Real-time measurement of DNA polymerase activity and inhibition with **switch**SENSE® and the **heliX**® biosensor

Keywords: nucleic acid-modifying enzymes I heliX I enzymatic activity | enzyme inhibition | surface energy transfer

Polymerases play a key role in all living cells for the replication and preservation of genetic information. They are also interesting drug targets for several diseases and viral infections, and they find applications in the biotechnology field, such as in PCR and in DNA sequencing.

Monitoring the real-time activity of these enzymes not only allows for their characterization but also facilitates inhibition studies and engineering to meet the pharmaceutical demand.

The heliX® biosensor, featuring a simple workflow and customizable chip, enables straightforward evaluation of enzymatic activity across various enzymes. Herein, we show how to perform real-time measurements of Taq polymerase elongation activity and inhibition.

Background

DNA polymerases are the enzymes responsible for DNA replication in every living cell. These enzymes catalyze the incorporation of mononucleotides into a growing strand (primer) using another strand (template) as a guide for DNA synthesis. Therefore, DNA polymerases are of great interest in biomedical research and for their application in the biotechnology field (e.g. DNA sequencing and PCR).

Here, we show how the **heliX**® biosensor can be used to measure the DNA binding of Taq polymerase (Pol) in real time, its DNA elongation activity, and its activity inhibition, using a customizable chip and simple **switch**SENSE® workflow (Figure 1). This setup can be easily translated to other types of enzymes (e.g. reverse transcriptases, nucleases, helicases, etc.), providing valuable information, such as association rate constants (k_{on}), binding affinity (K_d), catalytic rate (k_{cat}) and the Michaelis-Menten constant (K_M).

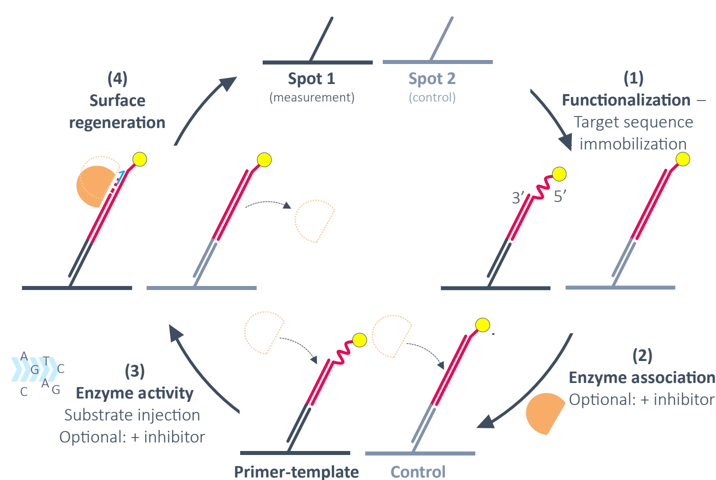

Figure 1 | switchSENSE® workflow for enzyme binding and activity study. 1) Immobilization of the Primer-Template sequence in spot 1 (measurement spot) and a dsDNA control sequence in spot 2 (control spot) on the biochip surface. 2) Association of Taq Pol to the DNA strands. 3) Injection of dNTPs at increasing concentrations (from 0.03 µM to 20 µM) to monitor the DNA elongation in real time. 4) Regeneration of the chip surface by injection of a high pH solution.

Methods

The experiments are performed on a **heliX**® biosensor using a standard **heliX**® adapter chip (ADP-48-2-0, Dynamic Biosensors GmbH). The enzymatic activity experiments are set up and analyzed using the **heliOS** software (Dynamic Biosensors GmbH). The biochip surface is functionalized with the DNA ligands of the Enzyme Activity kit (HK-EA-1, Dynamic Biosensors GmbH), in which spot 1 is functionalized with a template strand of 32 nt, while spot 2 is functionalized with a fully double-stranded template as a control. Primers are 96 nt long. DNA ligands are mixed following the instructions of the HK-EA-1 user manual. Activity duration and flow rate are 3 min at 50 µL/min. The biochip is regenerated and freshly functionalized before each concentration of a measurement series. For chip regeneration, a high-pH regeneration solution (SOL-REG-1-5, Dynamic Biosensors GmbH) is injected.

