dynamic BIOSENSORS

Application Note

# Kinetic measurements of oligodeoxynucleotide hybridization with switchSENSE® and comparison with Biacore and solution data

Keywords: Kinetics I DNA I fluorescence proximity sensing (FPS) I system comparison I repeatability/ reproducibility

## Background & Aims

The formation (hybridization) of double stranded DNA from two short single strands is an interesting model system for the investigation of binding kinetics. The duplex stability of short DNA strands (oligodeoxynucleotides, ODNs) increases with the number and types of base pairs formed and hence depends on the ODN length. In a recent SPR study, Palau and Di Primo investigated the interactions of 10 and 11 base pair ODNs featuring nano-molar dissociation constants using a Biacore™ T200 instrument [1].

Here, we report DNA hybridization measurements with the switchSENSE sensor using the same sequences as in [1] and determine on- and off-rates ($k_{ON}$, $k_{OFF}$) and the dissociation constants ($K_D$). The results are compared with SPR data and with stopped-flow measurements of the on-rate in the solution phase. We find very good agreement between values determined with switchSENSE, SPR, and stopped-flow. It is shown how any sequence of interest can be captured on a switchSENSE multipurpose chip (MPC) and that consistent kinetics data are obtained with different ligand designs using the Fluorescence Proximity Sensing (FPS) mode.

## Methods

In their 2012 Biacore SPR study [1], Palau and Di Primo immobilized the ligand sequence on the Biacore sensor chip by extending their 13 nucleotide (nt) target sequence by an A5 spacer and a triethyleneglycol (TEG) linker with a biotin tag (cf. 'Ligand seq. SPR' in Table 1). The ligand was captured via its biotin tag at a level of approximately 5 RU on a CM5 chip with a 100 nm thick dextran matrix that had been modified via amine coupling with streptavidin beforehand. Two DNA strands with 10 and 11 complementary bases to the ligand sequence were used as analytes (cf. Table 1). In order to improve the SPR signal change and signal-to-noise ratio upon binding, the analyte sequences were flanked (labelled) by overhangs comprising fifteen adenines (A15).

The SPR measurements were performed on a Biacore™ T200 instrument in single cycle kinetics mode: 3 analyte solutions (31 nM, 125 nM, 500 nM) were injected for 30 s at a flow rate of 25 µL/min, and were followed by dissociation phases of 30 s, 30 s, and 300 s, respectively.

**Table 1** | DNA sequences used in the switchSENSE (this work) and SPR (reference 1) assays

| switchSENSE assay | | | |
|---|---|---|---|
| Ligand swSE-A[†] (immob.) | 5′ | Q-AAA CTC ACA ACA GG AAA-cNLB | 3′ |
| Ligand swSE-B[†] (immob.) | 5′ | CTC ACA ACA GG AAA-cNLB | 3′ |
| 11 nt analyte | 3′ | GAG TGT TGT CC | 5′ |
| 10 nt analyte | 3′ | GAG TGT TGT C | 5′ |

| SPR assay, ref [1] | | | |
|---|---|---|---|
| Ligand SPR[†] (immob.) | 5′ | CTC ACA ACA GGC TAA AAA-TEG-biot | 3′ |
| 11 nt analyte – $A_{15}$[‡] | 3′ AAA AAA AAA AAA AAA | GAG TGT TGT CC | 5′ |
| 10 nt analyte – $A_{15}$[‡] | 3′ AAA AAA AAA AAA AAA | GAG TGT TGT C | 5′ |

[†] ligand sequences are flanked by $A_3$ or $A_5$ to minimize "end effects" (stacking interactions with bound analyte)

[‡] analyte sequences for the SPR study were flanked with $A_{15}$ tails for signal enhancement

nt…nucleotide TEG…triethyleneglycol linker; Q…black hole quencher; cNLB…complementary nanolever seq. B (48 nt)

Wash steps of 2 min with 20 - 40 mM NaOH were used to regenerate the surface. Data were analyzed with BiaEval™ 4.1 software and double-referenced (i.e. subtraction of reference channel signal and subtraction of blank injection).

