Bioassay Imaging

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Measuring Biomolecular Binding Events with a Compact Disc Player Device**

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Binding events in state-of-the-art parallel solid-phase binding assays, such as DNA arrays or protein chips, are quantified by using fluorescence labeling techniques.[1,2] Furthermore, immunoassays are one of the principal methods of measuring clinically relevant parameters in blood samples.[3] Herein, we demonstrate that inexpensive classical silver staining and standard compact disc (CD) reader technology can be combined into a novel binding-assay system as a substitute for the established, but expensive, signal tags and detection systems used for array-based assays. We constructed an assay reader using CD pickup technology, and determined the concentration of the important and versatile inflammation marker C-reactive protein (CRP)[4–6] in a test sample over a dynamic range of four orders of magnitude with a limit of detection (LOD) of 1 pM. We present a method of quantifying binding in a "binary" manner by counting individual binding events, potentially down to the single-molecule level, with an inexpensive readout system.

The principle of a CD or DVD (digital versatile disc) player relies on measuring the intensity of light reflected from a surface[7] (Figure 1). CDs have been applied in a biological context for some years.[8–16] However, most of these approaches merely use the CD as a cheap support medium and employ various detection mechanisms, such as colorimetry and external-signal or image processing. They do not adapt biological reactions to the high-density binary format of the information content of the CD, thus leaving the true density and simplicity of that system unused. In contrast, we demonstrate that the signal of a protein–protein interaction can be read in a format that is functionally identical to a CD information pit.

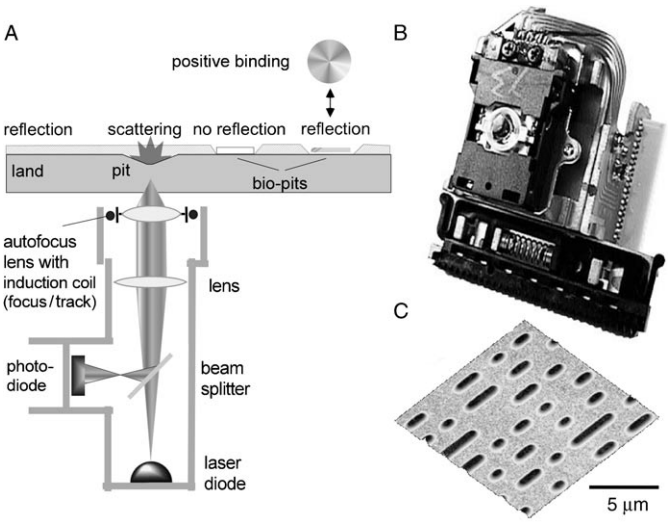

Figure 1. CD pickup for biomolecular-recognition detection. A) Operating principle. The light of a laser diode (standard wavelength of 780 nm) is passed through a beam splitter and focused through an electronically movable lens onto the disc surface. The metal-coated CD contains small impressions called pits, which encode the stored data in a spiral track. When following this spiral, the beam is reflected from the zones between the pits, and travels through the lens onto a photodiode. The intensity of the reflected light falls as a result of scattering when the beam of the pickup hits the sidewalls of the pits. An autotracking and autofocusing function, incorporated in the pickup head, keeps the beam centered on the spiral track. B) Photograph of a conventional CD pickup head. C) Topographic AFM image of a standard CD surface, which reveals the pit structure (the protective layer has been removed).

To obtain a versatile and flexible test system, we constructed the PickupImager, which was made of a CD reading head (pickup) mounted on the stage of an optical microscope,[17] and explored its application as a bioassay reader. As the CD reading principle is based on reflectivity (Figure 1 A), for a bioassay to be interpreted by original CD technology the perfect signal to be measured would be increased reflection, for example, from a tiny mirror. Therefore, we used silver precipitation catalyzed by colloidal-gold-labeled antibodies in a sandwich immunoassay format (Figure 2 A). The chemical process, called autometallographic precipitation,[18–20] is well-known from the staining of electrophoresis gels or tissue sections for electron microscopy. Silver reduction has already proved successful for use in DNA binding assays[21–23] and low-cost immunoassays.[24] We used specific antibodies coupled to colloidal gold particles with diameters of 1, 5, and 40 nm. However, the size of the

