

Application Note 13

Lifetime changes of the dyes Dy475-XL, Dy630 and Dy640 upon hydrophobic interaction with BSA

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Introduction:

The comprehensive understanding of protein behaviour in complex environments is a prerequisite for their use in the pharmaceutical drug development process. In addition to structural information aggregation, degradation, folding or surface hydrophobicity are important protein properties that may influence ligand-protein-interactions and protein integrity (1).

Bovine serum albumin (BSA) is a suitable protein for studying hydrophobicity or ligand-protein-interactions. Serum albumins are among the most abundant proteins in plasma having many physiological functions. They contribute to colloid osmotic blood pressure and are responsible for the maintenance of blood pH.(2) Another property of albumins is their ability to reversibly bind a large variety of endogenous and exogenous ligands. The binding involves hydrophobic, hydrophilic, and electro-static interactions. For the investigation of the pharmacokinetics, pharmacodynamics, and toxicology of drugs the interactions between albumin and drugs are very important. (3) The molecular interactions are often monitored using fluorescence spectroscopic methods.

Fluorescence spectroscopy is a highly sensitive tool for protein analysis. There are several ways to generate fluorescent signals for monitoring protein behaviour. Intrinsic protein fluorescence coming from the naturally fluorescent amino acids tryptophan and tyrosine can provide information on conformational changes of proteins (4). Covalently attached fluorescent dye molecules are routinely used for studying binding events with ligands or other proteins e.g. by exploiting Förster resonance energy transfer (FRET) processes (5). Another possibility is the use of extrinsic fluorescent dyes without covalently coupling as a method for protein characterization (1). Here noncovalent, hydrophobic or electrostatic interactions of the dye molecules with proteins and protein degradation products are used. Dye molecules that are intended to be used as sensitive and robust indicators for protein characterisation should exhibit a significant change of fluorescence intensity and fluorescence lifetime in relation to the investigated interactions. Especially the detection of the fluorescence lifetime supports the development of robust assays for protein characterisation in screening applications.

The focus of this application note is to characterise the green and red emitting fluorophores Dy475-XL, Dy630 and Dy640 with respect to their potential use as sensitive markers for protein hydrophobicity in screening applications especially with the focus on potential developments of robust assays based on fluorescence lifetime detection.

Material and Methods:

The dyes Dy475-XL, Dy630 and Dy640 have been obtained from Dyomics (Jena, Germany) as lyophilized samples. Stock solutions of the dyes with millimolar concentrations were made using dimethyl formamide (DMF, 40228, Sigma). Working solutions of the desired concentrations in pure PBS buffer (5368, Sigma, St. Louis, USA) and with bovine serum albumin (BSA, 4287, Sigma, St. Louis, USA) were diluted from the stock solutions.

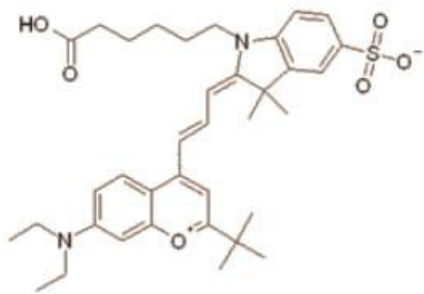


Fig. 1: Chemical structure of Dy630; structures of Dy475XL and Dy640 are unknown

Time-resolved fluorescence measurements have been performed in black 384 well microplates (Fluorac 200, Greiner, Frickenhausen, Germany) with 35 μ l sample volumes using the microplate reader **LF502 NanoScan FLT** (IOM, Berlin, Germany). Excitation wavelengths have been 488 nm and 633 nm for the Dy475-XL and the Dy630/Dy640 experiments, respectively. Detection occurred at 520 nm with 10 nm bandwidth and 700 nm with 30 nm bandwidth. Excitation laser energy was adjusted to 30% of maximum energy level. Averaging over 32 laser pulses was performed for reduction of noise.

Measurements of fluorescence spectra were done using the Fluoromax®-4 spectrometer (Horiba Jobin Yvon, Kyoto, Japan). Excitation and emission slits were typically set to 2 nm bandwidth. An integration time of 0,5 s was used.

Results:

Fig. 2: Fluorescence lifetimes of the dyes Dy475-XL, Dy630 and Dy640 with respect to an increasing concentration of BSA.

All three dyes showed a change of fluorescence lifetime and of fluorescence intensity with increasing concentration of BSA in the solution, however with different amplitudes. The largest lifetime change was observed for Dy640 from 2,8 ns at low BSA concentrations to 4,0 ns at 1 % BSA. Additionally, the absolute lifetime values of Dy640 are largest for all three dyes.

Dy475-XL changes its lifetime from 1,0 ns to 2,0 ns and Dy630 from 2,0 to 2,4 ns.

For Dy475-XL at 1 % BSA a saturated upper lifetime level was not yet observed.

