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Multiplexed Continuous Biosensing by Single-Molecule Encoded Nanoswitches

Rafiq M. Lubken, Arthur M. de Jong, and Menno W. J. Prins*

Abstract: Single-molecule techniques have become impactful in bioanalytical sciences because of their high detection sensitivity and digital quantitation. However, in the upcoming field of sensors for continuous biomolecular monitoring, the advantages of single-molecule methodologies are yet to be discovered. Multiplexing refers to the ability to measure multiple specific molecules in parallel. This is used to obtain comprehensive knowledge about biological systems and optimal diagnostic power in medical applications. Well-known methods for multiplexing are, for example, bead arrays, real-time PCR, and DNA microarrays. Here, samples are processed with mixtures of reagents and thereafter analyte-specific signals are measured in spectral channels or distinct positions. Such reagent-based multiplexing assays involve taking distinct samples and passing these through sequential processing steps. However, an ideal multiplexing methodology for real-time monitoring does not require reagents nor complicated sample processing. Such a methodology would allow the generation of a continuous and uninterrupted stream of measurement data, over a prolonged period of time, in a simple and cost-effective manner.

Here we describe a novel methodology to achieve reagentless, multiplexed, continuous biomolecular sensing by single-molecule encoded binary nanoswitches. The molecular design and measurement principle are sketched in Figure 1, exemplified with a DNA model system. Figure 1a shows a micrometer-sized particle bound to a substrate by a single nanoswitch. The nanoswitch comprises three components: (1) a single double-stranded DNA stem tethering the particle to the substrate, (2) a single probe attached to the DNA stem, and (3) multiple probes attached to the particle surface. The probe on the stem binds reversibly to target molecules that are captured from solution by the probes on the particle. The probe on the stem encodes the nanoswitch, because the interaction between this stem probe and the target molecules is designed to have a characteristic dissociation rate, which is the basis of the multiplexing functionality. In previous work, we studied sensor designs with less controlled numbers and orientations of probes on the substrate, giving variable responses within and between particles. In the nanoswitch design of Figure 1, every particle has only a single probe, in a well-defined orientation on the central stem. Combined with the smooth spherical particle, unambiguous concentric Brownian motion patterns are obtained (Figure 1b). These translate into time traces with binary transitions, from which unbound and bound state lifetimes and therefore association and dissociation kinetic rates are extracted at the level of individual particles. The dissociation kinetics of each particle are a unique signature that identifies to which kinetic subpopulation the particle belongs (index i or j). Hundreds of particles are measured simultaneously, each kinetically identified, and assigned to their specific subpopulation (Figure 1c). In each subpopulation, the association rates...
are continuously measured. The effective association rate depends on the amount of target molecules captured on the particle. Thus, by using a differently kinetic encoded nanoswitch per particle probes of type \( i \) (dark green) and a single stem probe of type \( i \) (green). Both probes bind reversibly to a single target molecule of type \( i \) (light green) present in solution. The inset shows schematically the DNA-based nanoswitch conjugated to the particle by a NeutrAvidin–biotin interaction and to the substrate by an antibody–antigen (digoxigenin) interaction. A detailed overview of the DNA sequences is given in the Supporting Information. (b) Target molecule binding to the nanoswitch causes the particle to exhibit either of two concentric motion patterns corresponding to the unbound (high mobility) and bound state (low mobility). (c) The radial position of a particle over time shows binary transitions caused by single-molecule binding and unbinding events. The distribution of observed bound state lifetimes per particle can be used to distinguish between, e.g., low-affinity (particles \( i \), blue) and high-affinity (particles \( j \), red) target-specific particle subpopulations, referred to as kinetic identification. Examples of raw data traces are shown in the Supporting Information. (d) Hundreds of particles, each functionalized with an encoded binary nanoswitch, are observed simultaneously. By kinetic identification based on the dissociation kinetics, each particle can be assigned to a target-specific particle subpopulation. For each particle subpopulation, the respective target concentration can be determined over time using the measured association kinetics.