In the switchSENSE assay, the ligand sequence was directly hybridized to the sensor surface of a multi-purpose chip (MPC2-48-G1R1-S). Hence, there was no need for a biotin capture tag or streptavidin immobilization. The ligand sequence was extended by a sequence complementary to the 48 mer nanolever 'B' of the MPC chip. Two versions of the ligand sequence were tested: sequence swSE-A featured a black hole fluorescence quencher (BHQ) at its surface-distal end, while target swSE-B was not modified (cf. Table 1). Note that the ligand sequence lengths were slightly different from the ones used in [1], i.e. 11 nucleotides long (instead of the 13 nt in [1]). The analyte sequences were label-free 10 nt and 11 nt. Signal-enhancing A15 tails like in the SPR assay were not used in the switchSENSE assay.

The switchSENSE experiments were performed with a DRX² instrument using the Fluorescence Proximity Sensing (FPS) measurement mode at a sampling rate of 1 Hz and a constant repulsive potential of - 0.1 V. The green fluorescence signal was used for detection and the illumination power was set to 3 or 4; photo-bleaching was not observed during the measurement time. Experimental workflows were planned and generated using the switchBUILD™ software. Analyte solutions were injected for 120 s at 5 different concentrations (6.25 nM, 12.5 nM, 25 nM, 50 nM, 100 nM), followed by dissociation phases of 600 s and 1200 s for 10 nt and 11 nt analytes, respectively. The flow rate was 50 µL/min. In between measurements, the sensor was regenerated by a 1 s NaOH wash step. Data were analyzed with the switchANALYSIS™ software: fluorescence signals were normalized to the baseline before analyte injections and a blank injection was used for reference subtraction. Kinetic rate constants were analyzed by fitting single-exponential functions (Eq. 1) to all association and dissociation curves of one run simultaneously (global fit).

For the SPR as well as the switchSENSE assays the binding/running buffer was 20 mM sodium phosphate, pH 7.2, 100 mM NaCl, 0.05% Tween-20, and the temperature was set to 23°C.

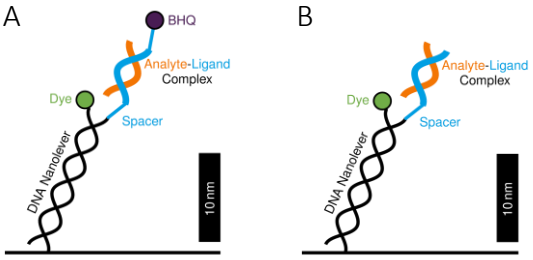


**Figure 1 |** Schematic drawings of surface modifications with the two different switchSENSE ligand designs. Ligand sequences are shown in blue, analyte sequences are orange. (A) and (B): For the functionalization of the switchSENSE detection spots the ligand sequence is extended by a sequence that is complementary to the immobilized nanolever sequence (black) of the multi-purpose chip. Ligand design A features a fluorescence quencher (BHQ), ligand design B does not.

## Results & Discussion

The switchSENSE binding kinetics measurements were performed using the Fluorescence Proximity Sensing mode (FPS, see also the 'Guide to switchSENSE Measurement Modes' on www.dynamic-biosensors.com). In an FPS assay, the fluorescence intensity of the dye, which is attached at the DNA nanolever's top end, is monitored over time while the DNA nanolever is kept in an upright orientation by applying a constant low repulsive potential to the electrode. Keeping the DNA orientation fixed has two implications here: on one hand, orienting the DNA nanolevers upright on the sensor surface ensures an optimal accessibility of the ligand sequence which protrudes from the top of the nanolever[1]. On the other hand, the long-ranged fluorescence energy transfer to gold surface, which is normally used for the time-resolved measurement of the DNA orientation switching, is a constant factor and therefore can be neglected in the interpretation of the fluorescence signal.

