ABSTRACT: Fluorescent dyes, for example, 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid dipotassium salt (bis-ANS) are extensively used to detect nonnative protein structures in therapeutic protein products, for example, during formulation development by monitoring the greatly enhanced dye fluorescence upon binding to nonnative species. Our aim was to characterize the level of heterogeneity of bis-ANS binding sites in a thermally stressed monoclonal antibody (IgG) formulation by steady-state fluorescence, time-resolved fluorescence, and isothermal titration calorimetry (ITC), and to obtain apparent dissociation constants ($K_d$) by data fitting. Because the methods differ in their underlying measurement principles, they provide different information on binding properties of bis-ANS to thermally stressed IgG. We found very heterogeneous bis-ANS binding sites on thermally stressed IgG, with apparent $K_d$ values ranging from as low as 50 nM (time-resolved fluorescence) to 63 μM (ITC). Steady-state fluorescence and ITC gave insight into an overall binding affinity of a wide population of dye binding sites with micromolar $K_d$ values. Time-resolved fluorescence was particularly sensitive to high-affinity binding sites with nanomolar $K_d$ values. The heterogeneity of the bis-ANS binding sites reflects the complex, heterogeneous nature of the heat-stressed IgG used in this study. To probe such heterogeneity adequately, one should apply complementary analytical methods under various experimental conditions as presented in this paper. © 2010 Wiley-Liss, Inc. and the American Pharmacists Association J Pharm Sci 100:1294–1305, 2011

Keywords: fluorescent dye; binding affinity; monoclonal antibody; fluorescence spectroscopy; ITC; steady-state fluorescence spectroscopy; time-resolved fluorescence spectroscopy; protein aggregation

INTRODUCTION

In the pharmaceutical field, fluorescent dyes have recently gained in importance as sensitive analytical tools for formulation development and stability testing of therapeutic protein products.1-8 One example is the polarity-sensitive fluorescent dye 4,4′-Dianilino-1,1′-binaphthyl-5,5′-disulfonic acid dipotassium salt (bis-ANS), which was first reported in 1969 by Rosen and Weber9 and has since then extensively been employed for the characterization of surface hydrophobicity, hydrophobic binding sites, aggregates, fibrils, folding intermediates, and structural variants of proteins.2,3,8,10 The emission spectrum of bis-ANS is sensitive to polarity due to the inhibition of an essentially nonfluorescent twisted intramolecular charge transfer state in nonpolar environments.11,12 This is reflected in an increase in quantum yield in hydrophobic environments, that is, from about 0.004 in water to 0.8 in dimethylformamide9 and an increase
in the fluorescence lifetime, that is, from 0.14 ns in water to 3.8 ns in dimethylformamide. In addition, a blue shift of the emission maximum of the dye occurs from about 550 nm in water to about 480 nm in hydrophobic solvents or when bound to hydrophobic parts of proteins or aggregates.

Because conformational changes, misfoldings, and aggregations of protein usually are associated with an increase in hydrophobicity of the protein's surface, bis-ANS can be used for a sensitive detection of structurally changed and aggregated protein. Bis-ANS interacts with proteins by non-specific hydrophobic and electrostatic interactions. Fluorescence intensity and the position of the emission maximum of bis-ANS in the presence of protein depend on the number of binding sites, as well as their properties, mainly with respect to polarity. The situation becomes even more complex for stressed protein formulations, which usually contain numerous structurally altered or aggregated species next to the native monomer, differing in size and physicochemical properties such as charge and hydrophobicity. Because of this, it is complicated to analyze the binding behavior of fluorescent dyes to proteins and to determine the corresponding binding constants. Nevertheless, this information is important for a better understanding and data interpretation, when using the dyes for protein characterization. Characterizing the heterogeneity of protein aggregates is also relevant for the field of immunogenicity, as it is not yet fully clear which types of aggregates induce an unwanted immune response.

Steady-state fluorescence spectroscopy and isothermal titration calorimetry (ITC) have mainly been used to study the interaction of bis-ANS and other extrinsic dyes with proteins, but circular dichroism spectroscopy, ultraviolet (UV) spectroscopy in combination with Scatchard analysis, chromatographic techniques, and equilibrium dialysis have also been used. As the listed methods are highly different in their underlying measurement principles, they may provide varying information concerning affinity and binding sites.