**RESULTS AND DISCUSSION**

Figure 2 illustrates the analytical performance and tunability of the stem probe sensor of Figure 1a (see the Supporting Information). Panels a and b of Figure 2 show the association and dissociation rates measured in both buffer (Figure 2a) and filtered blood plasma (Figure 2b) for a single-stranded DNA target with mid affinity to the particle probe. The mean bound state lifetime \( \tau_B \) (red), determined by fitting all observed bound state lifetimes by a single-exponential distribution (see the Supporting Information), is independent of the target concentration and of the matrix, which is the basis for the kinetic encoding strategy. In contrast, the mean unbound state lifetime \( \tau_U \) shows a clear concentration dependency (blue); an increasing target concentration in solution results in a shorter \( \tau_U \) as more target molecules are bound to the particle and therefore accessible for hybridization to the stem probe. In contrast to the dissociation kinetics, the association kinetics per particle show a broad distribution, indicating particle-to-particle variability. The mean unbound state lifetime \( \tau_U \) could be determined by fitting all observed unbound state lifetimes by a log-normal
multiexponential distribution (see the Supporting Information). This method gives large errors at low statistics, which is particularly visible at low concentrations (see inset of Figure 2b). Figure 2c shows dose–response curves for DNA targets with different affinities. The signal plotted on the y-axis is the switching activity, the average number of binding and unbinding events per particle per time interval. The dose–response curves exhibit an S-shape on a linear-logarithmic scale, which is characteristic for first-order affinity binding. The curves are fitted by the Hill equation.
with $A$ being the activity, $A_B$ the background signal, $A_A$ the activity amplitude (difference between the maximum signal and the background signal), $EC_{50}$ the half maximal effective concentration, and $[C]$ the target concentration in solution. The curves shift to lower concentrations for an increasing affinity between target and particle probes, showing the tunability of the system. Figure 2d shows a dose–response curve measured for the mid-affinity target in blood plasma filtered with a 50 kDa molecular weight cutoff. Here a similar $EC_{50}$ was found, but a higher background activity and larger uncertainty were found, compared to its counterpart in buffer. The higher background activity and larger uncertainty are caused by more nonspecific interactions and lower statistics, respectively.

The response to dynamic changes in target concentration is quantified in Figure 2e–g for the low- and mid-affinity targets in buffer (e, f) and the mid-affinity target in blood plasma (g). The response to a sudden drop in target concentration can be described with a single-exponential relaxation of the observed activity, with characteristic relaxation times of approximately 10 min for the low-affinity target and 40 min for the mid-affinity target. For the mid-affinity target, the single-exponential relaxation profiles in buffer and in blood plasma show comparable time scales within their uncertainty interval (Figure 2f,g).

The multiplexing functionality is shown in Figure 3, using two particle populations having different particle probes and equal stem probes, and two targets with comparable affinities.
to the particle probes and different affinities to the stem probes (see the Supporting Information). In Figure 3a–d, separate flow cells were used to determine the multiplexing specificity and sensitivity. Figure 3a shows the measured average bound state lifetimes for the two cases, that are clearly different and that are independent of target concentration, confirming that particle populations can be identified on the basis of kinetic dissociation rates. Each particle can in fact be considered as a single sensing entity. The distribution of the bound state lifetimes of all individual particles shows clearly two populations, as depicted in Figure 3b. The two populations can be separated by a combination of thresholding (indicated by the black line) and discarding the overlap of the distributions (indicated by the shaded area). The bound state lifetime distributions correspond to results from a simulation (see inset in Figure 3b). Due to the finite duration of the measurement, long bound state lifetimes are underestimated, causing the mean of the distribution of the longer lifetimes to be smaller than the ensemble bound state lifetime (Figure 3a). Increasing the measurement time from 10 to 30 min reduces this underestimation. Longer measurement times result in narrower distributions, which increases the ability to discriminate between the two populations. Figure 3c quantifies the performance of the kinetic identification by its sensitivity and specificity for the low-affinity target. The sensitivity is defined as the fraction of true positives of the total number of particles below the threshold, and the specificity as the fraction of true negatives of the total number of particles above the threshold. Both the sensitivity and specificity can be increased by discarding overlapping data. This is shown in the inset for the values at the position of the red dot in the graph. In Figure 3d, the cross-talk between two particle populations is shown. In this experiment, the low- and high-affinity DNA targets were added to both flow cells sequentially, as indicated in the target concentration profiles. For the mismatched target condition, only a small fraction of switching particles was observed, indicating a negligible cross-talk. For both particle populations, the number of switching particles and the activity per particle increased when the fluid-cell-specific DNA targets were added, confirming the selectivity and sensitivity of the system.