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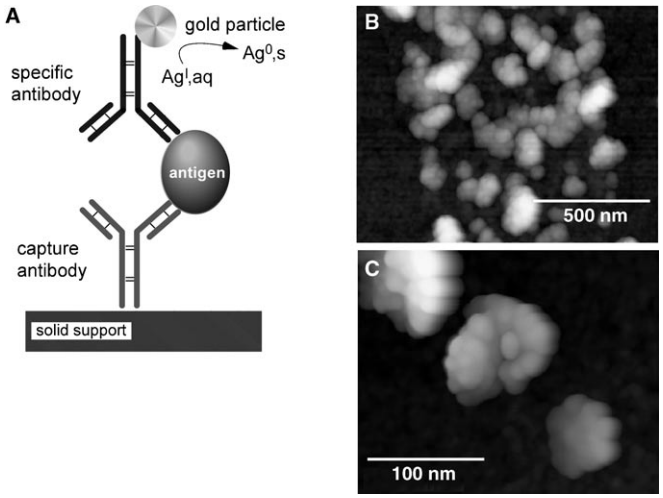

Figure 2. Signal enhancement using autometallography. A) Schematic of the sandwich immunoassay and signal generation. An immobilized capture antibody binds its target antigen. A gold-labeled specific detection antibody subsequently binds to the captured antigen and a silver grain is generated by gold-mediated catalysis. B) AFM image of silver grains grown on 1-nm colloidal gold particles in a patterned 1-μm-wide line of the immunoassay. C) Magnification of single growing silver grains reveals their shape and size.

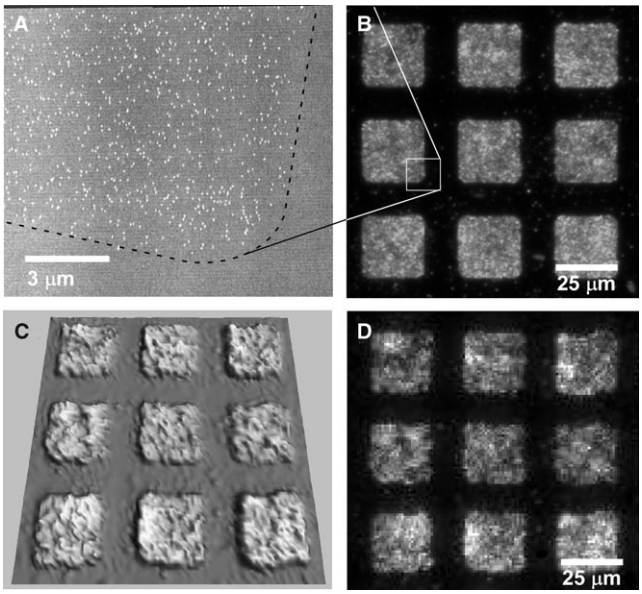

Figure 3. Imaging of the microcontact-printed recognition pads formed by colloidal-gold-labeled anti-biotin antibodies before and after silver development. A) Scanning electron microscope image of the gold beads in the binding assay before amplification by silver enhancement. B) Darkfield microscope image of the silver-enhanced array with a resolution of ca. 0.3 μm. C,D) CD-pickup image of the same array of recognition pads after silver enhancement with a resolution of approximately 1 μm (C) is an artificial 3D representation of the pickup data).

precipitated elementary silver particles, measured by AFM, was not significantly dependent on the size of the initiating colloidal gold particles attached to the antibodies: a larger size of the gold particles initiates and promotes a faster start and higher growth rate, but, from a certain size of the silver grains on the influence of the initial gold size fades and becomes negligible.

After brief metal enhancement (< 10 min), a particle-size distribution between 50 and 250 nm was observed (Figure 2B, C). Longer incubation times resulted in higher background noise and large clusters of grains. The average grain diameter achieved after prolonged enhancement (> 15 min) was (505 ± 140) nm under continuous exchange of the enhancement solution. Without exchange, the particles grew larger, to an average size of (795 ± 200) nm. Notably, grains grown under continuous exchange of the solution had a much smoother surface (see the Supporting Information).[17] Of practical relevance, we found no significant influence of ambient light exposure on the size of the silver grains.

To apply the PickupImager as a quantitative bioassay reader, we fabricated an array of squares (each 25×25 μm) of biotinylated bovine serum albumin (bBSA) by microcontact printing[25] onto a CD support made of polycarbonate, and blocked the surface with BSA to prevent nonspecific interactions. These squares acted as antigen-loaded test areas to bind Au-labeled anti-biotin antibodies from solution, and were subsequently enhanced by autometallography of the colloidal particles.

