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Probing concentration-dependent behavior of DNA-binding proteins on a single-molecule level illustrated by Rad51

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ABSTRACT

Low throughput is an inherent problem associated with most single-molecule biophysical techniques. We have developed a versatile tool for high-throughput analysis of DNA and DNA-binding molecules by combining microfluidic and dense DNA arrays. We use an easy-to-process microfluidic flow channel system in which dense DNA arrays are prepared for simultaneous imaging of large amounts of DNA molecules with single-molecule resolution. The Y-shaped microfluidic design, where the two inlet channels can be controlled separately and precisely, enables the creation of a concentration gradient across the microfluidic channel as well as rapid and repeated addition and removal of substances from the measurement region. A DNA array stained with the fluorescent DNA-binding dye YOYO-1 in a gradient manner illustrates the method and serves as a proof of concept. We have applied the method to studies of the repair protein Rad51 and could directly probe the concentration-dependent DNA-binding behavior of human Rad51 (HsRad51). In the low-concentration regime used (100 nM HsRad51 and below), we detected binding to double-stranded DNA (dsDNA) without positive cooperativity.

Single-molecule investigations of biomolecular behavior and interactions have received increasing interest during the past decade. The approach offers the possibility to explore heterogeneous and dynamic behavior as well as to decipher new mechanisms and rare events at an unprecedented detailed level [1,2]. One of the most commonly used detection methods for single biomolecules is fluorescence microscopy. Fluorescence microscopy has been widely used in combination with microfluidic channels because they offer a possibility of handling and manipulating both the biomolecules and the surroundings [3–5].

The behavior of biomolecules, especially proteins, is largely dependent on variations in the concentration of the biomolecule (protein) itself or of cofactors or ligands, such as ATP, Ca^{2+} and Mg^{2+}, or other small molecules. Deciphering the behavior of biomolecules, therefore, includes experiments using variable concentrations of reaction constituents. In microfluidic channels, the flow is extremely laminar due to low Reynolds numbers leading to diffusion-limited mixing between flow streams with variable contents and allowing for the creation of concentration gradients within the microfluidic channels [6,7]. Thus, the use of microfluidics offers the possibility of delivering reaction constituents in a concentration gradient manner. Combining such a microfluidic device with in vitro single-molecule detection, therefore, would provide a unique and powerful tool that can give single-molecule studies higher throughput as well as reveal heterogeneous and threshold behavior of the reaction constituents.

Microfluidic concentration gradients have been used previously for the study of cell behavior such as chemotaxis and protein expression [8,9], but single-molecule detection and handling *in vitro* puts other demands on the microfluidic devices. Such devices must allow for delivery and handling of the biomolecules while allowing the formation of a concentration gradient that is stable for hours. The gradient must also be narrow enough to fit into a single field of view at the highest possible resolution for fluorescence microscopy in order to be useful for single-molecule investigations in real time. In addition, for high throughput and usability, the concentration gradient must be controllable for various types of molecules in the range from small ions to large protein complexes.

In this study, we have designed and evaluated such a robust microfluidic flow channel system for the study of DNA–protein interactions on a single-molecule level. The design is based on a Y-shaped diffusive mixer that creates a concentration gradient that matches the field of view, which is 80 μm. The width of the gradient is controlled with the flow rate, and the gradient can be used for small ions and chemicals as well as for larger proteins.

One challenge with analysis of single biomolecules is the handling within the microfluidic flow system. In this study, the DNA

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molecules are attached to the bottom glass surface within the microfluidic device that is made of polydimethylsiloxane (PDMS)[1]. The surface functionalization strategy must be carefully considered, fulfilling two requirements: (i) it must allow attachment of biomolecules in such a way that their function is retained and (ii) it must strongly prevent nonspecific interactions. One surface functionalization strategy that meets these requirements is the use of supported lipid bilayer (SLB) membranes [10,11]. SLBs can be prepared on a glass or silica surface from small unilamellar lipid vesicles (SUVs) that, when exposed to the glass surface at a high enough surface concentration, spontaneously fuse and rupture, forming a continuous lipid bilayer. The SLB is fluid and allows lateral diffusion of lipids, and the lipid motion can be directed by hydrodynamic forces from a bulk flow above the SLB [5,12].

Single-molecule techniques suffer from a few important drawbacks; the experimental setups generally have high technical complexity and low throughput. To increase the throughput, the DNA molecules can be anchored to an SLB via a biotin–neutravidin–biotin coupling using biotinylated DNA and lipids, allowing dense DNA arrays to be formed [5]. The DNA strands, tethered to the lipids, are dragged within the fluid lipid bilayer using the bulk flow. The lipids that anchor the DNA strands move in the direction of the flow until they encounter a diffusion barrier that they cannot traverse. This results in an array of parallel DNA molecules on the diffusion barrier—a "DNA curtain." Such a dense DNA array allows for simultaneous investigation of a large number of individual DNA molecules, thereby increasing experimental throughput, and also offers the possibility of finding rare binding events.

In this study, we have combined the DNA array technology with the easy-to-use microfluidic diffusive mixer, described above, which allows for the formation of a stable concentration gradient across the measurement channel as well as rapid switching between two different flows. A DNA array stained with the fluorescent DNA-binding dye YOYO-1, applied to the array in a gradient manner, illustrates the method and serves as a proof of concept. Fluorescently labeled human Rad51 protein (HsRad51) bound to arrays of double-stranded DNA (dsDNA) confirms the applicability for studies of DNA–protein interactions.