In Figure 3e, the kinetic identification is demonstrated using two mixed particle populations in a single flow cell. The combined bound state lifetimes exhibit a double-exponential distribution, caused by the superposition of two single-exponential distributions of low-affinity and high-affinity dissociation. Using the threshold and window determined in Figure 3b,c, the two particle populations can be separated, resulting in two single-exponential distributions (see inset).

The simulations of Figure 3f support the multiplexing potential. Simulated data were generated from measurements of particles with different dissociation rate constants, corresponding to different interaction strengths between target and stem probe. The association rate constants of all six data sets were equal. The graph shows the resulting bound state lifetime distributions per particle, for a 30 min measurement duration. The width of the distributions is mainly determined by the stochastic binding and unbinding processes; increasing the length of the measurement decreases the width of the distribution. Therefore, longer measurements increase the multiplexing capabilities. To separate bound state lifetime distributions on a particle level, a high accuracy to determine the mean bound state lifetime per particle is not required when the distributions are distinguishable; i.e., the kinetic sensitivity and specificity should be high. Therefore, kinetic encoding potentially results in six levels of multiplexing within a measurement time of 30 min. The time window suitable for multiplexing can be extended by another decade into shorter time scales, by increasing the particle diffusivity (see the Supporting Information).

**CONCLUSION**

In this paper, we presented a sensor design with an encoded binary nanoswitch, enabling continuous sensing of target molecules at picomolar concentrations in human blood plasma, across a broad dynamic range. The ability to create and identify particle subpopulations with distinct dissociation properties allows multiplexed biosensing with high sensitivity and specificity. Multiplexing by single-molecule kinetic encoding does not require any reagents and is therefore suited for continuous sensing and real-time biomolecular monitoring, in contrast to multiplexing methods such as bead arrays,9-10 real-time PCR,11 and DNA microarrays.12 Kinetic encoding can be supplemented with orthogonal identification approaches, such as using particles with different colors (optical identification) and patterning of the sensor surface (identification by surface area imaging). Combining three identification approaches, each with six levels of multiplexing, would potentially give in total $6^3 = 216$ levels. In practice, a trade-off exists between the degree of multiplexing and the analytical performance of the biosensor. To maintain the precision of the concentration determination of multiple target molecules, the number of particles should scale linearly with the degree of multiplexing. Furthermore, while the functionality of kinetically encoded nanoswitches is demonstrated in this paper using DNA as a model system, other markers may be addressed using affinity binders such as aptamers and antibodies.13

In conclusion, single-molecule encoded nanoswitches open the perspective to gain accurate real-time insights into live biological systems by continuous monitoring of biomolecules with a high level of multiplexing, high sensitivity, and high specificity using single-molecule information.

**MATERIALS AND METHODS**

**Binary Nanoswitch Assembly.** All ssDNA oligonucleotides (IDT, standard desalting and HPLC purification for chemically modified DNA, stem probe: 5’-~TGC GAG AAC TCA GCA TAC ATC TA-3’) were diluted in TE buffer (10 mM Tris–HCl, 1 mM EDTA at pH 8.0) to a final concentration of 50 μM. The DNA strands were added together in equivalent amounts to a final concentration of 5 μM per strand in TE buffer with 50 mM NaCl. Using a thermal cycler (Bio-Rad, T100 Thermal Cycler), the mixture was heated to 95 °C and cooled down to 4 °C with a temperature decrease of 1 °C every 35 s. Analysis of DNA tethers was performed in a non-denaturing TBE gel (ThermoFischer Scientific, Novex TBE Gels, 4–20%). The TBE gel was assembled according to the supplier’s instructions, loaded with sample DNA mixtures in Nucleic Acid Sample Loading Buffer (Bio-Rad Laboratories) and an O’GeneRuler Ultra Low Range DNA Ladder (ThermoFischer Scientific), and ran in TBE buffer (89 mM Tris–HCl, 89
mM boric acid, 2 mM EDTA at pH 8.3). Subsequently, the gel was stained with SYBR Gold Nucleic Acid Gel Stain (Thermo Fischer Scientific, 10,000X concentrate in DMSO) in TBE buffer for 30 min. Finally, the TBE gel was visualized using an ImageQuant camera setup (GE Healthcare Life Sciences).