The electron microscope image (Figure 3A) of the treated surface before metal enhancement reveales that no bound gold particles were found outside the square pattern. As only the squares presented antigens (binding sites), the silver grains found outside these areas could be attributed to nonspecific background induced by autoprecipitation of silver. This array was recorded by the PickupImager with a precision and fidelity comparable to those of the optical microscope (Figure 3B–D). The smallest particles that were detectable and distinguished from background noise with the CD pickup had a diameter of roughly 300 nm (determined by AFM). For this type of single grain the acquired signal output voltage reached up to 2500 mV, within the full dynamic range of the output voltage of the PickupImager of 0–4000 mV.

Knowledge of the average density of binding partners on the surface allows the concentration of the analyte to be determined by counting a large population of single grains on a given surface area. The result will then be a concentration of grains (positive biotests) per unit surface area. At higher surface densities, when the silver grains become optically inseparable, the fractional surface coverage is determined by the PickupImager software.[17] To confirm this concentration dependence of the recorded signal, we patterned surfaces with a range of ratios of bBSA and unlabeled BSA molecules (Figure 4, insets), which conveniently simulated the varying density of captured antigens in a real-life assay.

Here, by using the ratio parameter bBSA/[bBSA+BSA], it might be appropriate to talk of single-molecule sensitivity: when decreasing the density of capture molecules to an average occupancy of the surface smaller than the size of the detecting silver grains, a single binding event is detected by an isolated grain. At the ratio of 0.01% (one bBSA molecule in 10000 BSA molecules, fractional coverage $= 0.16 \pm 0.01$, Figure 4) each bBSA ligand is separated from its nearest neighbor by about 1 μm, and at the ratio of 0.1% (0.23 ± 0.01) the average distance between ligands is still > 300 nm. Both

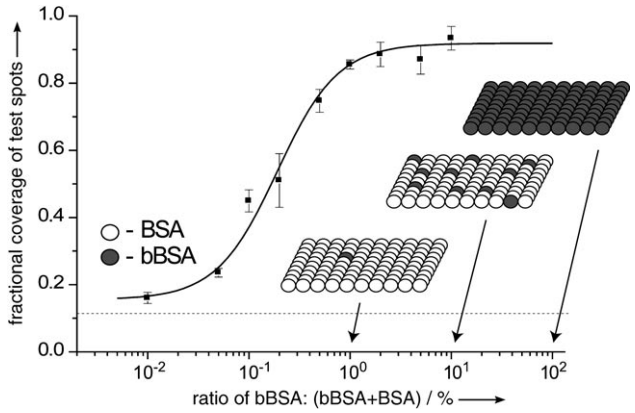

Figure 4. Two-component test assay set up by patterning surfaces with various ratios of bBSA molecules to the total number of BSA molecules. The fractional coverage of the test spots with silver precipitate was plotted against the surface ratio of capture molecules. The inset sketches illustrate different surface ratios of the molecules. At a ratio of 0.01 %, there is statistically one bBSA ligand on an area of 1000×1000 BSA molecules occupying ca. 1 μm^2 (size of BSA: $4\times4\times14$ nm^3). The dotted line marks the measured zero-dose value (0.13 ± 0.02), which is in good agreement with the parameter of the sigmoid fit ($A1=0.13\pm0.05$ and $A2=0.92\pm0.03$).

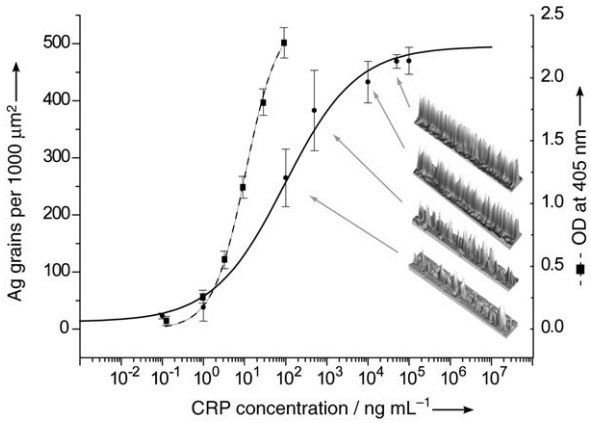

Figure 5. CRP-ELISA measured by the PickupImager and compared with standard ELISA. The number of counted silver grains per test area versus the antigen concentration (●) gives a classical binding curve (——). A classical standard CRP-ELISA measurement (■) with antigen concentration of 0.1–100 ng mL^{-1} was recorded photometrically (OD = optical density). The insets show line scans of dose–response experiments recorded by the PickupImager. Concentration-dependent reflectivity translates into a topographic map after applying different concentrations of CRP antigen and subsequent silver enhancement (100 ng mL^{-1}, 500 ng mL^{-1}, 1 µg mL^{-1}, and 5 µg mL^{-1}). The width of the structures is approximately 1 µm.

data points are above the zero-dose value (0.13 ± 0.02). Bearing in mind that the size of the average particle is 500–800 nm, these observations point toward single-molecule measurements.