Hence, changes in the observed fluorescence intensity over time indicate the association and dissociation of analyte molecules. These fluorescence changes arise because the presence of analyte alters the physico-chemical environment of the dye. Whether the binding of an analyte molecule increases or decreases the dye emission can have different causes. To illustrate this here, we chose two different ligand designs (cf. Figure 1 and Table 1): one ligand sequence (swSE-A) was modified with a Black Hole Quencher (BHQ), the other ligand (swSE-B) was unmodified. As will be shown in the following, both designs produced consistent real-time kinetics data.

Figure 2 shows association and dissociation phases for the interaction of 10 nt and 11 nt analytes with target sequence swSE-A immobilized on a multi-purpose chip. During the association phase, when analyte sequences bind to the ligands, the fluorescence increases; during dissociation of analyte from the sensor the fluorescence decreases again.

The signal change can be explained as follows: when the ligand sequence is single stranded, it is flexible and coil-like (the mechanical persistence length of ssDNA is less than 2 nm), which allows the quencher to approach the fluorophore at the top of the nanolever. The quencher diminishes the fluorophore emission through a combination of FRET (fluorescence resonance energy transfer) and static quenching (contact mediated). Upon

[1] The influence of the applied electrode potential at the ligand height can be considered negligible, since electric fields decay within a few nanometers in solution. For example, for the 120 mM salinity used here the Debye screening length is ~1 nm, while the nanolever length is 16 nm.

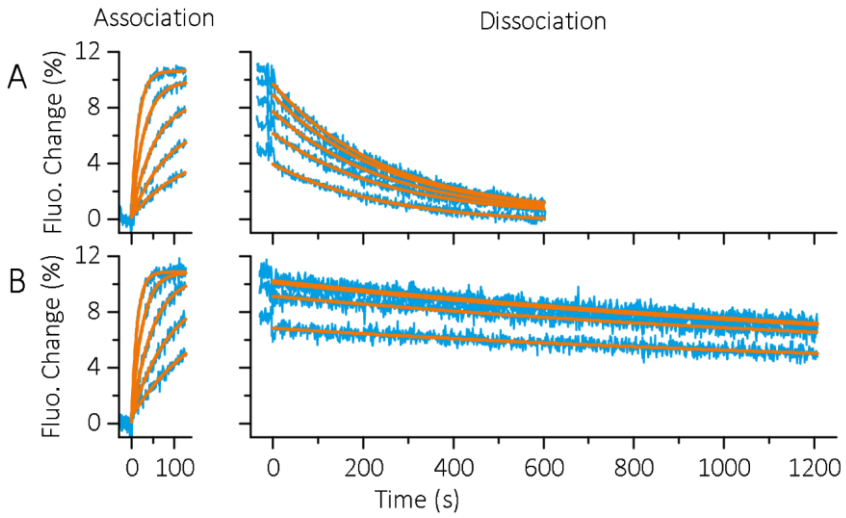


**Figure 2 |** switchSENSE association and dissociation kinetics measurements for oligodeoxynucleotides binding to ligand sequence swSE-A on a multi-purpose chip. (A) 10 nt analyte and (B) 11 nt analyte injected at concentrations of 6.25 nM, 12.5 nM, 25 nM, 50 nM, 100 nM. Data are referenced with a buffer injection. Solid orange lines are least-squares fits with single-exponential functions, Eq. 1 (simultaneous 'global' fit of all curves with switchANALYSIS™).

binding of the analyte sequence the ligand transforms to a rigid duplex which effectively separates the quencher from the fluorophore. As a result, the fluorescence emission increases. For complete saturation of the sensor with analyte (all ligand/analyte complexes are duplexes), a fluorescence enhancement of app. 11% can be observed; the intensity at any given point in time during the association and dissociation phases is proportional to the number of analyte molecules bound to the sensor (fraction bound).

The measured kinetics show the expected behavior of a reversible 1:1 interaction.