We selected steady-state fluorescence spectroscopy, time-resolved fluorescence spectroscopy, and ITC to elucidate the interactions of bis-ANS with aggregated IgG. In steady-state fluorescence spectroscopy, information on binding is gained indirectly from the increase in fluorescence intensity when the dye is binding to hydrophobic regions of a protein. Steady-state fluorescence has the drawback that it is difficult to evaluate the number of binding sites because different binding sites may not contribute equally to the measured increase in intensity. Moreover, populations of weakly interacting dye molecules and those whose binding is only related to minor increase in intensity, for example, of a small fraction of aggregates, might be overlooked. Those small amounts of strongly bound dye molecules can be detected by time-resolved fluorescence spectroscopy because of their clearly longer lifetimes, even if they only contribute up to a minor degree to the overall fluorescence intensity. The ability to distinguish the contribution of different populations, for example, free and bound dye molecules (obvious from the individual lifetimes and fractional contributions), to the total fluorescence signal was another reason for including time-resolved fluorescence measurements into the study. In ITC, dye binding to protein is measured by the heat released upon binding, independent of their fluorescence properties. Therefore, weakly binding fractions of dye that might contribute up to a minor degree to the overall fluorescence intensity can also be detected. However, ITC might be less sensitive to pick up small fractions of strongly binding and highly fluorescent dye molecules, which can be detected in steady-state, and more specifically in time-resolved fluorescence.

Our aim was to use the three techniques in parallel to study the interaction of bis-ANS with a humanized monoclonal antibody (IgG) and to compare the resulting binding affinities or constants. Combining these techniques makes it possible to cover a wide range of binding affinities and to obtain a comprehensive analysis of the binding behavior of bis-ANS. We focused our study on the interaction of bis-ANS with a thermally stressed IgG, which was previously shown to strongly interact with this dye. Gaining deeper insights into the binding behavior of bis-ANS will be highly relevant for the application of extrinsic fluorescent dyes for protein characterization, as it provides a scientific background for the setup of such experiments and the corresponding data interpretation. Our results show heterogeneous binding of bis-ANS to heat-stressed IgG with a wide range of binding strengths, which differ by several orders of magnitude.

**MATERIAL AND METHODS**

**Materials**

A recombinant humanized monoclonal antibody of the IgG1 subclass (IgG) with a molecular weight of 149 kDa, an isoelectric region between 9 and 10, and an aggregation temperature (T_agg = the onset of aggregation monitored by the optical density at 350 nm during a thermal scan) of about 81°C was used for the study. The IgG was filtered using 0.22 μm polyethersulfone low-binding syringe-driven filter units (Millex™ GP; Millipore, County Cork, Ireland) and formulated at a concentration of 1.0 mg/mL in 100 mM sodium phosphate buffer, pH 7.2, if not stated otherwise. After filtration, the protein content was determined from its UV absorbance at 280 nm (see below). The IgG preparation was heat stressed...
for 10 min at 80°C (heat-stressed IgG) to induce the formation of aggregates, as described earlier. Then, 1.5 mL of the formulation were stressed by heating at 80°C for 10 min in 1.5 mL reaction tubes (Eppendorf, Hamburg, Germany) using a thermomixer (Eppendorf). Bis-ANS (Sigma, Steinheim, Germany) was used as aqueous stock solution.

**UV Spectroscopy**

A diode array Agilent 8453 UV-Vis spectrometer (Agilent, Waldbronn, Germany) was used to determine the IgG content in the formulations, using an extinction coefficient of 1.49 for a 1.0 mg/mL solution. The spectra were recorded from 240 to 360 nm using an integration time of 15 s and a resolution of 1 nm. To determine the concentration of the bis-ANS stock solution, a molar extinction coefficient of 16,760 M⁻¹ cm⁻¹ at 385 nm in water was used. The spectra were recorded from 240 to 600 nm, using an integration time of 5 s and a resolution of 1 nm. All spectra were corrected for the absorbance of the particular solvent.

**Steady-State Fluorescence Spectroscopy**

A Tecan Infinite M1000 plate reader (Tecan Benelux BVBA, Giessen, the Netherlands) was used to record the emission spectra of bis-ANS by top reading in black polypropylene 96-well plates (Greiner Bio-One B.V., Alphen a/d Rijn, the Netherlands). One hundred microliter of the IgG–dye mixture per well (n = 3) was measured within 30 min after dye addition. Bis-ANS was excited at 385 nm and the emission scanned from 400 to 650 nm with slits of 5 nm, steps of 2 nm, and flashes of 4.8 J. Under these experimental settings, no inner filter effect and no disturbance by scattered excitation light was observed. By subtracting the background signals of the respective dye-free controls from the emission spectra of the dye-containing samples, the contribution of light scattering at the solvent, manifested as Raman peak, was removed from the spectra.