Taq Pol (NEB GmbH) and deoxynucleotide (dNTP) solution mix (NEB GmbH) are diluted in the Polymerase running buffer (50 mM Tris pH 8.3, 75 mM KCl, 3 mM $MgCl_2$, 0.05 % Tween20) at the specified concentrations. The inhibitor $CaCl_2$ (Merck KGaA) is diluted in the Polymerase running buffer at 50 mM and added to the Pol and dNTP mix solutions at the specified concentrations.

Results and Discussion

Polymerase activity. After functionalizing the biochip (Figure 1, step 1), Taq Pol is injected at a fixed concentration of 5 nM (Figure 1, step 2). A previous binding kinetic study of Taq Pol (not shown here) interacting with the templates (ss and dsDNA) showed that at this concentration a 1:1 interaction with the ligands can be expected, avoiding the interaction of multiple proteins with a single strand. As reference, the concentration was chosen close to the K_d value of the interaction. As shown in Figure 2, the binding of Taq Pol to the Primer-Template in spot 1 (Figure 2A) and dsDNA in spot 2 (not shown here), results in a reproducible quenching of fluorescence. By fitting the association curves, the association rates (k_{on}) of Taq Pol binding to the Primer-Template and to the dsDNA can be obtained, resulting in 6.45 ± 0.05 E+6 $M^{-1}s^{-1}$ and 5.03 ± 0.05 E+6 $M^{-1}s^{-1}$, respectively.

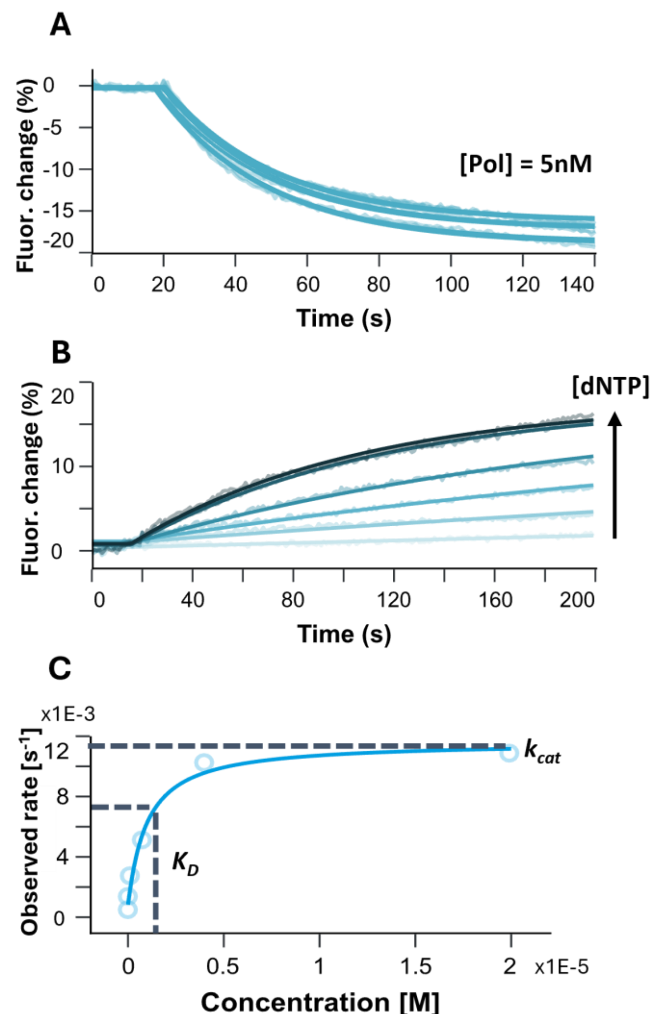

Figure 2 | Association and enzymatic activity of Taq Pol. A) Association to Primer-Template ligand in spot 1. B) Elongation activity in spot 1 upon injection of increasing concentrations of dNTPs. C) Plot for catalytic rate determination.

To measure the enzymatic activity (Figure 1, step 3), increasing concentrations of dNTPs (from 0.03 µM to 20 µM) are injected. As shown in Figure 2B, the injection of substrate results in an increase in fluorescence in spot 1. The elongation of the primer results in the stiffening and stretching of the dsDNA, effectively increasing the distance of the dye from the quenching gold surface (a mechanism called *Surface Energy Transfer*[1,2]) and thus increasing its fluorescence emission. The fluorophore is quenched linearly with increasing proximity to the gold surface within a region extending about 100 nm from the surface[2]. In spot 2, increasing concentrations of dNTPs

do not induce an increase in fluorescence (data not shown here), since the dsDNA cannot be elongated further by the Taq Pol. The fluorescence traces increasing during the elongation in spot 1 can be fitted with a mono-exponential model (see Supplementary information). Plotting the substrate concentration versus the observed rates yields a catalytic plot (Figure 2C). A hyperbolic equation is used to fit the data and extrapolate the Taq Pol catalytic rate ($k_{cat} = 12.0 \pm 0.2\ E\text{-}3\ s^{-1}$) and the affinity ($K_d = 1.86 \pm 0.1\ \mu M$) of Taq Pol for the substrate dNTPs.