**Table 2** | Association and dissociation rate constants ($k_{ON}$, $k_{OFF}$) and dissociation constant ($K_D$) for 10 bp and 11 bp duplexes determined with switchSENSE (this work) and SPR Biacore™ (Palau and Di Primo [1]).

| Number of base pairs | System | $k_{ON}$ ($10^5$ $M^{-1}s^{-1}$) | $k_{OFF}$ ($10^{-3}$ $s^{-1}$) | $K_D$ (nM) |
|---|---|---|---|---|
| 10 bp | switchSENSE[1] | 5.86 ± 0.09 | 3.97 ± 0.03 | 6.77 ± 0.11 |
| | Biacore[2] | 2.98 ± 0.08 [4] | 3.46 ± 0.04 | 11.6 [4] |
| 11 bp | switchSENSE[1] | 7.81 ± 0.14 | 0.57 ± 0.04 | 0.73 ± 0.05 |
| | Biacore[2] | 3.33 ± 0.04 [4] | 0.59 ± 0.01 | 1.8 [4] |

[1] Errors are fit errors from a global analysis of 5 association/dissociation curves analyzed with switchANALYSIS (switchSENSE)

[2] cf. Figure S5 and Table S2 in the Supporting Information of reference [1]; sequences are labelled A', C10, and C11 in [1], corresponding to SPR ligand, 10 nt, and 11 nt here. Errors are from 3 association/dissociation 'single cycle' curves analyzed with BiaEval (SPR).

[3] The solution phase error is the standard deviation of three measurements.

[4] The on-rates were underestimated in this SPR study due to interference of the $A_{15}$ tail, see text. Consequently, the $K_D$'s are overestimated.

Association and dissociation kinetics follow single-exponential time courses

$$F(t) = a \cdot \left(1 - exp\{-k_{ON}^{obs} \cdot t\}\right) \quad (1a)$$

$$F(t) = a \cdot exp\{-k_{OFF} \cdot t\} \quad (1b)$$

indicating that potential measurement artefacts like mass-transport limitations or rebinding effects do not occur [2-5]. The observable rate constants of the association curves scale with the analyte concentration $k_{ON}^{obs} = c \cdot k_{ON} + k_{OFF}$ (with $k_{ON}$ being the intrinsic molecular on-rate) while all dissociation curves feature the same off-rate $k_{OFF}$, independent of the saturation level reached after association. After the dissociation phases, the signals return to their initial values (cf. Figure 1 A, right panel), which confirms that irreversible (unspecific) adsorption of analyte to the sensor surface does not occur, i.e., the sensor response is well-behaved.

The results for $k_{ON}$ and $k_{OFF}$ and the dissociation constant $K_D = k_{OFF}/k_{ON}$ from a global fit analysis with switchANALYSIS™ are given in **Table 2**. The table also lists the kinetic rates obtained by Palau and Di Primo in their Biacore SPR study.

*Comparison with Biacore SPR measurements*

The off-rates determined by switchSENSE and SPR are in very good agreement: absolute $k_{OFF}$ values deviate by less than 15% from each other. Both measurement systems show the same trend for analyte sequences of different length, namely an approximately 6-fold increase in stability for the 11 bp duplex over the 10 bp duplex.

The on-rates show the same tendency of marginally higher on-rates for the 11 bp duplex; however, for both analytes, the switchSENSE $k_{ON}$ values are two-fold higher than the SPR values reported in [1].

When discussing our results with the authors of the SPR study, Carmelo Di Primo hypothesized that the reason for the slower on-rate in the SPR analysis might be that the A15 tails which were used for signal enhancement in the SPR study might have hampered the base pairing reaction of the dedicated binding part to some extent. Carmelo Di Primo kindly offered to optimize the SPR assay and repeat the measurements without the A15 tail. In fact, he found that the on-rates without the A15 tail were two to three-fold faster compared to the previous SPR results with A15 (personal communication). This corroborates the notion that the A15 tail hampers base-pairing of the target sequence and explains the differences between the on-rates reported in the initial SPR study. The results of the switchSENSE and newer SPR measurements are in good agreement.