Rad51 is a key protein in the DNA repair pathway homologous recombination in eukaryotes [13–15]. *In vitro*, it binds to single-stranded DNA (ssDNA) or dsDNA, forming a helical filament and thereby elongating the DNA by approximately 50% compared with its normal B-DNA conformation [16,17]. At lower concentrations of Rad51 on dsDNA, patches of bound protein, so-called nucleation sites, can be observed [18,19].

The elongated nucleoprotein filament is considered to be the functioning assembly for DNA repair, and the Rad51–DNA complex has, due to its important role in the mechanism of DNA repair, attracted great interest in single-molecule studies [4,20,21]. Experimental techniques used in these studies include optical trapping and total internal reflection fluorescence (TIRF) microscopy, approaches that require specialized, and expensive, equipment. The microfluidic device presented here facilitates visualization of DNA-bound HsRad51 using a standard epifluorescence microscope, thanks to the possibility of rapid and convenient switching between the different flows in the measurement channel.

The described method combines the investigation of single molecules in real time with the concentration dependence of biomolecular interactions and offers a powerful tool for monitoring reaction dynamics and heterogeneity as well as the discovery of rare events and threshold behavior. The applicability of the method is confirmed by probing the concentration-dependent binding behavior of HsRad51 to dsDNA.

[1] *Abbreviations used:* PDMS, polydimethylsiloxane; SLB, supported lipid bilayer; HsRad51, human Rad51 protein; dsDNA, double-stranded DNA; ssDNA, single-stranded DNA; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; biotin–PE, biotin–phosphatidylethanolamine; bp, base pair(s); RT, room temperature; EMCCD, electron-multiplying charge-coupled device.

Materials and methods

Fabrication of microfluidic flow channel systems

The microfluidic flow channel system was made of PDMS using replica molding. An aluminum mold for the simultaneous fabrication of 10 systems was custom-made. The channel height was 200 μm, and the widths of the inlet channels and combined channel were 200 and 400 μm, respectively. Access holes were created by placing needles at the appropriate positions during molding. A PDMS replica was prepared from a mixture of Sylgard 184 base and curing agent (Dow Corning) at a 10:1 weight ratio, which was allowed to cure at 90 °C for at least 2 h. The PDMS replica was bonded to a microscope cover slip, 24 × 60 mm and 0.13–0.19 mm thick (Mentzel Gläser), that had been cleaned in a 2% (v/v) Hellmanex solution (Hellma Analytics) for 30 min followed by thorough rinsing in MilliQ water and drying with N_2(g). For the subsequent preparation of DNA arrays in the flow channels, diffusion barriers were mechanically etched on the microscope cover glass using a diamond drill bit. The glass slide and the PDMS were placed in a plasma cleaner (Harrick Plasma) for 30 s prior to bonding, which was accomplished by placing the surfaces in contact. The systems were then stored at room temperature. Prior to use, polytetrafluoroethylene (PTFE) tubings (0.25 × 0.76 mm, inner and outer diameters, Cole–Parmer) equipped with syringe connectors were fitted into the access holes in the PDMS.

Lipid vesicle preparation

Lipids, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine (DOPC), and biotin–phosphatidylethanolamine (biotin–PE) (Avanti Polar Lipids) were stored in chloroform at −20 °C. Lipid vesicles were prepared from pure DOPC lipids or from 0.5% biotin–PE and 99.5% DOPC. The chloroform solution containing the appropriate lipid or lipid mixture was applied to a round-bottom flask, and the solvent was evaporated under a stream of nitrogen gas. The lipid film was further dried under nitrogen for at least 2 h before it was resuspended in buffer A (10 mM Tris [pH 8.0] and 100 mM NaCl) at a concentration of 20 mg ml^{-1}, vortexed, left at 4 °C overnight, and then vortexed again. If not used directly for extrusion, the lipid solutions were stored at −20 °C. The lipids were mixed and diluted with buffer A to obtain a final concentration of 10 mg ml^{-1} lipids and 0.05% biotin–PE before extrusion through a polycarbonate filter with 100-nm pores (Avanti Polar Lipids). The resulting lipid vesicles were stored at 4 °C and used within 1 week of preparation.

Biotinylation of DNA

A biotinylated oligonucleotide (5′-pAGGTCGCCGCCC-TEG-Biotin, Eurogentec) was annealed to the 12-nucleotide overhang at the right end of the 48,502-bp (base pair) bacteriophage λ-DNA (Roche). A 20-fold molar excess of oligonucleotide was mixed with the λ-DNA in T4 DNA ligase buffer (Promega), heated to 80 °C for 10 min, and then slowly cooled to room temperature (RT). The mixture was briefly (10–15 min) cooled on ice before the addition of T4 DNA ligase, and the reaction was then incubated at RT for at least 2 h. After the completed reaction, the ligase was inactivated by heating to 70 °C for 10 min, and excess oligonucleotide was removed by overnight dialysis against TE buffer (10 mM Tris [pH 8.0] and 1 mM ethylenediaminetetraacetic acid [EDTA]).