**Time-Resolved Fluorescence Spectroscopy**

Time-resolved fluorescence measurements were performed on a LifeSpec-ps fluorimeter (Edinburgh Instruments Ltd., Livingston, UK) using a PMT detector module. As excitation source, a 375 nm PDL 800-B picosecond pulsed diode laser (Picoquant, Berlin, Germany) operating at a frequency of 10 MHz was used. On the emission side, a cut-on filter of 495 nm was used. The temperature was kept constant at 25°C. Samples (900 μL) were measured in half-micro quartz fluorescence cuvettes (Hellma). The fluorescence decays were measured over 100 ns, up to a peak count of 10,000 using 2048 channels. The dynamic instrumental response function (IRF) was recorded by measuring the “decay” of a diluted LUDOX™ LS colloidal silica (Sigma Aldrich, Steinheim, Germany) solution in water. To deconvolute the IRF and to fit the intensity decay, the FAST software package (Edinburgh Instruments Ltd., Livingston, UK) was used. Calculations therein are based on a combination of global least square analysis minimization and non-negative singular value decomposition. The fluorescence decays were fitted using global analysis, with three exponentials (lifetimes) and the lifetime of the free dye, τa, fixed to 0.14 ns. The goodness of the fits was evaluated by the reduced χ² criterion and the residuals of the fit. The average lifetime was calculated as:

\[
\text{average lifetime} = \frac{\sum_{n=1}^{n} \tau_n f_n}{100}
\]

where, \(\tau\) is a discrete lifetime component, \(n\) is the number of lifetime components, and \(f_n\) is the fractional contribution (in %) of lifetime \(n\).

**Data Fitting Fluorescence Spectroscopy to Determine Binding Affinities**

The data of steady-state fluorescence intensity at the emission maximum plotted against bis-ANS or IgG concentration, respectively, were fitted with a Langmuir-type model assuming one average binding constant using GraphPad Prism, version 5.02 (GraphPad Software, San Diego California USA). To fit the data derived from titrations of bis-ANS to a fixed amount of IgG, Eq. 2 was used:

\[
I_{\text{meas}} = \frac{I_{\text{max}} [\text{bis} - \text{ANS}]}{K_{d-ss} + [\text{bis} - \text{ANS}]}
\]

where, \(I_{\text{meas}}\) is measured fluorescence intensity, [bis-ANS] is bis-ANS concentration in μM, \(I_{\text{max}}\) is maximum fluorescence intensity, and \(K_{d-ss}\) is equilibrium dissociation constant from steady-state fluorescence spectroscopy in μM. To fit the data derived from titrations of IgG to a fixed amount of bis-ANS, Eq. 3 was used:

\[
I_{\text{meas}} = \frac{I_{\text{max}} [\text{IgG}]}{K_{d-ss} + [\text{IgG}]}
\]

Fitting was also performed by using Eq. 4, which is derived from Eq. 3 by introducing a heterogeneity index \(h\), which is generally used to describe heterogeneity, for example, in binding of polyclonal antibody
with antigen or hapten.$^{30-32}$

$$I_{\text{meas}} = \frac{I_{\text{max}}[\text{IgG}]^h}{K_{d-\text{ss}}^h + [\text{IgG}]^h}$$

(4)

where, $I_{\text{meas}}$ is measured fluorescence intensity, $[\text{IgG}]$ is IgG concentration in $\mu$M, $I_{\text{max}}$ is maximum fluorescence intensity, $K_{d-\text{ss}}$ is equilibrium dissociation constant from steady-state fluorescence spectroscopy in $\mu$M, and $h$ is heterogeneity index.

From time-resolved fluorescence measurements, the average lifetime ($\tau_{\text{ave}}$) and fractional contributions $\tau_n$ plotted against the IgG concentration were fitted. For data fitting, a model with one binding site was used as shown in Eq. 5.

$$Y_{\text{meas}} = \frac{Y_{\text{max}} \times [\text{IgG}]}{K_{d-\text{tauave}} + [\text{IgG}]} + Y_0$$

(5)

where, $Y_{\text{meas}}$ is $\tau_{\text{ave}}$ or $f$ for the particular IgG concentration, $Y_{\text{max}}$ is maximum $\tau_{\text{ave}}$ or $f$; $Y_0$ is $\tau_{\text{ave}}$ or $f$ without IgG added, $[\text{IgG}]$ is IgG concentration in nM, and $K_{d-\text{tauave}}$ is equilibrium dissociation constants calculated from $\tau_{\text{ave}}$ or $f$ in nM.

