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Ligand Binding Assays on the Basis of Fluorescence Anisotropy

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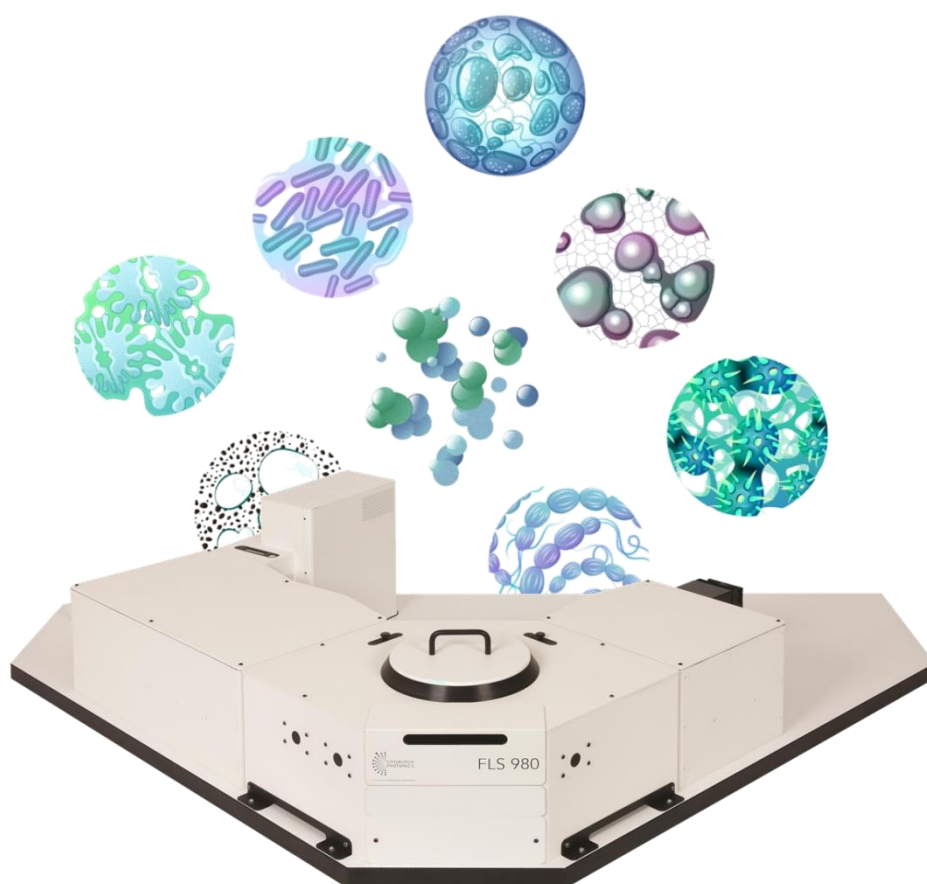
Introduction

Fluorescence anisotropy or polarisation provides a sensitive tool to measure the binding of ligands to proteins when a fluorophore is attached to the ligand. This method is particularly useful if no changes in other fluorescence properties are seen. Changes in the anisotropy are caused by changes of the mobility of the fluorophore. This is the case when a small ligand binds to a macromolecule (*e.g.* proteins) that moves much slower than the ligand free in solution. If a fluorescence-labelled ligand is used only as a probe in competition experiments, dissociation constants for label-free compounds under investigation can be obtained. This assay can provide useful data even for low affinity ligands and can be automated with a titration device.

Drug discovery and mechanistic biological studies on proteins require quantitative ligand binding data. A multitude of methods are available to obtain these data and the choice is often directed by the amounts and quality of target protein available, the number and nature of ligands to test, the binding kinetics and affinities[1]. Often it is desirable to apply several independent methods for cross validation. Fluorescence has the advantage that only small amounts of target protein and ligands are required, that it can easily be automated and that even low affinity binding can be characterised. Beside changes in the fluorescence intensity, changes in the emission peak position and steady-state anisotropy may also change with the binding of a ligand which can be used to obtain binding constants[2–5].

This application note describes as example the binding of a label-free ligand in a competition experiment shown for the regulatory domain of the potassium efflux system (Kef) from *Shewanella denitrificans*[7]. Kef protects Gram-negative bacteria against toxic electrophilic compounds.

Methods & Materials

Polarised emission spectra were recorded in an FLS980 Fluorescence Spectrometer equipped with double excitation and emission monochromators. Calcite polarisers were used in the excitation and emission, while for detection a photomultiplier tube detector (Hamamatsu, R928P) with 0.2 s dwell was used.

For anisotropy measurements linearly polarised light is used for excitation with a polariser placed between light source and sample and the emission intensity is measured dependent on the polarisation plane by using a second polariser between sample and detector[6]. The anisotropy is then obtained as shown in Equation 1 where the first subscript indicates the position of the excitation polariser, the second of the emission polariser and $G(\lambda_{em})$ is an instrumental correction factor $G(\lambda_{em})=I_{HH}(\lambda_{em})/I_{HV}(\lambda_{em})$. The polarisation either vertical (*V*) or horizontal (*H*):

$$r = \frac{G(\lambda_{em})I_{VV}(\lambda_{em}) - I_{VH}(\lambda_{em})}{G(\lambda_{em})(\lambda_{em})I_{VV}(\lambda_{em}) + 2I_{VH}(\lambda_{em})} \qquad (1)$$

The anisotropy is sensitive to the mobility of the fluorophore as it may move between excitation and emission resulting in a changed polarisation plane of the emitted light. Thus anisotropy measurements are especially useful when the ligand is labelled because the ligand is immobilised upon binding.

