Smaller Structures Taking the Lead - Analysis and Simulation of Structure Size Influences on Binding Kinetics Down to the Single Molecule Level

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ABSTRACT
This paper describes a method for the quantitative detection of biochemical binding events onto microstructured and functional surfaces on a truly single molecular level. The classic Streptavidin-biotin system is used to provide the last detection step and the binding is visualized via gold-nanoparticles in a SEM. We showed that this allows a spatial resolution down to the nanometer scale. It also allowed us to proof the spot size dependence of binding kinetics according to the theorem of Ekins in one single experiment. The method allows to analyze any binding event on a planar surface and is enabling to measure surface densities of functional groups like the amount of BSA molecules on a blocked glass surface.

INTRODUCTION
Binding kinetics of biomolecules in microstructured systems for genetic- or immuno-assays (e.g. microarrays) are of vast interest in Life Sciences aiming for faster binding and higher yield. Ekins expressed ~20 years ago, that "in theory" smaller spot should bind more molecules per area than larger ones in the same time[1]. This theorem - also initiating the miniaturization of assays to microarrays- is broadly accepted and the validity was shown qualitatively in 2007 by Dandy *et.al.[2]* but still lacks proper quantification especially for structures less than 500 microns. Binding kinetics on surfaces are usually monitored by fluorescence. But fluorescence detection bears several severe problems such as bleaching, quenching on the surface, selfquenching and a spatial resolution of typically several microns [3]. The other typical detection method is the use of enzymes. This is a quite sensitive method but typically the enzyme substrates are soluble and as such their diffusion will lead to a spatial resolutions much worse than with fluorescence. Additionally the "supply" of the enzyme with the substrate is driven by diffusion and as such will be restricted also by the spot size[4].
To overcome these shortcomings we used nanogold-labelling of the detection antibody instead of labeling with an enzyme or fluorophore. The gold particles are visualized with scanning electron microscopy (SEM imaging) with an resolution of 5 nm and the microstructures were generated with microcontact printing[5].

ASSAY PRINCIPLE
Typically the last binding step in an biological assay is the binding between biotin and Streptavidin [4]. The typical immunoassay (fig.1a) was changed by Lange *et. al.*[5] in the final against a gold nanoparticle (fig.1b) and enabled a quantitative ELISA. We reduced the complex immuno-assay to its basic binding step (fig.1c). We structured the surface with bovine serum albumine (BSA) and biotinylated BSA (bBSA). This allowed us to precisely "binding" and "non-binding" surfaces.

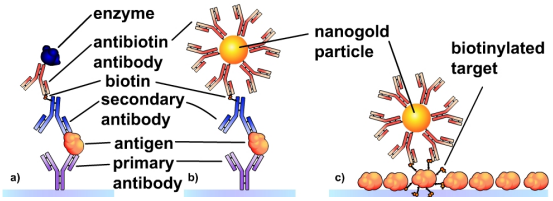

Figure 1: "Evolution" from standard immunoassay ELISA (a) to detection via gold nanoparticles [5] (b) into the simple binder/no-binder structure.

The kinetic of the binding is a simple counting all nanoparticles bound to the structure. The ratio between accessible interaction volume (by diffusion of binders onto surface) to surface area is important for the amount of binding events per area unit. Smaller structures have a higher ratio and as such shall bind (by theory) more gold particles in the same time.

FABRICATION
The generation of the microstructures have been realized by softlithography (fig.2). A planar PDMS-stamp was "inked" with water containing 5 mg/ml bBSA (fig.2a) for 30 min leading to an adsorbed monolayer of bBSA. The supernatant was removed (fig.2b) and the PDMS with the bBSA monolayer was brought into close contact to a microstructured master (photoresist) (fig.2c) leading to a transfer of bBSA to the master. Thus on the PDMS a bBSA "negative" of the master remains (fig.2d), which was finally transferred to a wafer (fig.2e+f) and stored dry in the dark until use.