**Fractionation of Heat-Stressed IgG Aggregates by High-Performance Size Exclusion Chromatography**

High-performance size exclusion chromatography (HPSEC) was performed on an Agilent 1200 with UV and fluorescence detection (Agilent Technologies, Palo Alto, California) combined with a Wyatt Eclipse (Wyatt Technology Europe GmbH, Dernbach, Germany). A TSK Gel 3000 SWXL column (Tosoh, Tokyo, Japan) ($300 \times 7.8$ mm) was used. Fifty microliter of heat-stressed IgG at 10 mg/mL was injected, and separation was performed at a flow rate of 0.5 mL/min. The running buffer was composed of 100 mM phosphate and 100 mM Na$_2$SO$_4$ at pH 7.2. UV detection was performed at 280 nm. Aggregates eluting from 12 to 14 min were collected and used for time-resolved fluorescence measurements (data not shown). Reanalysis of the fractionated aggregates by HPSEC proved that the fractions exclusively contained aggregates and that these remained stable within the time frame of the experiments (data not shown).

**Isothermal Titration Calorimetry**

ITC experiments were carried out using a VP-ITC calorimeter (MicroCal, Northampton, Massachusetts) with a sample cell volume of 1.41 mL. All experiments were carried out at 30°C using a 250 $\mu$L syringe and a stirring speed of 307 rpm. Samples were degassed prior to use. In each case, injections were preceded by one injection of 1 $\mu$L; interval between injections was 5 min. Raw data were analyzed with Origin 7.0 software (OriginLab, Northampton, MA, USA), assuming multiple identical binding sites.

A solution of 460 $\mu$M bis-ANS was injected to a solution of 1 mg/mL (6.7 $\mu$M) or 0.2 mg/mL (1.32 $\mu$M) IgG. The injection sequence consisted of 27 injections of 10 $\mu$L. Alternatively, 10 mg/mL (67 $\mu$M) IgG was injected to a solution of 47 $\mu$M bis-ANS. The injection sequence consisted of nine injections of 30 $\mu$L.

**RESULTS**

Steady-state fluorescence spectroscopy, time-resolved fluorescence spectroscopy, and ITC were used to study the interaction of bis-ANS with IgG stressed for 10 min at 80°C (heat-stressed IgG). For the nonstressed IgG and fractionated monomer, negligible interactions between bis-ANS and IgG were found with all the three techniques. This is in agreement with earlier observations showing that bis-ANS predominantly interacts with IgG aggregates induced by thermal stress.$^2$ Binding of bis-ANS to nonstressed IgG or fractionated monomer was too weak to determine $K_d$ values.

**Steady-State Fluorescence Spectroscopy**

Titrations of bis-ANS with a constant amount of heat-stressed IgG (Fig. 1) and titrations of heat-stressed IgG with a constant amount of bis-ANS (Fig. 2) were performed, and the relative bis-ANS fluorescence intensity was plotted as a function of the bis-ANS or IgG concentration, respectively. The emission maximum of bis-ANS is located at about 490 nm (Fig. 1a), indicating that the dye interacts with hydrophobic parts present on the heat-stressed IgG.$^{11,12}$ With increasing dye concentrations added to IgG, the bis-ANS fluorescence intensity gradually rose (Figs. 1a and 1b). The titration was performed up to 47.5 $\mu$M bis-ANS; measurements at higher concentrations would be impeded by the inner filter effect due to a too high optical density at the excitation wavelength.

To fit the bis-ANS fluorescence intensity as a function of the bis-ANS concentration, a simple Langmuir-type model assuming one average binding constant (apparent $K_{d-\text{ss}}$; Eq. 2) was found to be most suitable. From the one binding site model, an apparent $K_{d-\text{ss}}$ of 6.7 $\mu$M was calculated for the titration of bis-ANS with heat-stressed IgG (Table 1).