A soluble construct of the ligand binding domain of Kef from *S. denitrificans* was purified for binding experiments[7]. In the shown experiment a high concentration of 54 µM Kef was used which provided low noise levels, but requires corrections during the analysis for the depletion of the ligands.

In this example, a specific fluorescence probe was synthesised, but general probes are commercially available. Kef is activated by adducts of glutathione and electrophiles. These adducts are formed when electrophiles enter the cell. Therefore, a fluorescence probe was developed with the fluorophore dansyl attached to a glutathione backbone: S-{[5-(dimethylamino)naphthalen-1-yl]sulfonylaminopropyl}glutathione (DNGSH) (synthesised by Conway *et al*., Oxford). The dansyl group was chosen as it is a small fluorophore which reduces the chance of steric clashes during binding to Kef. It was established that this probe binds to Kef and a dissociation constant of K_d=6 µM was determined[7]. Twice the Kef concentration (100 µM) of DNGSH was used in the competition experiment.

The binding of the ligand S-Octan-3-on-1-yl glutathione (OctSG; adduct of the electrophile 1-octen-3-one and glutathione) was used as an example[7] (synthesised by Conway *et al.,* Oxford). This ligand, as well as DNGSH, was dissolved in the measuring buffer.

A micro fluorescence cuvette (Hellma, 105.254-QS) with 3x3 mm light paths was used to minimise the required sample volume to 100 µl. The temperature was kept constant at 20°C.

It was a prerequisite to establish the K_d for the fluorescence probe DNGSH beforehand (see above). In addition, it is required to approximate properties of the bound DNGSH before the competition experiment can be started. Therefore, a reverse titration of a small amount DNGSH with increasing concentration of Kef was performed. It was established that the anisotropy of DNGSH bound to Kef is r=0.180 and that the fluorescence intensity is 4 times as high as for the free ligand (Q=4). The anisotropy of the free DNGSH was measured directly r=0.020.

DNGSH was added to the Kef sample and the anisotropy was recorded. Then the ligand OctSG was added stepwise, the sample was equilibrated for 5 min, and the anisotropy was recorded.

Results - Discussion

The raw data of the OctSG titration are shown in Figure 1. To simplify analysis, mean values for the anisotropy were calculated over the recorded wavelength range for each titration step. These are shown as data points in Figure 2.
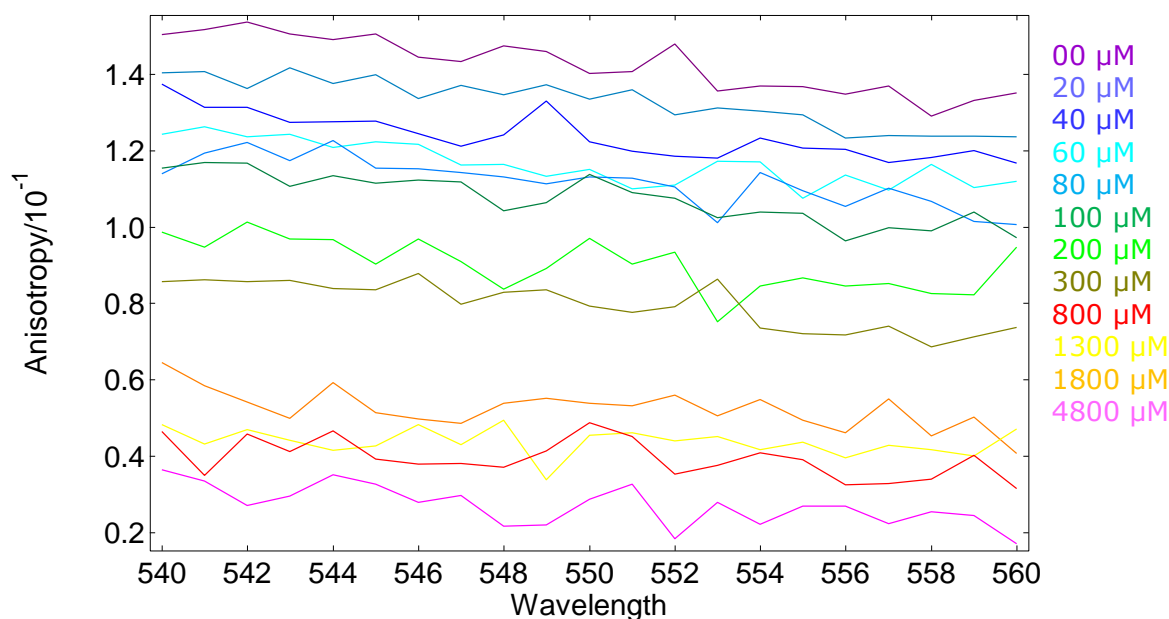

Figure 1: OctSG titration to Kef in the presence of DNGSH. Anisotropies were recorded in the emission range from 540 nm-560 nm. The excitation wavelength was set to 340 nm for excitation of the dansyl group. The anisotropy was measured over the emission range of 540 nm-560 nm where the dansyl group shows strong fluorescence. Slit widths of $\Delta\lambda_{exc}$=2 nm and $\Delta\lambda_{em}$=3 nm, step=1 nm and t_{integr}=0.2 s were used.

