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Structural control of dye–protein binding, aggregation and hydrophilicity in a series of asymmetric cyanines

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ABSTRACT

Aiming at the rational design and the identification of brilliant fluorescent reporters for targeted optical probes and fluorescence assays in biological matrices, we systematically assessed the correlation between dye–protein binding, dye aggregation, and dye hydrophilicity for bioanalytically relevant fluorescent labels. Here, we report on the influence of sulfonic acid groups on dye aggregation and dye–serum protein interactions exemplarily for a family of NIR-emissive cyanine dyes, the DY-67x fluorophores. For highly hydrophobic dyes like DY-675 and DY-676, which show a strong tendency for aggregation in phosphate buffer saline solution, the dye–protein binding constants determined spectroscopically using a 2-state binding model, which considers only protein-bound and unbound dye molecules, can be influenced by the dimerization of the unbound dyes. To consider and quantify this influence, we expanded this common photometric method to a 3-state model that accounts for the presence of dye aggregates in the binding studies. Our results can be exploited for the screening of fluorescent reporters, efficiently providing information on the size of dye–protein interactions and on maximally achievable fluorescence quantum yields in biological systems.

1. Introduction

Near infrared (NIR) absorbing and emitting fluorophores are gaining more and more attention in analytical, biological, and biomedical research, ranging from fluorescent reporters in fluorescence assays performed in complex matrices like scattering and absorbing body fluids to nanosensors and sophisticated optical probes for measurements in biological systems [1–8]. One of the most rapidly developing area of application of NIR dyes presents the design of contrast agents and targeted probes for NIR fluorescence (NIRF) imaging for the detection of very early stages of diseases such as cancer, monitoring of disease progression, and evaluation of the effect of drugs [1–3,5,6,8–13]. For use as a signaling moiety, NIR fluorophores should reveal an intense absorption and emission in the spectral range of 650–900 nm under application-relevant conditions, i.e., in an aqueous environment, as well as sufficient thermal and photochemical stability. For *in vivo* applications, a low cytotoxicity and optimum pharmacological properties are mandatory. The latter include minimum non-specific binding and an adequate retention time in the body preferably followed by fast excretion [9,10]. Drawbacks of many NIR reporters, which need to be eventually overcome, are a low fluorescence quantum yield and a low stability in aqueous media [14,15] as well as strong binding to plasma proteins [16,17], resulting in rapid elimination through the liver as encountered e.g., for indocyanine green (ICG), the only NIR fluorescent dye clinically approved by the Food and Drug Administration (FDA) at present [9,18–21]. Hence, criteria are desired for the efficient choice of optimum fluorescent reporters and the prediction of their performance in different environments based upon a minimum number of measurements.

One of the most important parameters to be assessed for all applications involving body fluids, cells, and tissue is the size of the interactions of fluorescent reporters with plasma proteins like albumins [22]. This plays a significant role for the transport, biodistribution, and deposition of small molecules in mammals [22]. Serum albumin, which is the most abundant protein in blood and plasma, can e.g., increase the solubility of hydrophobic molecules and can thus modulate their delivery to cells *in vitro* and *in vivo*. Accordingly, serum protein binding of different ligands ranging from potential drugs to fluorescent reporters is of great scientific interest and many ligand–plasma protein binding studies have been performed with the overall goal to derive protein–ligand affinity or binding constants (K_P) for specific chemical classes of compounds to estimate potential effects on pharmacokinetics and

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pharmacodistribution and to obtain more structural information on albumin binding sites [23–25]. Moreover, binding of dyes to serum proteins can significantly alter their spectroscopic properties, especially their fluorescence quantum yields, which are typically increased [26–32]. Although often advantageous, such effects must be also considered, e.g., for the design of homogeneous FRET assays (Foerster resonance energy transfer) in body fluids using non- or low-emissive acceptor dyes as the emission of these quenchers can be altered similarly by dye–serum protein interactions. This is of special importance since the chemical structures of such chromophores and the processes responsible for the radiationless deactivation of the excited singlet state are often not reported.

For the study of interactions with serum proteins, typically bovine serum albumin (BSA) is used as model system. BSA consists of three homologous domains (I,II,III), with the specific drug binding sites, the warfarin site and the diazepam site, which bind small heterocyclic or aromatic carboxylic acids, being located in the subdomains IIA and IIIA [29,33].

Ligand–protein binding constants (K_P) can be determined with a broad variety of methods ranging from optical spectroscopy e.g., measurements of absorption or fluorescence intensities or fluorescence lifetimes as well as light-scattering techniques and IR spectroscopy, over NMR, mass spectroscopy, and microcalorimetry to chromatographic techniques [34–36]. Among the most widely employed methods for the measurement of K_p are photometry and fluorometry due to their ease of use and speed of analysis and in the latter case, also high sensitivity. The photometric determination of K_P relies on binding-induced changes in absorbance and requires a strongly absorbing ligand like a dye [27,31,32,37–39]. The fluorometric determination of K_P exploits either ligand binding-induced quenching of the fluorescence of an intrinsic fluorophore such as tryptophan [40–42], or changes in the emission of extrinsic fluorophores like environmentally sensitive chromophores [24], the displacement of site-specific fluorescent probes like warfarin or dansylamide [41,42], or less common, specific and selective fluorescent probes for certain albumins [36,41,43–47].