We then applied our PickupImager to a sandwich immunoassay of CRP, for which the capture antibodies were deposited by microcontact printing onto glass or polycarbonate carriers. A range of different concentrations of analyte (100 pg mL^{-1}–100 µg mL^{-1} CRP spiked into serum) was applied, each on a separate carrier. The resulting reflective pattern of the precipitated metal particles was imaged with the PickupImager, and the measured density was plotted against the applied antigen concentration (Figure 5, filled circles). Significantly, these measurements showed that different analyte concentrations could easily be distinguished over at least four orders of magnitude. This is a truly remarkable value compared to the two orders of magnitude for conventional ELISA (Figure 5, filled squares). The sensitivity (LOD) of the CD pickup–ELISA was 0.1 ng mL^{-1} ((23 ± 5) grains per 1000 μm^2, which is distinguishable from the zero-dose value of (2.7 ± 0.8) grains per 1000 μm^2). This result corresponds to a LOD of about 1 pM for a molecular mass of 114 kDa.

The adaptation of a biological test to the CD format with pit structures that are 0.6 um wide and 1.5–6 µm long (Figure 1 C) was our final goal. A CD for molecular diagnostics should be readable in a standard PC laptop CD player. The ability to comply with the standard Philips CD coding format[7] requires that the bioactive receptor sites are of the same size (see the Supporting Information).[17] We also achieved this by microcontact printing. The capture antibodies for CRP were printed onto a polycarbonate disc by using stamps that consisted of parts of the pattern of a complete CD relief. Then a CRP assay was performed on the disc at a concentration of 100 ng mL^{-1} antigen, subsequently silver-enhanced, and analyzed (Figure 6). The measured concentration value corresponded well with that obtained from the same assays performed on glass slides (Figure 5).

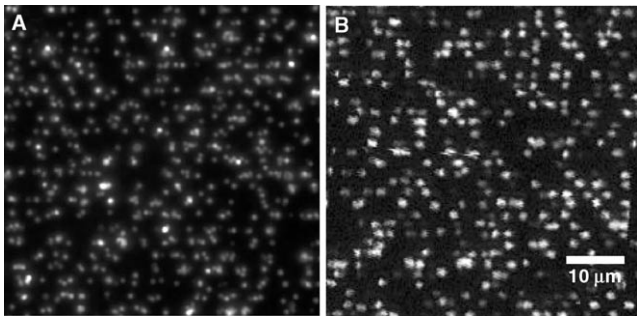

Figure 6. Microscope image (A) and pickup image (B) of a microcontact-printed CRP assay in CD format after Au enhancement. The two images show different areas on the same surface at the same magnification. Both images reveal the pit structure created by the printing stamp. This CRP disc (100 ng mL^{-1}) analyzed by the PickupImager yielded a value of (270 ± 30) grains per 1000 μm^2.

In conclusion, we have demonstrated that a CD pickup can be used to measure biomolecular binding quantitatively within a large dynamic range, and ultimately down to the single-molecule level. The lowest concentration detected with the PickupImager and distinguished from the background signal (samples without antigen) was 1 pM for the CRP antigen. Microcontact printing of proteins[25] or DNA[26] generates large numbers of separate immunoassay "spots". In a regime with a low concentration of analytes adjusted to yield statistically less than one detected binding event per

individual spot on the surface, the number of silver grains detected would in principle allow quantification as a "binary decision"—that is, either a spot carries a silver grain or it does not. The number of "positive" spots measured in a series of many (> hundreds) identical spots then directly correlates to the concentration of the analyte in solution. As the number of binary information units available on a single CD exceeds six billion, the implementation of multiplex tests seems to be limited only by the logistics of receptor-molecule application and fluidic handling. In light of the demand for parallel analysis of multiple binding partners, this "binary biochemistry" detection method could add a novel approach to the microarray technology available today.

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