The titration of IgG with a constant amount of bis-ANS resulted in an apparent $K_{d-\text{ss}}$ of 2.1 $\mu$M, when using the one binding site model (Eq. 3) to fit the data (Fig. 2, Table 1). The data were also fitted with a modified Langmuir model including a heterogeneity index, $h$ (Eq. 4), which might serve to fit binding of bis-ANS to heterogeneous IgG aggregates as well. When introducing a heterogeneity index into the Langmuir model, the fit was not further improved and the fitting results ($h = 0.94 \pm 0.06; \chi^2 = 0.299; R^2 = 0.993$) did not significantly differ from the Langmuir model without a heterogeneity index ($h = 1; \chi^2 = 0.295$;
Figure 1. (a) Titration of 0.1 mg/mL (0.66 M) heat-stressed IgG with bis-ANS (final concentration 0–47.5 M). Steady-state fluorescence emission spectra of bis-ANS excited at 385 nm, (b) plot of fluorescence intensity against bis-ANS concentration (n = 3) with fitted curve (Eq. 2), and (c) the residuals of the fit.

$R^2 = 0.993)$. The resulting $K_{d\text{-ss}}$ for the two Langmuir fits were 2.2 and 2.1 $\mu$M, respectively, which confirm that the binding can be adequately described by the one binding site model.

From the steady-state fluorescence measurements, an apparent $K_{d\text{-ss}}$ value of about 6.7 $\mu$M was determined for the titration of heat-stressed IgG with bis-ANS, whereas a value of about 2 $\mu$M was obtained for the titration of bis-ANS with heat-stressed IgG. This difference is indicative of binding site heterogeneity,\textsuperscript{33} which, however, did not become apparent from either of the individuals titrations (Figs. 1 and 2). For this reason, the second titration (bis-ANS titrated with heat-stressed IgG) was repeated at several different bis-ANS concentrations (Fig. 3). The resulting apparent $K_{d\text{-ss}}$ values increased with bis-ANS concentration (Fig. 3c), which is again reflective of binding site heterogeneity. The most likely explanation is that weaker binding sites are only sampled at the higher bis-ANS concentrations.\textsuperscript{24} Another possibility is that various types of binding sites enhance bis-ANS fluorescence to a different extent, masking the heterogeneity if only a single concentration of bis-ANS is considered (see also Discussion).

### Time-Resolved Fluorescence

A titration of bis-ANS with IgG was impractical for time-resolved fluorescence due to very long measurement durations for the low bis-ANS concentrations. The opposite titration of heat-stressed IgG with a fixed amount of bis-ANS could be performed without

![Figure 2](image2.png)

**Figure 2.** (a) Titration of 5 $\mu$M bis-ANS with heat-stressed IgG (0–8.9 mg/mL; 0–60 $\mu$M), data fitting using a one binding site model (Eq. 3) and a one binding site model with a heterogeneity index $h$ (Eq. 4) and (b) the residuals of the fits.

<table>
<thead>
<tr>
<th>Experiment</th>
<th>IgG ($\mu$M)</th>
<th>bis-ANS ($\mu$M)</th>
<th>$K_{d\text{-ss}}$ ($\mu$M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Titration of bis-ANS to heat-stressed IgG</td>
<td>0.66$^a$</td>
<td>0–47.5</td>
<td>6.7 (0.4)$^b$</td>
</tr>
<tr>
<td>Titration of heat-stressed IgG to bis-ANS</td>
<td>0–60</td>
<td>5$^a$</td>
<td>2.1 (0.3)</td>
</tr>
</tbody>
</table>

$^a$Fixed amount, $^b$standard deviations shown between parentheses.
difficulty (Fig. 4a). The resulting bis-ANS fluorescence decays were fitted by global analysis, using three exponentials while fixing the lifetime of the free dye (tau1) to 0.14 ns,13 with the results for fitting the lifetime decays by global analysis summarized in Table 2. Although the best fitting results (with respect to reduced \( \chi^2 \) values and residuals) were obtained with three exponentials, the limited physical relevance of the individual lifetimes should be pointed out. Only the lifetime of the free dye (tau1) can be attributed to a distinct population within the sample. Tau2 (ranging from 0.5 to 5.6 ns) and tau3 (ranging from 6 to 10.1 ns) reflect bis-ANS interacting with the stressed IgG molecules, mainly aggregates; however, a clear assignment to certain subpopulations is not possible. The fractional contribution \( f_1 \) for tau1 was rapidly declining during the titration and fell below 5% at concentrations above 600 nM IgG for the heat-stressed IgG formulation. The fractional contributions \( f_2 \) and \( f_3 \), as well as \( \tau_{ave} \) increased during the titration. Note that the fractional contributions represent the relative contributions of the individual components to the total fluorescence decay. Therefore, fractional contributions cannot be directly related to a concentration, for example, of free dye for tau1.