**Silica Particle Functionalization.** Carboxyl-functionalyzed silica particles (Bangs Laboratories, 1 μm mean diameter) at a concentration of 10 mg mL⁻¹ were activated with EDC (Sigma-Aldrich, final concentration of 4.3 mM) and NHS (Merck, for synthesis, final concentration of 10 mM) in MES buffer (0.1 M MES at pH 5.0) for 30 min at room temperature. After activation, the particles were centrifugally washed at 6,000 × g for 5 min using a tabletop spinner (Eppendorf MiniSpin) and resuspended in MES buffer. NeutrAvidin (ThermoFischer Scientific) was dissolved in Milli-Q (ThermoFischer Scientific, Pacific AFT 20) at a concentration of 10 mg mL⁻¹ and added to the activated particles at a final concentration of 500 μg mL⁻¹. The protein functionalization was performed overnight at room temperature. The NeutrAvidin-functionalized silica particles were twice washed in TBS-Tween buffer (25 mM Tris-HCl, 0.15 mM NaCl, 0.05 vol % Tween-20) and twice in 0.1 wt % BSA in PBS-Tween buffer (130 mM NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄, 0.05 vol % Tween-20, at pH 7.4). The binding capacity was determined using a fluorescence supernatant assay with Atto655-biotin and was approximately 800 pmol per mg of particles. The NeutrAvidin-functionalized silica particles were stored at 10 mg mL⁻¹ in PBS-Tween at 5 °C for up to 2 months until use.

**Flow Cell Experiments.** Glass slides (25 × 75 mm, #5, Menzel-Gläser) were cleaned by 15 min of sonication in methanol (VWR, absolute), isopropanol (VWR, absolute), and methanol (VWR, absolute) baths. After each sonication step, the glass coverslips were dried under nitrogen flow. A custom-made fluid cell sticker (Grace Biolabs) with an approximate volume of 24 μL was attached to the glass slide. A flow cell was made by inserting tubing (Freudenberg Medical, monolumen) into the fluid cell sticker and connecting the tubing to a syringe pump (Harvard Apparatus, Pump 11 Elite). First, the flow cell was pretreated with PBS (130 mM NaCl, 7 mM Na₂HPO₄, 3 mM NaH₂PO₄, at pH 7.4) at a flow speed of 500 μL min⁻¹ for 2 min. Functionalization of the glass substrate was performed by physisorption of 83 ng mL⁻¹ anti-digoxigenin antibodies (ThermoFischer Scientific) in PBS for 60 min. Finally, the glass substrate was blocked by incubation with 1.0 wt % casein (Sigma-Aldrich, casein sodium salt from bovine milk) in PBS for 60 min. After each incubation step, the fluid cells were flushed with PBS (250 μL min⁻¹ for 1 min).

NeutrAvidin-functionalized silica particles were incubated in bulk with a 10 nM nanoswitch for 10 min. Subsequently, the particles were coated with ssDNA by an incubation with 40 μM biotin-labeled single-stranded oligonucleotide (IDT, standard desalting, S'-TAG TCA GGT TGG ATG TCT AC-3'-biotin). The particles were thrice centrifugally washed in 1.0 wt % BSA (Sigma-Aldrich, lyophilized powder, essentially globulin free, low endotoxin, ≥98%) and 0.05 vol % Tween-20 (Sigma-Aldrich) in PBS at 6,000 × g for 5 min using a tabletop spinner (Eppendorf MiniSpin). Finally, the particles were resuspended in PBS/BSA/Tween-20 to a final concentration of 0.17 mg mL⁻¹ (0.26 μM) and sonicated using an ultrasonic probe (Hielscher). The particles were added to the flow cell at a flow speed of 50 μL min⁻¹ for 5 min and incubated for 30 min. After incubation, the fluid cell was reversed and subsequently flushed with PBS/BSA/Tween-20 at a flow speed of 50 μL min⁻¹ for 5 min to remove unbound particles. A ssDNA target (IDT, standard desalting, low affinity: S'-AAC CTG ACT AAA AAT AGA TGT ATG-3', mid affinity: S'-CAA CCT GAC TAA AAA TAG ATG TAT G-3', high affinity: S'-CCA ACC TGA CTA AAA ATA GAT GTA TG-3') at the required concentration in PBS/BSA/Tween-20 was added at a flow speed of 50 μL min⁻¹ for 5 min and incubated for 20 min to reach equilibrium.