*Comparison with stopped-flow measurements in solution*

In order to investigate the on-rates further with an orthogonal technique, we performed association measurements in solution using a stopped-flow fluorescence assay (Figure 3). As dissociation rates cannot be measured in a stopped-flow experiment, only $k_{ON}$ but neither $k_{OFF}$ nor $K_D$ values can be compared to the biosensor data. The 11 nt analyte sequence was injected under intense stirring at equimolar ratio into a cuvette containing swSE-A sequences, while the fluorescence increase upon association of the two reactants – like in the switchSENSE measurement – was monitored over time using a Shimadzu RF5301 spectrofluorophotometer. The fluorescence signals were fitted with the equation

$$F(t)=F_\infty - (F_\infty - F_0)/(1+c \cdot k_{ON} \cdot t) \quad (2)$$

which describes the association kinetics of two equimolar reactants in the solution phase [6]. The

association rate was determined from three injections, $k_{ON}$ = 14 ± 5 × $10^5$ $M^{-1}$ $s^{-1}$ (mean ± standard deviation). We note that the coefficient of variation (COV= std. dev./mean) for repeated solution measurements is relatively high, i.e. COV = 36 %. A single experiment with 10 bp DNA gave a similar result, $k_{ON}$ ≈ 20 × $10^5$ $M^{-1}s^{-1}$ (data not shown).

The on-rates measured in the solution phase are in reasonable agreement with on-rates determined for surface bound ligands in the switchSENSE experiment.

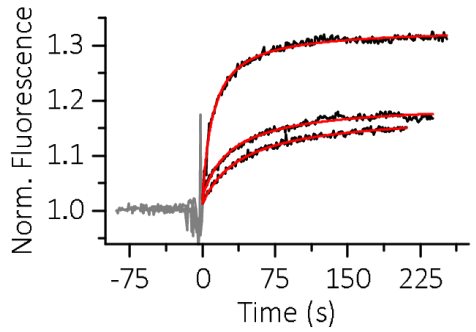


**Figure 3** | Solution phase stopped-flow measurements of the association rate between sequences swSE-A and 11 nt analyte. Measurements have been performed in triplicates with reactant concentrations of 25 nM, 25 nM, and 50 nM. Solid red lines are least-square fits with Eq. 2.

*Influence of ligand design*

In addition to ligand swSE-A, another ligand design, swSE-B, was investigated in order to, (i) exemplify the effect of different chemical environments on the FPS signal, and (ii) to evaluate the reproducibility of the kinetic analysis for different ligands and repeated measurements. Ligand swSE-B features the same binding sequence as swSE-A, but lacks the fluorescence quencher at its surface-proximal 5' end. As analytes, the same label-free 10 nt and 11 nt sequences were used.

The binding of analyte again causes clear changes in the fluorescence signals and the association and dissociation kinetics can be analyzed from fitting Eq. 1 to the data shown in Figure 4. Generally, the on- and off-rates of both ligands agree well, cf Table 3. Values from swSE-A and swSE-B ligands differ by less than 25%, except for the off-rates of the 10 nt analyte, which differ by 60%. Taking into account that the presence of the black hole quencher might slightly alter the binding affinity of the ligand-analyte strands, we consider the kinetic rate constants obtained with the two ligand designs consistent.

With regard to the FPS signal, it is interesting to examine a few notable differences in the fluorescence signals with and without quencher (Figures 2 and 4, respectively): In contrast to the ligand with a quencher (swSE-A), the fluorescence decreases upon binding of analyte to the 'bare' ligand swSE-B. In the latter case, suppression of fluorescence by nearby nucleobases is the dominating effect [7]. The formation of base pairs in the proximity of the dye quenches its fluorescence, in particular, the more G-C base pairs are formed close to the fluorophore position, the stronger the quenching effect. Figure 5 shows 4.5% quenching for the 11 nt analyte where 3 nucleotides (A3) towards the dye remain unpaired, and 3% quenching for the 10 nt analyte, where 4 nucleotides (CA3) towards the dye remain unpaired. When the A3 spacer was omitted and the ligand-analyte sequence was only 1 nucleotide away from the dye (cf. Table 1 and Figure 1), even stronger quenching was observed (data not shown). The type of nucleobases, especially the presence of guanine (which is active in photoinduced electron transfer), the base pairing state, as well as the