Data fitting was performed for \( \tau_{ave} \) as well as the fractional contributions \( f_1, f_2, \) and \( f_3 \) using Eq. 5, with the resulting apparent \( K_d \) values summarized in Table 3. The results of the fractional contributions are in the same order of magnitude and confirm those of the \( \tau_{ave} \). However, because the physical meaning of the fractional contributions is unclear, we limit our discussion on the results obtained for \( \tau_{ave} \) (Fig. 5a).

The apparent dissociation constant of about 50 nM for the titration of heat-stressed IgG with bis-ANS suggests that time-resolved fluorescence probes subpopulations with higher affinity binding sites for bis-ANS as compared with the populations detected by steady-state fluorescence (cf. Table 1 and 3). Those higher affinity sites are expected to be mainly present on heat-stressed IgG aggregates.
Table 2. Results from Global Analysis of Time-Resolved Fluorescence Data of 5 μM bis-ANS Titrated with 0 to 1.0 mg/mL Heat-Stressed IgG

<table>
<thead>
<tr>
<th>IgG Concentration (nM)</th>
<th>τave (ns)</th>
<th>f1 (%)</th>
<th>f2 (%)</th>
<th>f3 (%)</th>
<th>τave (ns)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0.46</td>
<td>77.3</td>
<td>14.0</td>
<td>1.11</td>
<td>0.14</td>
</tr>
<tr>
<td>1.32</td>
<td>1.17</td>
<td>55.7</td>
<td>38.0</td>
<td>2.71</td>
<td>0.14</td>
</tr>
<tr>
<td>13.2</td>
<td>1.86</td>
<td>45.0</td>
<td>42.2</td>
<td>3.22</td>
<td>0.14</td>
</tr>
<tr>
<td>66</td>
<td>3.58</td>
<td>28.1</td>
<td>49.3</td>
<td>4.8</td>
<td>0.14</td>
</tr>
<tr>
<td>132</td>
<td>4.05</td>
<td>17.9</td>
<td>52.9</td>
<td>5.66</td>
<td>0.14</td>
</tr>
<tr>
<td>330</td>
<td>4.71</td>
<td>6.4</td>
<td>55.4</td>
<td>6.69</td>
<td>0.14</td>
</tr>
<tr>
<td>660</td>
<td>5.24</td>
<td>2.5</td>
<td>51.7</td>
<td>7.2</td>
<td>0.14</td>
</tr>
<tr>
<td>1320</td>
<td>5.49</td>
<td>2.0</td>
<td>47.0</td>
<td>7.4</td>
<td>0.14</td>
</tr>
<tr>
<td>3300</td>
<td>5.56</td>
<td>0.5</td>
<td>52.7</td>
<td>7.85</td>
<td>0.14</td>
</tr>
<tr>
<td>6600</td>
<td>5.58</td>
<td>0.1</td>
<td>54.5</td>
<td>8.08</td>
<td>0.14</td>
</tr>
</tbody>
</table>

The first lifetime component (τ1) was fixed to 0.14 ns. Global χ² = 1.07.

To prove this assertion, aggregates were fractionated by HPSEC and analyzed in a titration with a constant amount of bis-ANS. The resulting decays of the fluorescence lifetime (Fig. 4b), as well as the fitting of these decays by global analysis (using three exponentials and τ1 fixed to 0.14 ns, results summarized in Table 4), were very similar to the results from the nonfractionated heat-stressed IgG formulation. The resulting apparent dissociation constants for the fractionated aggregates of 7.5 nM obtained from the average lifetime (Fig. 5b) were lower, but still in the same order of magnitude as those of the nonfractionated heat-stressed IgG formulation (Table 3). This shows that time-resolved fluorescence mainly detects bis-ANS interacting with heat-stressed IgG aggregates. Saturation of binding is reached at clearly lower IgG concentrations during the titration as compared with steady-state fluorescence spectroscopy, suggesting that the lower affinity binding sites contribute up to a minor degree to the fluorescence lifetime.