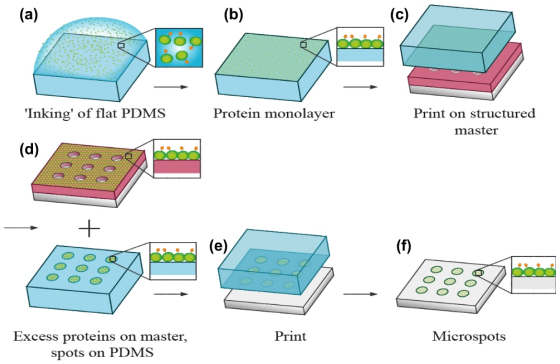

Figure 2: Schema of "inverse" microcontact printing according to [6]

Binding assay

Direct before the assay the microcontact printed wafer was blocked with a 1% (w/w) solution of bovine serum albumine (BSA) to reduce non-specific binding reactions. The wafer was blown dry with nitrogen and than was covered immediately with a solution containing biotin-binding molecules.

In case of pure visualization the wafer was covered with a 200 µg/ml Streptavidin-FITC and the readout was performed in a fluorescence microscope. For the single-binding event assay the wafer was covered with a solution containing 17 µg/ml nanogold-particle labeled antibodies (GAB 20, BBInternational, Cardiff, UK). The readout was performed with a electron microscope (SUPRA 60VP, Zeiss, Germany) by measurement of back-scattered electrons.

SIMULATION

A simulation of the binding kinetics was done via a numerical simulation with CFD-ACE+ from ESI-Group (Eschborn, Germany). The calculations were performed with time steps of 100µs and a diffusion constant of $4.5*10^{-12}$ m^2s^{-1} (measured by dynamic laser scattering). A mesh with a rotational symmetry and roughly 200,000 cells was arranged. The simulated spot diameters ranged from 2 to 120 micron in a tubular volume of 650 µm diameter and 1000 µm height. It was assumed, that every gold-particle interacting with the "binding spot" will bind with a probability p_{bind} of

$$p_{bind} = \frac{n_{bound}}{n_{bound} + n_{free}}$$

Where n_{bound} is the number of already bound gold particle and n_{free} is the number of still free binding positions. It is experimentally assumed that with the used nanogold particles a n_{bound} of 100 particles per μm^2 could be provided.

RESULTS AND DISCUSSION

The layout of the lithographic master (according to fig.2c) covered single dots in a size from 2 up to 120 µm and centered within a hexagon. Each hexagon had an diameter of 650 µm. Within one stamp of in the size of 1 to 1 cm we realized roughly 100 dots.

Fluorescence assay

To visualize the binding direct the biotin was stained with Streptavidin labeled with FITC. This allowed us to control single wafer from the whole production batch. As depicted in fig.3 we obtained very homogeneous dots.

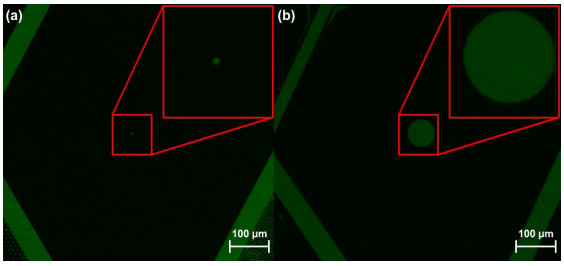

Figure 3: Fluorescence image of a 3 µm (a) and a 76 µm (b) dot. On the edges of the picture the frame of the hexagon is visible.

Single molecule binding assay

The arrangement of the dots allowed that in the first 45 min the dots were not influenced by the hexagon by competition for binding of gold nanoparticles.

Due to the high atomic number of gold the nanogold particles are easily visible in the SEM as "white dots". As depicted in fig.4 the binding specificity was high and in the worst case 1 non-specific bound particle to more than 800 specific bound. In ideal cases we obtained a signal-to-noise ratio of 1:10,000!

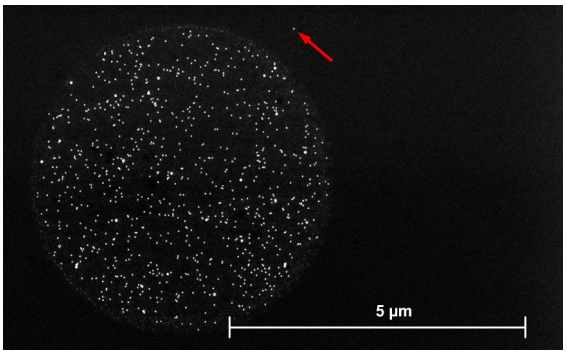

Figure 4: Nanogold particles (white dots) bind specific to the bBSA (dark grey circle). Only one non-specific bining is visible (red arrow).