**Table 3** | Comparison of different ligands: Association and dissociation rate constants ($k_{ON}$, $k_{OFF}$) and dissociation constant ($K_D$) for 10 bp and 11 bp duplexes determined with switchSENSE ligands swSE-A (with quencher) and swSE-B (without quencher).

| Number of base pairs | Ligand | $k_{ON}$ ($10^5$ $M^{-1}s^{-1}$) | $k_{OFF}$ ($10^{-3}$ $s^{-1}$) | $K_D$ (nM) |
|---|---|---|---|---|
| 10 bp | swSE-A | 5.86 ± 0.09† | 3.97 ± 0.03† | 6.77 ± 0.11† |
| | swSE-B | 7.13 ± 0.54‡ | 6.38 ± 0.55‡ | 9.01 ± 0.12‡ |
| 11 bp | swSE-A | 7.81 ± 0.14† | 0.57 ± 0.04† | 0.73 ± 0.05† |
| | swSE-B | 8.90 ± 0.34† | 0.72 ± 0.06† | 0.81 ± 0.08† |

† global fit analysis of 5 associations & dissociations from one measurement run, global fit value ± fit errors
‡ global fit analysis of 5 associations & dissociations from four measurement runs, mean value ± std. dev (n=4).

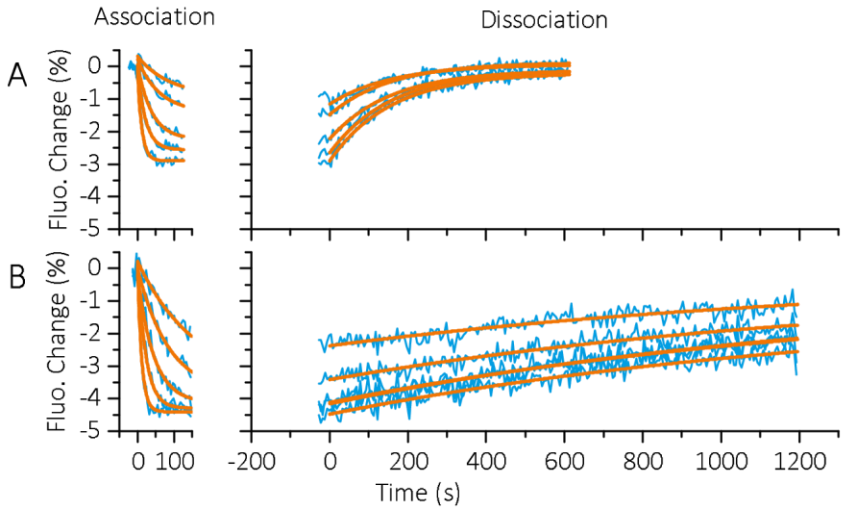


**Figure 4** | switchSENSE association and dissociation kinetics measurements for oligodeoxynucleotides binding to ligand sequence swSE-B on a multi-purpose chip. (A) 10 nt analyte sequence injected at concentrations of 7.5 nM, 15 nM, 30 nM, 60 nM, 120 nM and (B) 11 nt analyte sequence injected at concentrations of 6.25 nM, 12.5 nM, 25 nM, 50 nM, 100 nM. Data are referenced with a buffer injection. Solid orange lines are least-squares fits with single-exponential functions, Eq. 1. The sampling rate was 0.2 Hz.

distance and the spatial arrangement (orientation) with respect to the fluorescence probe all contribute to the nucleobase-mediated quenching effect.