The addition of OctSG solution causes an increase of the total volume and stepwise dilution of Kef and DNGSH. The accurate concentrations were calculated for each step.

Data were analysed for a single site binding model considering the depletion of the probe DNGSH and the ligand OctSG due to binding to Kef[8]. The experimental observed anisotropy r_{obs} can be expressed as a sum of anisotropies of the free and bound probe, r_L and r_{ML}, under consideration of their fluorescence intensities, I_{ML} and I_L:

$$r_{obs} = \frac{r_{ML}I_{ML}+r_L I_L}{(I_{ML}+I_L)} \qquad (2)$$

Intensities are defined with the fraction of bound ligand $f_B=[ML]/[L]_0$ and the quantum yields as $I_{ML}=\Phi_{ML}f_B$ and $I_L=\Phi_L(1-f_B)$. This can be substituted into Equation 2 and solved for f_B using the ratio of quantum yields $Q=\Phi_{ML}/\Phi_L$:

$$f_B = \frac{1}{1+Q\frac{(r_{obs}-r_{ML})}{(r_L-r_{obs})}} \qquad (3)$$

Equation 3 can be rearranged for use in the fitting together with expressions for f_B.

$$r_{obs} = \frac{\frac{r_L}{f_B Q}-\frac{r_L}{Q}+r_{ML}}{\left(1+\frac{1}{f_B Q}-\frac{1}{Q}\right)} \qquad (4)$$

Definitions for the dissociation constants DNGSH and OctSG lead together with mass conservations to a cubic equation of the DNGSH-Kef complex con-centration, [ML]. One meaningful solution can be obtained and substituted as f_B into Equation 4 (for details see suppl. information ref[7]). A MATLAB program was written for fitting of the apparent number of binding sites, n, and the disso-ciation constant for OctSG, K_d(OctSG). A dissociation constant of Kd=7.1 μM for OctSG was found.

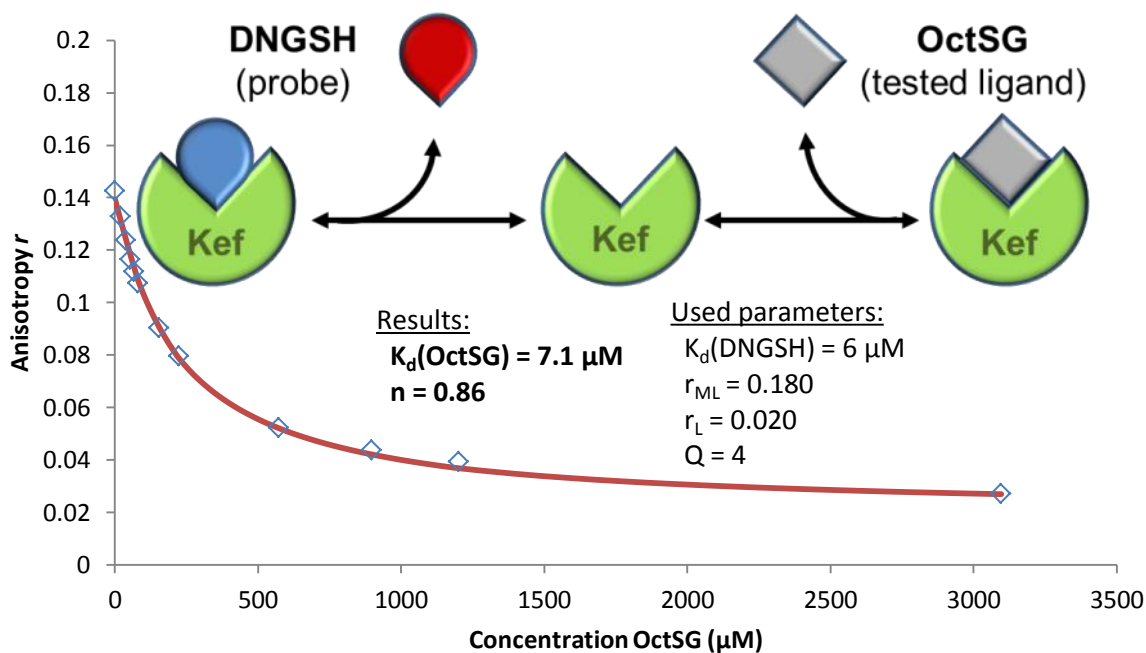Figure 2: Fit of the dissociation constant for the titration concentrations used in the experiment. The inset shows an overview of the experiment.

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