Interaction of BODIPY dyes with bovine serum albumin: a case study on the aggregation of a click-BODIPY dye†

Laramie P. Jameson, Nicholas W. Smith, Onofrio Annunziata and Sergei V. Dzyuba*

BODIPY dyes are among the most useful and versatile small molecule fluorescent probes, and a wide range of applications has been attributed to the dyes' high thermal and chemical stabilities, high quantum yields, extinction coefficients, as well as tunable spectroscopic properties.1–3 In regard to biomolecular processes, BODIPY dyes have been primarily used to label ligands to address ligand–receptor interactions.4,5 Recently, several reports suggested that BODIPY dyes could interact directly with a variety of proteins and peptide assemblies, acting as fluorescence-based sensors.6,7

Fluorophore–albumin interactions are of interest, since serum albumins are the major small molecule-binding proteins, which are considered suitable models for various in vitro studies on ligand–protein interactions.8 In addition, due to its size and collection of binding sites, bovine serum albumin (BSA) could be viewed as a viable model for non-specific binding. Although a number of fluorophores have been shown to bind to albumins, only a few BODIPY dyes have been investigated.9–11 Notably, a BODIPY dye was identified (out of a library of 137 dyes) that exhibited ca. 200-fold emission enhancement in the presence of BSA, while exhibiting high specificity towards BSA over serum albumins from other species (human, porcine, rat, and sheep).11,12 Significantly, specifically substituted BODIPY-based fluorescent probes were shown to be viable sensors of protein hydrophobicity.14 Furthermore, several common BODIPY dyes, including a water-soluble derivative, were recently suggested to interact with albumins15 as was evidenced by an increase in the emission intensity.

We previously demonstrated that the incorporation of a triazole moiety on the BODIPY dye scaffold afforded probes that had a significant affinity towards soluble oligomers of amyloid peptides,16 thus illustrating the possibility for click-BODIPY dyes to act as biosensors.

Here, in order to expand on the utility of BODIPY dyes, we examined the interactions between triazole-containing BODIPY dyes, so-called click-BODIPY dyes, (Fig. 1) and BSA. The incorporation of the triazole group onto the BODIPY scaffold was accomplished in a straightforward manner using an alkyne-containing BODIPY scaffold (ESI†). Dye 2 has a triazole moiety, and the presence of the methyl group, rather than the benzylic group, assures that dye 2 is less hydrophobic than dye 3.

During the initial screening, the fluorescence of dyes 1, 2, and 3 was measured in the presence of a fixed amount of BSA (39.2 μM) and a notable enhancement in the fluorescence of the dyes in the presence of BSA was observed (Fig. 2).

At the highest experimental dye concentration (1 μM), krypto-BODIPY dye 1 exhibited a ca. 20-fold increase in its fluorescence.

Department of Chemistry and Biochemistry, Texas Christian University, Fort Worth, TX 76129, USA. E-mail: s.dzyuba@tcu.edu
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Fig. 1 Structures of krypto-BODIPY (1) and click-BODIPY (2 and 3) dyes.
intensity in the presence of BSA, while the introduction of the methyl-triazole moiety, dye 2, resulted in only a 10-fold increase. Remarkably, in the presence of BSA, dye 3 exhibited a significantly larger enhancement, ca. 40-fold. Notably, the fluorescence enhancement of dye 3 compared to that of dyes 1 and 2 could be viewed as even more remarkable at lower dye concentrations (e.g., 0.2 μM), since the fluorescence of dye 3 saturated at dye concentrations greater than 0.5 μM. It should also be pointed out that the absorption spectra of the dyes were not drastically different in the absence and presence of BSA (Fig. S1–S6, ESI†).

Furthermore, the fluorescence intensity of dye 3, in the presence of BSA, was found to increase with time, t, towards its asymptotic equilibrium value. This was likely related to the kinetics of protein–dye association and the corresponding desolvation effects of the dye and its aggregates. This behavior was not observed in the case of the other two dyes, i.e., no time-dependent increase in the emission was observed upon addition of dyes 1 and 2 to the solution of BSA. For dye 3, the fluorescence intensity at equilibrium, \( I_F(\infty) \) was obtained by fitting the time-dependent fluorescence, \( i_F(t) \), to the first-order kinetic expression \( I_F(\infty) [1 - a \exp(-bt)] \), where \( I_F(\infty) \), a, and b are fitting parameters. A representative profile is shown in Fig. 3.

The aforementioned observations may be related to the presence of the benzyl group on dye 3, which increases the hydrophobicity of the dye, and as such the dye’s interaction with hydrophobic binding pockets of the protein should be favored. However, the saturation of the fluorescence signal was taking place at dye concentrations of ca. 0.5 μM (Fig. 2C). In order to gain insight into the BODIPY–BSA interactions, we carried out more detailed titration experiments at dye concentrations that were below the saturation point using fluorescence spectroscopy.