Table 3. Apparent Dissociation Constants Derived from Time-Resolved Fluorescence Using the Average Lifetime (τave) and the Fractional Contributions (f1–f3) for Data Fitting (n = 3)

<table>
<thead>
<tr>
<th>Experiment</th>
<th>IgG (nM)</th>
<th>Keqave (nM)</th>
<th>Kd1 (nM)</th>
<th>Kd2 (nM)</th>
<th>Kd3 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Titration of heat-stressed IgG with 5 μM</td>
<td>0–660</td>
<td>50 (9)</td>
<td>37 (3)</td>
<td>103 (13)</td>
<td>29 (8)</td>
</tr>
<tr>
<td>bis-ANS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Titration of fractionated IgG aggregates</td>
<td>0–600</td>
<td>7.5 (1.8)</td>
<td>13 (2)</td>
<td>47 (13)</td>
<td>1 (0.5)</td>
</tr>
<tr>
<td>with 5 μM bis-ANS</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

*Standard deviations between parentheses.
Table 4. Results from Global Analysis for Time-Resolved Fluorescence of 5 μM bis-ANS Titrated with 0–0.09 mg/mL Fractionated Heat-Stressed IgG Aggregates

<table>
<thead>
<tr>
<th>IgG Concentration (nM)</th>
<th>0</th>
<th>8</th>
<th>17</th>
<th>33</th>
<th>66</th>
<th>132</th>
<th>198</th>
<th>296</th>
<th>330</th>
<th>396</th>
<th>462</th>
<th>592</th>
</tr>
</thead>
<tbody>
<tr>
<td>tau1 (ns)</td>
<td>fixed to 0.14</td>
<td>0.44</td>
<td>1.95</td>
<td>3.28</td>
<td>3.42</td>
<td>4.58</td>
<td>4.61</td>
<td>5.11</td>
<td>5.03</td>
<td>4.97</td>
<td>5.51</td>
<td>5.08</td>
</tr>
<tr>
<td>tau2 (ns)</td>
<td>6.33</td>
<td>6.92</td>
<td>7.86</td>
<td>7.83</td>
<td>8.72</td>
<td>8.73</td>
<td>9.31</td>
<td>9.22</td>
<td>9.08</td>
<td>10.01</td>
<td>9.27</td>
<td>10.05</td>
</tr>
<tr>
<td>f1 (%)</td>
<td>61.0</td>
<td>41.4</td>
<td>22.6</td>
<td>15.6</td>
<td>7.6</td>
<td>4.1</td>
<td>2.3</td>
<td>1.5</td>
<td>0.8</td>
<td>0.5</td>
<td>0.5</td>
<td>0.3</td>
</tr>
<tr>
<td>f2 (%)</td>
<td>9.6</td>
<td>13.3</td>
<td>21.9</td>
<td>25.4</td>
<td>38.6</td>
<td>37.9</td>
<td>46.4</td>
<td>42.7</td>
<td>41.2</td>
<td>56.6</td>
<td>43.3</td>
<td>53.2</td>
</tr>
<tr>
<td>f3 (%)</td>
<td>29.4</td>
<td>45.3</td>
<td>55.6</td>
<td>59.0</td>
<td>53.8</td>
<td>58.0</td>
<td>51.3</td>
<td>55.8</td>
<td>58.0</td>
<td>42.9</td>
<td>56.2</td>
<td>46.4</td>
</tr>
<tr>
<td>τave (ns)</td>
<td>1.99</td>
<td>3.45</td>
<td>5.12</td>
<td>5.51</td>
<td>6.47</td>
<td>6.82</td>
<td>7.15</td>
<td>7.30</td>
<td>7.32</td>
<td>7.41</td>
<td>7.41</td>
<td>7.63</td>
</tr>
<tr>
<td>χ²</td>
<td>1.09</td>
<td>1.11</td>
<td>1.13</td>
<td>1.11</td>
<td>1.09</td>
<td>1.03</td>
<td>1.07</td>
<td>1.05</td>
<td>1.05</td>
<td>1.09</td>
<td>1.05</td>
<td>1.08</td>
</tr>
</tbody>
</table>

The first lifetime components (tau1) was fixed to 0.14 ns. Global $\chi^2 = 1.09$.

0.2 mg/mL IgG, respectively. However, it must be emphasized that these values are probably not very reliable because $n$ and $\Delta H$ compensate in the fitting model, and a substantial range of values of $n$ and $\Delta H$ yielded acceptable fits. In addition, as bis-ANS mainly interacts with aggregated IgG (compare with time-resolved fluorescence), the real stoichiometries for certain populations are most likely even higher.

Titration of heat-stressed IgG with bis-ANS (Fig. 7) resulted in a binding isotherm from which apparent binding parameters could be reliably calculated. The apparent dissociation constant $K_{d-ITC}$ of 5 μM (Table 5) is in good agreement with the values obtained from steady-state fluorescence under comparable conditions. Again, as observed in steady-state fluorescence (Fig. 3), the apparent dissociation constant found using this model depends on the range of concentrations used to determine the binding profile and the direction of the titration (IgG with bis-ANS versus bis-ANS with IgG), indicating heterogeneous binding sites.

