% and 30% following the application of the greater forces reflects gradual breaking of the Ab−Ag tethers. This is consistent with a typical strength of antibody−antigen interactions.[16] It also suggests that the number of tethers attaching individual beads is relatively low. We can only crudely estimate that number to be between 1 and 100 (see below). This estimate is supported by similar experiments in which a significantly larger numbers of tethers were formed. That was accomplished by using the undiluted antibody solution in the Ab surface binding experiments.[12] In those cases, unlike in Figure 3 (red data points), we observed a minimal change in the microbead population regardless of the applied force, including 90 pN. This suggests that the number of tethers per bead was high enough that the applied force per single tether was insufficient to even sequentially break the tethers and release beads.

The other two sets of data in Figure 3 correspond to two different blank experiments. In those cases FBRS is ~30−40% and does not appreciably decrease with increasing force. This behavior is consistent with covalent, nonspecific binding of the microbeads to the sensor surface. Indeed, in the limit of a high force and long times, FBRS in the first set of data corresponding to the Ab−Ag tethered beads reaches the same common limit of ~30−35% indicating that about half of this bead population was attached via difficult to control, nonspecific binding.

While determination of the number of tethers and the mechanism by which these tethers break is certainly important, the crucial experiment in testing the performance of these devices involves addition of free antigen capable of engaging in a competitive exchange with Ag−Ab tethers as illustrated by the reaction in eq 1 and Figure 1. Typical results of such an experiment are shown in Figure 4 where we examine a similar population of microbeads attached to a device surface with a similarly low number of tethers as those in Figure 3. During the first 200 s of this experiment, the protocol of Figure 3 was followed with a 5 pN force applied. The initial population of 469 microbeads decreased to 235. Subsequently, a 10 μL aliquot of free cardiotroponin (4.8×10^{-16} mol) was added to the device resulting in a final antigen concentration of around 16 pM. The mere act of this addition resulted in the detachment of a fraction of the beads due to injection induced convection. At $t = 202$ s, the remaining population of 225 microbeads was rescaled to FBRS = 1.0. As can be seen in Figure 4, about 70 s following the cardiotroponin addition, some beads began to be released. FBRS decreased to ~0.93 in about 170 s. This corresponds to a release of 16 microbeads.

There are a number of notable features of this set of data. First, the beads were released at a force of only 5 pN. Knowing that the equilibrium in eq 1 is not effective in breaking tethers in the absence of the external force, this result implies either a rather

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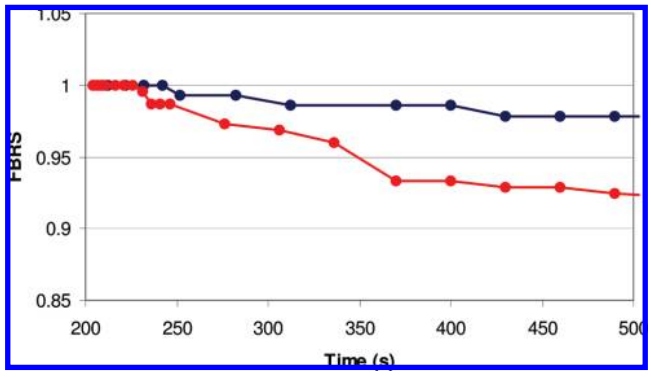

Figure 4. The fraction of beads remaining on the surface (FBRS) when 10 μL of 1.05 ng/mL cardiotroponin solution (red, lower data set) or 10 μL 1.0 mg/mL BSA solution (blue, upper data set) was added to a PBS buffer solution covering microbeads. The microbeads were attached with a low number of cardiotroponin antibody−antigen tethers as were those in the experiment of Figure 3. The injection took place at $t = 200$ s with a 5 pN force acting on the beads. The data are normalized to the remaining number of the microbeads (141 and 225 in the BSA and ITC experiments, respectively) after some beads were convectively dislodged and removed.

long distance over which the force was effective (eq 2) or a greater sensitivity to force than expected. If we interpreted the result of Figure 4 as indicating that the equilibrium constant of the reaction in eq 1 was increased to $K = 100$, the fact that this was possible at $F = 5$ pN would imply, in view of eq 2, that the force was effective over 8.1 Å. This is plausible; while antibodies have a shallow binding pocket, the applied force could deform the extended structure of an antibody, thus increasing the distance over which it is applied. Clearly, this is one of the aspects of this system's behavior which we focus on in our current research.

Additionally, knowing the time delay of the beads release following the cardiotroponin injection provides an upper bound of the number of tethers holding those beads to the surface. This is obtained by assuming that the reaction of the cardiotroponin with its antibody is diffusion limited. As the bound antibodies are spread over the entire sensor surface, the transport of cardiotroponin to the surface following the initial rapid concentration equilibration is expected to obey linear diffusion. Thus, an integrated flux of antigen, $N(t)$, obtained by solving standard diffusion equations can be used as the total amount of cardiotroponin that reached the device surface over a given time:

$$N(t) = \frac{2AD_c^{1/2}C_c t^{1/2}}{\pi^{1/2}} \quad (4)$$

Assuming the diffusion constant, D_c, of cardiotroponin to be 1×10^{-6} cm^2/s and the area A to be the area under a single 4.5 μm bead and using the known initial antigen concentration C_c (16 pM), approximately 20 cardiotroponin molecules diffused and became involved in the reaction in eq 1 at each bead during 170 s (see Figure 4). Therefore, in view of this rather idealized model, only the beads that were bound with no more than 20 tethers could have been released in the course of this experiment.

The sensor model outlined in the introductory text suggests that the device should exhibit a response proportional to the analyte concentration. Efforts were made to construct a dose−response curve. However, because of difficulties with reproducibly fabricating devices, the sensor was only determined to possess a present/not present type response, with a sensitivity of at least 16 pM and an integration time of around 3 min. While this sensitivity is already comparable to the currently established immunoassay techniques, it is possible to envision numerous possibilities to optimize this sensor's performance and to substantially increase its sensitivity.

CONCLUSIONS

A new class of sensing devices for biological molecules has been described. These sensors rely on the release of tethered beads as a result of competitive exchange reactions. In the presence of an analyte, the tethers holding the beads to the surface are broken and the beads are released. The use of superparamagnetic beads and of magnetic tweezers apparatus allows a small force to be applied to the tethers, providing a driving force for the competitive exchange reaction. Additionally, the same force serves to rapidly remove the untethered beads from the surface. This functions as a very sensitive signal transduction mechanism which, in principle, can signal capture of a single analyte molecule. A sensor was developed using a cardiotroponin−anticardiotroponin system. In the preliminary set of data, we achieved a sensitivity of 16 pM for cardiotroponin with the sensor, while blank BSA solutions generated no response.

ACKNOWLEDGMENT

We express our appreciation to the National Science Foundation for supporting this research under Grant CHE-0719334.

Received for review May 17, 2009. Accepted July 10, 2009.

AC9010759