The titration conditions were chosen such that the total concentration of protein P (\( C_P \)) was large enough when compared...
to the total concentration of the dye D (C_D). Thus, it could be
assumed that only a 1:1 complex of protein–dye (PD), would form
appreciably, irrespective of the number of binding sites of BSA.
This reversible interaction could be represented as P + D ⇄ PD,
with the following mass action law (eqn (1)):

$$ K = \frac{[PD]}{[P][D]} $$ (1)

where $K$ is the corresponding association constant and $[P]$, $[D]$, and
$[PD]$ are the concentrations of the three reaction species at
equilibrium, which are linked to the total concentrations by the
mass balances: $C_P = [D] + [PD]$ and $C_D = [P] + [PD]$. To determine $K$,
we examined the effect of $C_P$ at a constant $C_D$, on the observed
fluorescence enhancement, $F = I_F/I_F^0$, where $I_F$ and $I_F^0$ are the
fluorescence intensities in the presence and in the absence
of BSA, respectively. Assuming that fluorescence intensity is
directly proportional to fluorophore concentration, it follows:

$$ F = 1 + (R - 1)\alpha $$ (2)

where $R$ is the fluorescence ratio of bound to free dye (fluorescence
gain) and $\alpha$ is the fraction of bound dye (defined as $\alpha \equiv [PD]/C_D$).
Eqn (3) is obtained from the mass balances and eqn (1):

$$ \alpha = \frac{1 + \frac{K_C P + K_C D}{2K_C D}}{\sqrt{(1 + \frac{K_C P - K_C D}{2K_C D})^2 + 4K_C D}} $$ (3)

The titrations were performed by varying the protein concentration
$C_P$ while keeping the dye concentration $C_D$ constant at 0.20 μM.
The results were subsequently fitted using eqn (2) and (3), and the
results are shown in Fig. 4 and Table 1. The binding constants for
all dyes were very similar, and therefore a drastic increase (a 34-fold
increase of the fluorescence of the dye) all dyes were very similar, and therefore a drastic increase (a 34-fold
increase of the fluorescence of the dye 3 as compared to dyes 1 and
2, which showed 1.5 and 2.9 fold increases, respectively) could not
be explained by a difference in the binding affinities.

The observed saturation in the fluorescence behavior of
dye 3 (Fig. 2C) required additional explanation. At 0.5 μM of
the dye, the protein was present in a large excess as compared to

![Image](14x290 to 26x354)

**Table 1.** Binding constants and fluorescent enhancement upon dye binding to BSAa.

<table>
<thead>
<tr>
<th>Dye</th>
<th>$K$/μM$^{-1}$</th>
<th>$R^b$</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>0.34 ± 0.03</td>
<td>2.9 ± 0.1</td>
</tr>
<tr>
<td>2</td>
<td>0.29 ± 0.15</td>
<td>1.5 ± 0.1</td>
</tr>
<tr>
<td>3</td>
<td>0.12 ± 0.01</td>
<td>34 ± 1</td>
</tr>
</tbody>
</table>

* a Buffer: 10 mM TRIS (0.1 M NaCl, pH 7.4). b Fluorescence ratio of bound to free dye.

In regard to the dye–BSA interaction, the dye aggregation
could be assessed when fluorescence intensity (expressed as
$I_F - I_F^0$, where $I_F^0$ is the fluorescence intensity of the protein-free
system) is plotted as a function of $C_D$ at constant $C_P$ (Fig. 4). At
low protein concentrations, $I_F - I_F^0$ reached a plateau as the
dye concentration was increased. This plateau decreased as the
protein concentration increased. It is important to note that the
protein concentration was significantly larger than that of
the dye in all cases, which precludes the saturation of the
protein’s binding sites. In this case, the protein likely acted as a
solubilizer for the dye, and thereby reduced the amount of dye
aggregates in solution. Such solubilization by BSA could also
explain the disappearance of the plateau in the titration
experiments at higher protein concentrations (Fig. 5).

In order to quantitatively describe the observed behavior,
dye reversible aggregation could be represented as $nD = D_n$
with the following mass action law (eqn (4)):

$$ \beta = S_D^{(n-1)} = \frac{[D_n]}{[D]^n} $$ (4)

where, for simplicity, it is assumed that only one monodisperse
mesoscopic aggregate, $D_n$, is formed with aggregation number $n$,
and $\beta$ is the corresponding association constant. When $n > 1$,
the aggregate could be treated as a separate phase, and $S_D$ in eqn (4)
represents the solubility of monomeric D with respect to the aggregates in water.19,20 For a given $n$, either $\beta$ or
$S_D$ could be equivalently utilized to characterize aggregation
thermodynamics. In our case, we choose to use $S_D$ due to its
more direct graphical identification. Since the contribution of
dye aggregates to fluorescence was neglected, the difference in
intensity, $I_F - I_F^0$ could be described as follows:

$$ I_F - I_F^0 = k_F(R[PD] + [D] - [D]_0) $$ (5)

where $k_F$ is the proportionality constant describing the effect
of free monomeric dye on fluorescence intensity and $R = 34$ (Table 1).
At a given $C_D$, $[D]$ and $[D]_0$ represent the concentrations of free
monomeric dye in the presence and absence of BSA, respectively.
Note that $[D] - [D]_0$ is small compared to $R[PD]$ in eqn (5). The
concentrations $[PD]$, $[D]$, and $[D]_0$ can be related to $C_D$ and $C_P$ by
the mass balances:

$$ C_D = [D] + K_C C_P (1 + K_C D) + nS_D ([D]/S_D)^n $$ (6)
The interaction of several BODIPY dyes with BSA has been demonstrated that BSA could play a dual role: (a) disaggregate the dye’s aggregates and (b) subsequently bind monomeric BODIPY. Notably, a similar disaggregation phenomenon was also reported for aza-BODIPY dyes. Our results suggest that, at least in some cases, the fluorescence enhancement upon a dye–protein interaction might not be exclusively attributed to the binding event. Potentially the disaggregation of click-BODIPY dyes could be used as a detection event as well as a sensor for the hydrophobic surfaces of the proteins.

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Notes and references