*Repeatability*

We tested the repeatability of the kinetic measurements with the more demanding of the two ligand systems, swSE-B. Because the signal-to-noise level of this ligand was lower than for swSE-A, we expected that reproducible results were harder to achieve for swSE-B and hence the

**Table 4** | Repeatability of $k_{on}$ and $k_{off}$ analysis of the swSE-B ligand and the 10 nt sequence as analyte. Measurements were repeated on different measurement electrodes in different flow channels, running 5 analyte injections and dissociations each.

| Run # | $k_{ON}$ ($10^5$ $M^{-1}s^{-1}$) | $k_{OFF}$ ($10^{-3}$ $s^{-1}$) | $K_D$ (nM) |
|---|---|---|---|
| 1 | 6.99 | 6.99 | 10.0 |
| 2 | 7.44 | 6.49 | 8.72 |
| 3 | 6.43 | 6.38 | 9.92 |
| 4 | 7.66 | 5.65 | 7.37 |
| **Mean value** | 7.13 | 6.38 | 9.01 |
| **Standard deviation** | 0.54 | 0.55 | 0.12 |
| **Coefficient of variation†** | **7.6 %** | **8.6 %** | **13.7 %** |

† Coefficient of variation COV = std. deviation/mean

obtained coefficients of variation (COV) would represent upper limits. Measurements were repeated on different detection spots in different flow channels using the same instrument (electrode-to-electrode and channel-to-channel variation). For each measurement, five association and dissociation curves were recorded and on- and off-rates were analyzed with a global fit analysis using switchANALYSIS. The coefficients of variation for the on-rate and off-rate were found to be better than 10% (cf. Table 4).

## Conclusions

It was shown that standard multi-purpose chips can be used in a straightforward manner to investigate hybridization reactions of oligodeoxynucleotides of any sequence.

Functionalization (immobilization) and regeneration is possible using a simple workflow by extending the ligand sequence of interest with an 'anchor' sequence which is complementary to the DNA immobilized on the multi-purpose biochip.

Duplex formations between immobilized ligand sequences and label-free 10 nt and 11 nt analyte sequences were monitored successfully by switchSENSE in real-time using the FPS mode.

The association and dissociation rate constants were analyzed and it was found that while the on-rates are similar ($k_{ON}$ = 6 - 8 × $10^5$ $M^{-1}s^{-1}$), the 11 bp duplex was >6 times more stable than the 10 bp duplex ($k_{OFF}$, 10bp = 4 × $10^{-4}$ $s^{-1}$ vs. $k_{OFF}$, 11bp = 0.6 × $10^{-4}$ $s^{-1}$).

The $k_{ON}$, $k_{OFF}$ values determined with the switchSENSE sensor agree very well with SPR measurements performed on a Biacore T200 instrument (reference [1] and personal communication of recent measurements by C. Di Primo). The on-rates also agree well with $k_{ON}$ values determined by stopped-flow measurements in solution, indicating that surface-induced measurement artifacts did not occur.

A comparison of two different ligands of the same sequence, with or without a fluorescence quencher, respectively, gave consistent results regarding the analyzed kinetic rate constants. A

superior signal-to-noise ratio was observed for the quencher-modified ligand. The repeatability of the binding kinetics was investigated regarding spot-to-spot and channel-to-channel variation. Consistent $k_{ON}$ and $k_{OFF}$ values were obtained with coefficients of variations of less than 10%.

## Acknowledgements

We are very thankful to Carmelo Di Primo (Univ. Bordeaux and INSERM) for helpful advice and discussions, his critical review of this Application Note and his willingness to perform additional SPR experiments to elucidate the influence of the poly-A tail used in [1] on the observed association rate.

Authors I Felix Kröner, Prayanka Rajendran, Ralf Strasser, Ulrich Rant
Contact I info@dynamic-biosensors.com
Dynamic Biosensors GmbH I December 2016

## References

[1] W Palau and C Di Primo, *Biochimie* 94, 1891-1899 (2012)
[2] RW Glaser, Anal. Biochem. 213, 152-161 (1993)
[3] P Schuck and AP Minton, Anal. Biochem 240, 262-272 (1996)
[4] P Schuck, Biophys. J. 70, 1230-1249 (1996)
[5] L Nieba, A Krebber, A Plückthun, Anal. Biochem. 234, 155-165 (1996)
[6] Theory Note "Association rate of two equimolar reactants in solution" on www.dynamic-biosensors.com/switchsense/application-library/
[7] Principles of Fluorescence Spectroscopy, Joseph R. Lakowicz, Springer (2006)