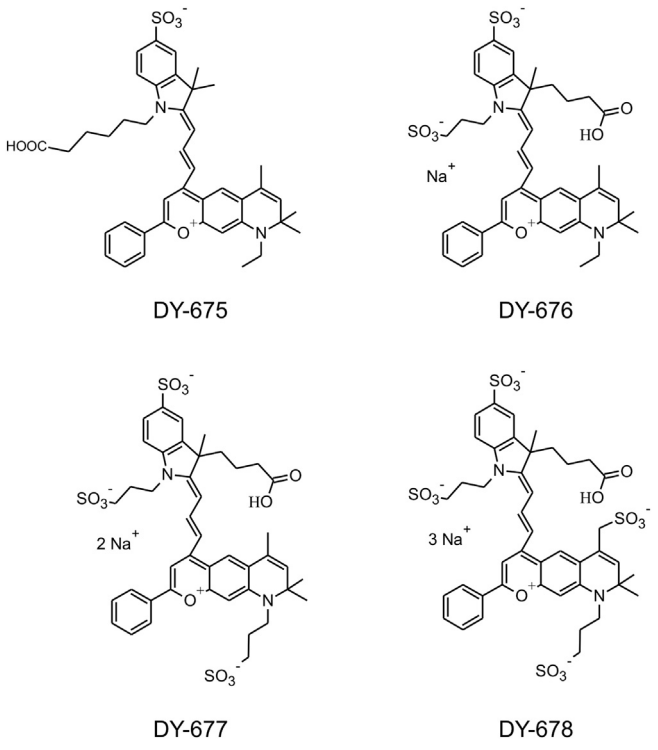

Fig. 1. Chemical structures of the DY-67x dyes.

The search for new diagnostic tools for the construction of targeted optical probes encouraged us to systematically assess the correlation between signal size-determining optical properties like the fluorescence quantum yield (Φ_f) and dye hydrophilicity, aggregation tendency, and dye–serum protein interactions for different fluorophores. This included hemicyanines from DYOMICS, Cy dyes, i.e., cyanine dyes from GE Healthcare Life Sciences, stimuli-responsive CypHer 5 (GE Healthcare Life Sciences), and recently also a new family of symmetric pentamethines, the xS-IDCC series from Mivenion GmbH equipped with a linker for bioconjugation in the middle of the dye's polymethine chain [13,48–50]. In this study, we focus on the influence of the number of charged groups in the dye molecule on the binding strength to bovine serum albumin (BSA) and quantify the influence of dye aggregation tendency and hydrophilicity on K_P values exemplarily for the DY-67x dyes (Fig. 1) comparing the conventional 2-state binding model and a 3-state model considering aggregation of the unbound dye and BSA-induced deaggregation. Such effects have been neglected until now. To the best of our knowledge, this is the only recent study where the BSA complexes of structurally very similar NIR dyes have been investigated [51,52] and the first study where the influence of BSA-induced dye deaggregation on optically measured K_P values is quantified.

2. Experimental

2.1. Materials

The asymmetric trimethine dyes DY-675, DY-676, DY-677, and DY-678 were provided by Dyomics GmbH (Jena, Germany). The quantum yield standard oxazine 1 (quantum yield in 96% ethanol 0.15 [53]) was obtained from Lambda Physik GmbH (Goettingen, Germany). Phosphate buffered saline (PBS, 7.7×10^{-4} mol/L, pH 7.4) was purchased from GIBCO Life Sciences (Paisley, Scotland) and bovine serum albumin (BSA; fraction V) from Merck KGaA (Darmstadt, Germany).

2.2. Methods

2.2.1. Spectroscopic studies

The absorption and fluorescence spectra of the DY-67x dyes were determined in duplicate in air-saturated PBS (pH 7.4) and in an air-saturated solution of 5 mass-% (7.5×10^{-4} mol/L) BSA in PBS (BSA/PBS) at $T = (25 \pm 2)°C$ in 1 cm-quartz cells. Typically, a dye (or species) concentration of 1×10^{-6} mol/L was used, except for the BSA-titration measurements at low dye concentration where the dye concentration was about 2×10^{-8} mol/L. The absorption spectra were recorded on a calibrated CARY 5000 spectrometer (Varian Inc., Palo Alto, USA). The fluorescence spectra were measured with a calibrated Spectronics Instruments SLM 8100 (Spectronics Instruments, Westbury, USA) spectrofluorometer [54]. All fluorescence measurements were carried out with Glan Thompson polarizers set to 0° in the excitation and 54.7° in the emission channel (magic angle conditions) [55] to eliminate any influence of an anisotropic emission that is commonly observed for NIR dyes and protein-bound dyes. The emission of the DY-67x dyes was excited always at the blue vibronic shoulder of the longest wavelength absorption band using absorbances of 0.02–0.06 at the excitation wavelength to minimize inner filter effects. The fluorescence quantum yields were calculated from blank and spectrally corrected and integrated emission spectra as previously described [54,56].