While for Dy630 and Dy640 the analysis of fluorescence lifetimes was done with a single-exponential decay model (one fluorescence lifetime only), for Dy475-XL a double-exponential curve fit with a linked second lifetime was performed. The second, long lifetime value was fitted to be 3,9 ns. The first lifetime component ranged between 0,2 and 0,9 ns. Upon increasing BSA concentration the weight of the second lifetime increased thus increasing the calculated "mean lifetime" values.

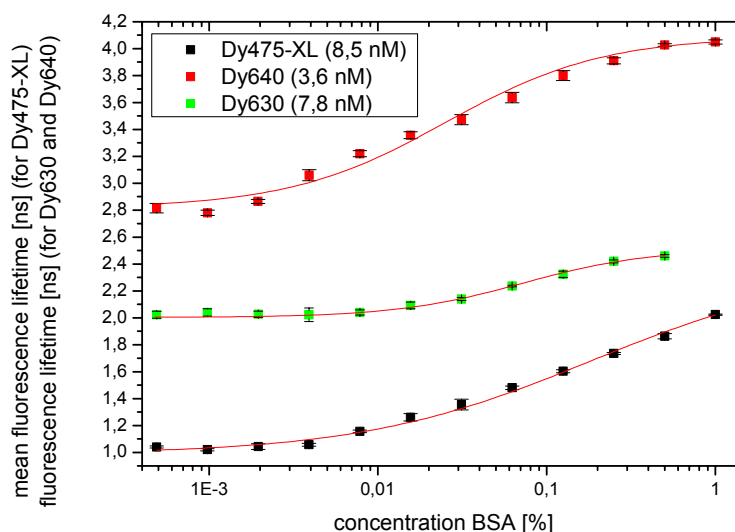
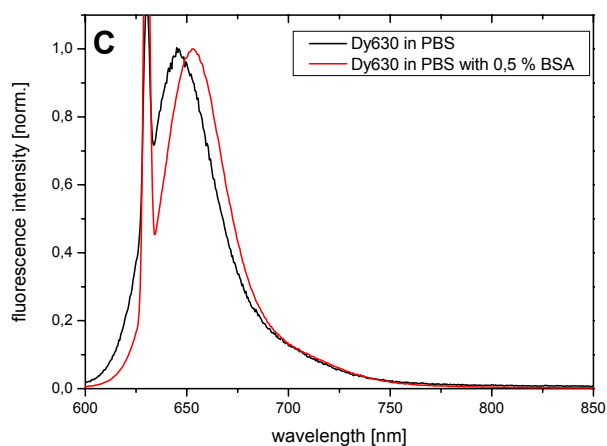
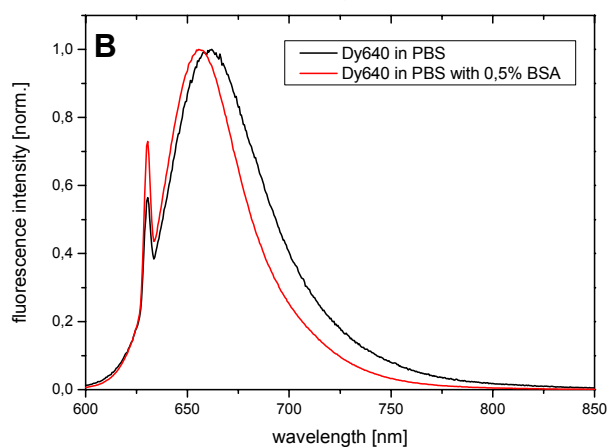
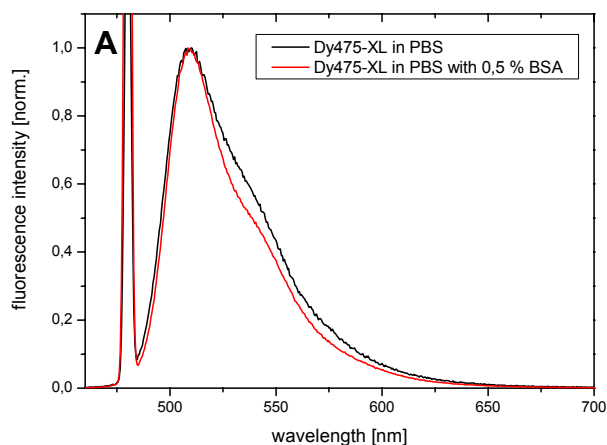
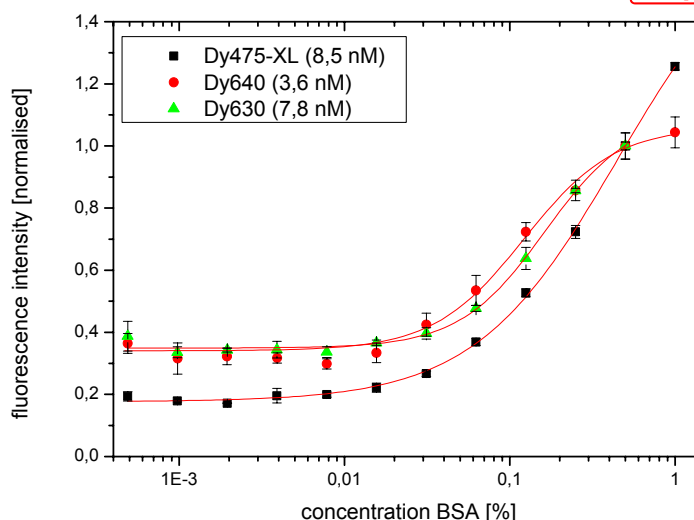