The obtained SEM images have been analyzed by a self written program based on NIH/Scion Image. The single nanogold particles were identified, counted and recalculated into binding events per μm^2.
Within one single experiment it was possible to proof the theorem of Ekins [4]. It predicts that small dots will "take the lead" and more binding events will encounter there. As example in fig.5 a experiment with a 1.8 nM nanogold particle solution and a incubation time of 10 min is depicted. It is obvious that small structures indeed bind more than the larger ones and to cite Ekins **"smaller structures taking the lead"**.

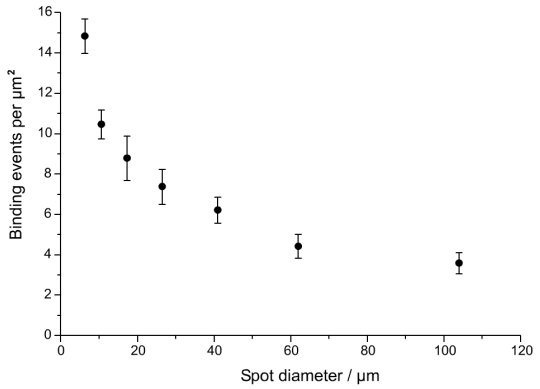

Figure 5: Experimental data of bound particles in dependence of spot size. A reduction of size will lead to more binding events.

The experimental data have been used to adjust the simulations and allow now a prediction of the binding kinetics. This simulation will be used in future for the prediction of binding kinetics in our centrifugal microstructures and microfluidics like the BioDisk. As example (fig.6) a 10 µm structure will saturate within 2000 seconds if a 1.8 nM solution of the nanogold particle is applied whilst a 100 µm structure will not even have generated 20% of the maximum signal. For a "real" assay this effects will be of importance for reducing the incubation time.

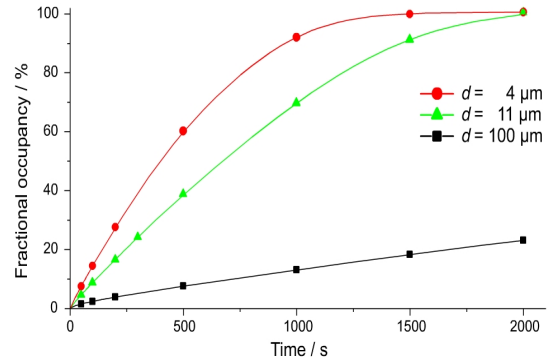

Figure 6: Simulation of binding of a 1.8 nM nanogold particles onto different sized spots.

It has to be taken into account that many biomolecules provide a diffusion constant in the range of 10^{-10} to 10^{-11} m^2s^{-1} and as such are roughly 10-fold faster (in case of antibodies) than the nanogold particles. This means that a 10 µm structure will saturate for a biotin-antibiotin antibody system within 3 minutes, which is indeed a incubation time realized in many *in-vitro* diagnostic assays. But a 100 µm structure will only yield a 20% of the maximum signal.
The simulation also allows us to visualize the temporal progression of the change in the concentrations of the binding molecule (fig.7). Again it is to mention that the reaction times have to be reduced by a factor of 10 for antibodies without nanogold labeling.

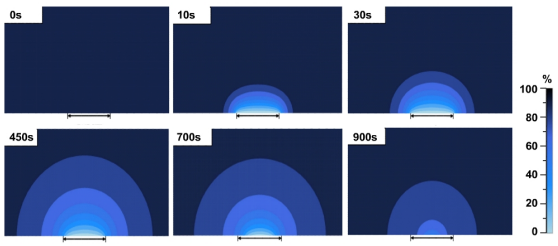

Figure 7: Simulation of temporal concentration progression of nanogold particles onto a 27 µm dots. After 450 s a steady-state is reached and after 700 s the depletion of the media is regenerated by the surrounding media.

CONCLUSION AND OUTLOOK

The presented method to visualize a biological binding event with nanogold particles onto microstructured surfaces allowed us a true insight into the single molecule binding kinetics. It enabled us to proof Ekins theorem within each single experiments. All experiments together allowed us the simulation of binding events onto microstructures. Due to the nanogold particles the diffusion was slowed down and facilitated us to monitor the binding which would have been 10-fold faster than for antibodies (and not visualizable). Now we have a tool in hand to proof the binding and to simulate an according setup for the "real" binding without the nanogold particle. This will enable us to predict binding kinetics in microstructures and microfluidics.

ACKNOWLEDGEMENT

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