# *Abstract*

The formation of non-covalent complexes constitutes a majority of biochemical processes in living systems. The strength of the interaction in the formed complex – and its stability – is determined by its equilibrium constant ($K$). Several analytical techniques have been developed to quantify $K$. However, only fluorescence-based methods are sensitive enough to measure $K$ in solutions of low concentrations (< 1 nM), in small volumes (< 1 µL), and without immobilization of one of the reactants on the surface (as in e.g., microbalance technique or Surface Plasmon Resonance).

This Ph.D. thesis shows the discovery and development of a new fluorescence method to determine $K$. The method is based on observation of molecular brightness under change of local environment, further called "brightness analysis method". The Fluorescence Correlation Spectroscopy (FCS) determines the molecular brightness as the number of photons displayed by the molecules in time before and after the complex formation. Monitoring the change in photon counts enables the determination of the concentration of formed complexes in the system, hence the $K$. The obtained results were verified with the Fluorescence Resonance Energy Transfer (FRET) method.

Brightness analysis simplified the FRET analysis approach to operating **by using only one intrinsically fluorescent (or labeled) substrate** without losing its generality. As a model reaction of complex formation, I chose hybridization of complementary DNA oligonucleotides. The sensitivity of the method enabled us to determine the $K$ for samples even at 80 pM. In such examples, the change of only 100 emitted photons (compared to the control sample) enabled us to analyse the result quantitatively. This improvement not only allows for **quick initial tests for determining the interaction of ligands with drugs or biomolecules** but also paves a way towards quantitative study of complex formation in living systems.

The experimental framework can be divided into three parts. The initial part studies kinetics and equilibrium constants of hybridization of oligonucleotides pairs by FRET analysis. This part determines the time after the equilibrium is being established in a wide range of concentrations of reagents (10

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pM to 100 µM). Those experiments are followed by the determination of association, dissociation, and equilibrium constants at different conditions, i.e., temperature and ionic strength. The second part is directly related to the consequence of observing photophysical changes during the reaction, and development of the brightness analysis method. There, the theoretical basis was validated, as well as experimental factors influencing the measurement were determined. These results were specified and described in detail. Finally, the brightness variation study method was used to determine ion complexation by various molecular crowders.

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