Polymerase inhibition. The same workflow employed to study Taq Pol activity can be used to test its inhibition induced by $CaCl_2$. The influence of $CaCl_2$ can be observed in Figure 3. Taq Pol is injected at a fixed concentration (5 nM) to observe the effect of increasing concentrations of $CaCl_2$ (from 0.16 mM to 10 mM) on the binding to DNA. As seen in Figure 3A, Taq Pol is able to bind to the DNA ligands despite the presence of the inhibitor. In the next step, the same increasing concentrations of $CaCl_2$ are mixed with a fixed concentration of dNTPs (100 µM) to observe the inhibitory effect on the Taq Pol elongation activity (Figure 3B). At increasing concentrations of $CaCl_2$, the Taq Pol activity is gradually reduced, and it is completely inhibited at 10 mM $CaCl_2$. Similarly to the analysis done for the activity study, it is possible to plot an inhibition curve by analyzing the observed activity rates at each inhibitor concentration. By normalizing the rates, the resulting activity inhibition (%) at each inhibitor concentration can be plotted (Figure 3C). This provides the half maximal inhibitory concentration (IC_{50}), in this case found to be equal to 0.50 ± 0.03 mM, which is the concentration of inhibitor required to inhibit the activity of Taq Pol by 50 %.

Conclusions

This simple and innovative design to study enzymatic activity of polymerases can provide all important biophysical parameters to characterize them (k_{on}, k_{cat}, K_d). The key advantages of this workflow are: *i)* the full customizability of primer and template sequences by the user, *ii)* the ability to separate the binding event of the polymerase from its activity (this is especially useful when screening for inhibitors), *iii)* the possibility to perform inhibition assays, *iv)* the automated workflow with minimal user input, and *v)* the adaptability of the system to study different types of nucleic acid modifying enzymes.

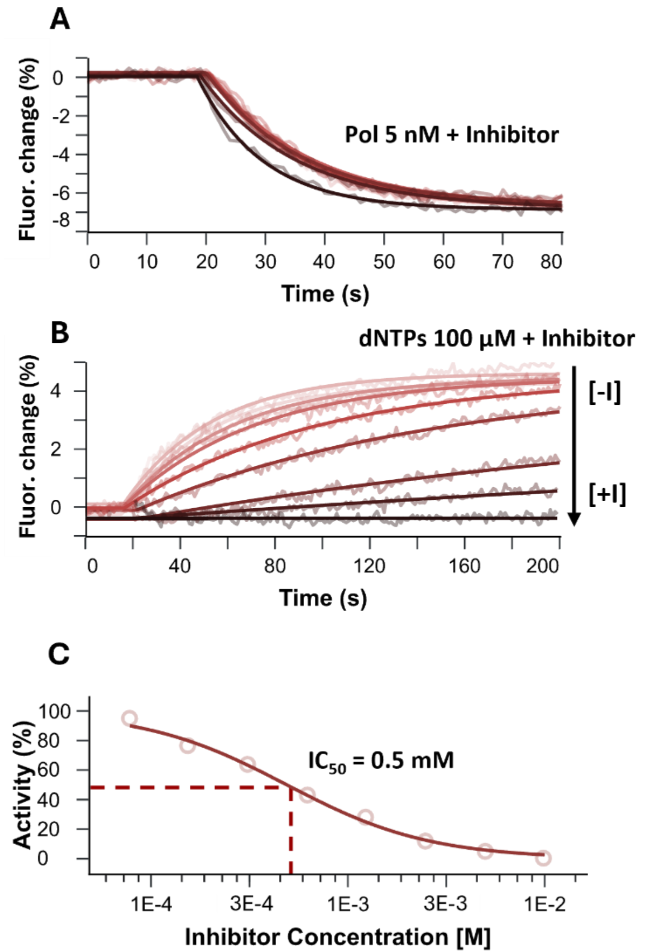

Figure 3 | Inhibition of Taq Pol. A) Binding inhibition. $CaCl_2$ is mixed together with Taq Pol (5 nM) before injection. Increasing concentrations of $CaCl_2$ do not inhibit Taq Pol binding to DNA. B) Activity inhibition. $CaCl_2$ (from 0.16 mM to 10 mM) is mixed with dNTPs (100 µM) before injection. A decrease in fluorescence levels related to the inhibition of Taq Pol activity can be observed at increasing $CaCl_2$ concentrations. C) Plot describing inhibition assay, yielding IC_{50}.