Fig. 3: Fluorescence intensity of the dyes Dy475-XL, Dy630 and Dy640 with respect to an increasing concentration of BSA

The fluorescence intensity of the dyes in the presence of BSA shows a similar behaviour as the lifetime. With increasing BSA concentration a rise of the measured intensity values is observed. Again, for Dy475-XL there was no signal saturation observed. Also, in comparison to the other two dyes the increase in intensity is significantly larger.



In order to get a more comprehensive knowledge of the dye-BSA-interaction absorption and fluorescence spectra of all dyes with and without BSA have been recorded. The concentrations of Dy475-XL, Dy640 and Dy630 for these measurements were 0,85 μM , 0,71 μM and 0,079 μM respectively.

For all dyes it was found that the wavelength of maximum absorption increases in the presence of BSA (see table 1).

The fluorescence of Dy475-XL was excited at 480 nm. For Dy640 and Dy630 the excitation wavelength was 630 nm.

Interestingly all three dyes showed a different behavior. Upon interaction with BSA the fluorescence maximum of Dy475-XL is nearly not shifted and remains at 509 nm. For Dy640 the fluorescence maximum shifts towards shorter wavelengths from 661 nm to 656 nm.

For Dy630 a significant bathochromic shift from 645 nm to 653 nm was observed.

A summary of important spectroscopic properties of the investigated dyes are listed in table 1.

Fig. 4: normalized fluorescence spectra of Dy475-XL (A), Dy640 (B) and Dy630 (C) in PBS buffer (pH 7,4) and in PBS + 0,5% BSA

table 1: spectroscopic properties of the dyes with and without BSA interaction

	λ_{Abs} [nm]	λ_{Em} [nm]	τ [ns]
Dy475-XL in PBS	492	509	1,01 \pm 0,05
Dy475-XL in PBS with 0,5 % BSA	495	509	2,02 \pm 0,02
Dy640 in PBS	626	661	2,85 \pm 0,03
Dy640 in PBS with 0,5 % BSA	638	656	4,05 \pm 0,02
Dy630 in PBS	631	645	2,00 \pm 0,05
Dy630 in PBS with 0,5 % BSA	637	653	2,46 \pm 0,02

Discussion:

The investigated dyes Dy475-XL, Dy640 and Dy630 are known to be relatively hydrophobic. With respect to their interaction with the protein BSA they all have in common to show an increase in fluorescence quantum yield and fluorescence lifetime in the presence of BSA which is in accordance with data published earlier (6). However, the extents to which fluorescence lifetime and intensity values change are different.

The largest relative intensity and lifetime change was observed for Dy475-XL. The intensity increases by more than a factor of five and lifetime by a factor of two.

The largest absolute lifetime increase of 1,2 ns was measured for Dy640. It is accompanied by an intensity increase of a factor of 3. Considering the relatively long lifetime of approx. 4 ns Dy640 appears to be most suited for a possible application for probing protein surface hydrophobicity or ligand-protein-interactions. This is due to the facts that longer lifetimes of 4 ns can be measured with better precision than shorter lifetimes of 1 ns giving better data statistics and that the long excitation and emission wavelengths lead to reduced perturbations due to compound fluorescence.

Dy630 seems to be less suited for protein probing via lifetime measurements since the absolute and relative lifetime change is rather small.

The hydrophobic interaction between dye molecules and BSA may happen at more than one binding site per protein molecule (4) whereas the binding strength normally is unequal. It is likely that due to this noncovalent binding the inner flexibility of the dye molecules is hindered which at least for Dy630 is given by the polymethine backbone. Thus, the efficiency of intramolecular quenching processes like (twisted) internal charge transfer (T)ICT or the formation of excited state complexes is lowered. As a result fluorescence intensity and lifetime increase (1). The results of the spectral measurements indicate that the internal quenching mechanisms of the dyes in aqueous environment may be different.

Summary:

- **Dy640 seems to be most suited of all three investigated dyes for use in robust fluorescence lifetime-based assays to probe protein surface hydrophobicity or ligand-protein-interactions**
- **Dy640 has a relatively long fluorescence lifetime and avoids perturbations due to compound fluorescence because of the long excitation and emission wavelengths**
- **Dy475-XL is a green emitting label showing a 100% increase of fluorescence lifetime. Upon binding to BSA, it showed the highest increase in fluorescence intensity and may be profitably used in dual read-out assays using fluorescence lifetime and intensity**

Literature:

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