**Flow Cell Experiments with Blood Plasma.** Single-donor human blood plasma (Sanquin, The Netherlands, citrate stabilized, healthy volunteer) was filtered through a 50 kDa molecular weight cutoff centrifugal filter (Merck Millipore, Amicon). The plasma filtrate was collected and spiked with ssDNA at the required concentration. The measurements were then performed as described in the previous section.

**Particle Imaging and Tracking.** Samples were observed under a white light source using a microscope (Leica DM6000M) using a dark field illumination setup at a total magnification of 20× (Leica objective, N PLAN EPI BD, 20×, NA 0.4). A field-of-view of approximately 400 × 400 μm² was imaged using a CMOS camera (Grasshopper 2.3 MP Mono USB3 Vision, Sony Pregius IMX174 CMOS sensor) with an integration time of 10 ms and a sampling frequency of 30 Hz. The silica particles were tracked with a 3 nm accuracy using the center-of-intensity of the bright particles on the dark background. Trajectory parameters were calculated which describe the motion pattern and were used to select single-tethered particles.

**State Lifetime Analysis.** Particles that showed strong irregularities in their motion pattern (e.g., strongly confined or asymmetrical) or no switching behavior were excluded from further analysis. The measurements were performed in a flow cell setup in which the target concentration was increased sequentially by means of buffer exchange. After 20 min incubation, the measurement was performed. Trajectory analysis was performed only on particles showing a bimodal distribution in the averaged radial position. In order to detect binding and unbinding events, a dual thresholding method was implemented in which the threshold was set on the (local) minimum between the two peaks of the bimodal distribution. A dual threshold with a 12.5% offset was found to yield accurate event detection with 91% sensitivity and 96% specificity (data not shown here). Based on the detected events, the bound (low mobility) and unbound states (high mobility) could be identified. The lifetimes of the two states were plotted in a cumulative distribution function for different target concentrations to extract the association and dissociation rate constants (see the Supporting Information).

**Simulations.** Data were simulated using experimental positional data of bound and unbound particles. For each simulation, two single-exponential distributions were generated: one with a given mean bound state lifetime and one with a given mean unbound state lifetime. The particle traces...
were reconstructed block-by-block with each block length according to the two predefined single-exponential distributions. Nonspecific interactions and inter- and intraparticle heterogeneity were neglected. Subsequent time-dependent analysis was performed as if experimental data were analyzed.

**ASSOCIATED CONTENT**

1. **Supporting Information**

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.nanolett.9b04561.

Details on used DNA sequences, quantifying single-molecule affinity kinetics, observed heterogeneity in association kinetics, and the potential of multiplexing by kinetic encoding (PDF)

Comprehensive visualization of multiplexing by kinetic encoding (MP4)

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**Author Contributions**

All authors conceived and designed the methodology, measurement system, and experiments. R.M.L. performed the experiments and the data analysis. All authors interpreted the data, discussed results, and co-wrote the paper. All authors approved the submitted version of the manuscript.

**Notes**

The authors declare the following competing financial interest(s): M.W.J.P. is listed as one of the inventors on patent application WO/2016/096901 (Biosensor based on a tethered particle). M.W.J.P., A.M.d.J., and R.M.L. are listed as inventors on a filed patent application relating to the data presented in this paper. M.W.J.P. is cofounder of Helia Biomonitoring BV that has a license to these patents. All authors declare no further competing interests.

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