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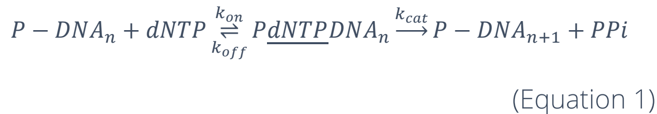
Supplementary information

The analysis of the enzymatic activity of a polymerase on surface biosensors[3] can be described with the Michaelis-Menten model[4] applied to polymerases:



$$P - DNA_n + dNTP \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} P\underline{dNTP}DNA_n \xrightarrow{k_{cat}} P - DNA_{n+1} + PPi$$

(Equation 1)

where P-DNA_n is the polymerase in complex with the target sequence (Primer-Template), dNTP is the substrate, P<u>dNTP</u>DNA_n is the complex of polymerase/target sequence/substrate, P-DNA_{n+1} is the product formed (polymerase/target sequence elongated by one nucleotide) and PPi is the pyrophosphate as a result of the catalytic step. The rate constants k_{on}, k_{off} are the association and dissociation rate constants of the substrate binding to the enzyme. The k_{cat} is the catalytic rate (or turnover number) of substrate conversion to product. The solution to this equation for the product formation over time is:

$$[P - DNA_{n+1}] = [P - DNA]_{max} \cdot (1 - e^{-k_{obs} \cdot t}) \quad \text{(Equation 2)}$$

where the observed rate is defined as:

$$k_{obs} = \frac{k_{cat} \cdot [S]}{K_d + [S]} \quad \text{(Equation 3)}$$

Equation 2 underlines the exponential dependency of the product formation. Therefore, after fitting the elongation curves with Equation 2, the observed rate of the exponential k_{obs} can be extracted for each substrate concentration. If the substrate concentration is plotted versus the observed rates k_{obs}, Equation 3 can be used to fit the data and extract the catalytic rate k_{cat} and the K_d of the substrate binding to the enzyme.

This model undertakes the following assumptions:

i. Enzyme concentration is constant. A full binding kinetic must be performed before, so that the dissociation constant of the polymerase from the DNA strands is negligible within the time course of the experiment;

ii. Product formation is irreversible: the product is not transformed back into a substrate, or if it is, the forward reaction is much faster than the backward reaction ($k_{cat} >> k_{-cat}$);

iii. Substrate concentration is constant. The **switch**SENSE® assay encompasses a constant flow of substrate during the activity, thus this condition is fulfilled. In contrast, for in-solution assays the analysis has to be limited to the initial phase of the reaction, when the substrate concentration can be considered constant;

iv. No allostericity or cooperativity: if the enzyme has more than one binding site, which interact with one another, the Hill equation is used to fit the catalytic plot of [S] *vs* k_{obs};

v. Rapid equilibrium of substrate binding to and unbinding from the enzyme, so that they are in equilibrium before the catalytic step takes place. If this assumption is not fulfilled, the K_M (Michaelis-Menten constant) is obtained instead of the K_d.

In practical terms, after normalization of the elongation curves, a mono-exponential fit extracts the characteristic k_{obs} per substrate concentration with the following equation:

$$y = y_0 + A \cdot \left(1 - e^{-\boldsymbol{k_{obs}} \cdot (x - t_{act})}\right) \quad \text{(Equation 4)}$$

where y_0 is the baseline level, A is the amplitude, k_{obs} is the observed rate of the exponential, x is the time and t_{act} is the starting time of the activity curve. Lower substrate concentration leads to slower rates and higher substrate concentration leads to faster rates until a plateau is reached (see Figure 2B). Finally, plotting the substrate concentration versus the observed rates yields a catalytic plot (Figure 2C). The hyperbolic Equation 3 is fitted to the data points to determine the k_{cat} and the K_d (affinity between enzyme and substrate, under the rapid-equilibrium approximation).

References

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