DISCUSSION

We used steady-state fluorescence spectroscopy, time-resolved fluorescence spectroscopy, and ITC to study the interaction between bis-ANS and heat-stressed IgG. The three techniques differ in their underlying principle used to study binding and the covered binding affinities, which has been described in detail.
Table 5. Calculated Parameters for Binding of bis-ANS to Heat-Stressed IgG as Determined by Isothermal Titration Calorimetry

<table>
<thead>
<tr>
<th>Experiment</th>
<th>$K_{d-ITC}$ (μM)</th>
<th>Stoichiometry ($n$)</th>
<th>$\Delta H$ (10$^4$ kcal/mole bis-ANS)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Titration of bis-ANS with 6.7 μM (1.0 mg/mL), IgG ($n = 4^a$)</td>
<td>63 (9)$^b$</td>
<td>9 (3)</td>
<td>$-2.0$ (0.5)</td>
</tr>
<tr>
<td>Titration of bis-ANS with 1.3 μM (0.2 mg/mL), IgG ($n = 3$)</td>
<td>23 (3)</td>
<td>5.4 (0.7)</td>
<td>$-2^c$</td>
</tr>
<tr>
<td>Titration of IgG with 47 μM bis-ANS ($n = 2$)</td>
<td>5 (1)</td>
<td>7.1 (0.3)</td>
<td>$-0.72$ (0.05)</td>
</tr>
</tbody>
</table>

$^a n = X$ represents number of experiments.

$^b$ Standard deviations between parentheses.

$^c$ Value fixed in fit.

Figure 7. Titration of heat-stressed IgG with 47 μM of bis-ANS, with the molar ratio of IgG to bis-ANS on the x-axis.

in the Introduction. The intrinsically heterogeneous nature of heat-stressed IgG formulations composed of various types of aggregates$^2,3$ complicates quantitative analysis of binding. The selected approach of combining the three techniques allows covering different binding affinities as wide as possible. The characteristics of the IgG formulation used in this study (heat-stressed for 10 min at 80°C), with respect to aggregation profile and protein structure, have been described in detail in one of our earlier publications.$^2$

Steady-state fluorescence spectroscopy and ITC have been widely used to analyze binding of dye molecules to protein.$^{14,22-24}$ Usually, different concentration ranges are used for the two methods due to the discussed limitations of inner filter effect in fluorescence spectroscopy on the one hand and low signals of weakly binding populations in ITC on the other hand.

Within our study, comparable concentration ratios of bis-ANS to heat-stressed IgG were employed for the different techniques as far as possible. This was possible as the steady-state fluorescence measurements were performed in a plate reader system with top reading and a short path length, where the inner filter effect is less pronounced than in a cuvette.

Fitting the data from steady-state fluorescence spectroscopy worked best using a simple Langmuir one binding site model, resulting in one average binding constant $K_{d-ss}$. Fitting the data with a Langmuir model including a heterogeneity index proved the suitability of this simple mode (Fig. 2b). Introducing a second or third binding constant did not improve the fit (data not shown) and does not necessarily better reflect the complex situation present in the analyzed samples. The heat-stressed IgG formulation is highly polydisperse and contains, besides monomeric IgG, a complex mixture of aggregates differing in size, structure, and affinity to bis-ANS. Information on the number of bis-ANS molecules binding to each of this species, as well as the individual $K_d$ values, cannot be obtained as it is practically impossible to collect fractions of all species present in the formulation. Therefore, giving an average $K_d$ value for the whole formulation is the more appropriate approach.

When comparing the three techniques included within our study, steady-state fluorescence spectroscopy and ITC resulted in binding constants of similar order of magnitude, that is, for a comparable titration of IgG with bis-ANS, the apparent $K_{d-ss}$ was 2.1 μM and the apparent $K_{d-ITC}$ was 5 μM. These binding constants are comparable to those found in the literature for binding of extrinsic dyes to aggregates, for example, 1.6 μM for 9-dicyanovinyl juloidine (DCVJ), 2.4 μM for 8-anilinonaphthalene-1-sulfonic acid (ANS), and 6.8 μM for thioflavin T to oligomers of transthyretin determined by steady-state fluorescence spectroscopy$^8$ and proteins with dye-binding sites in the native state, for example, 0.5 μM for ANS and 7.6 μM for 2-p-tolu- idynaphthalene-6-sulfonate (TNS) to human tear lipocalin.$^{34}$ Higher apparent $K_{d-ITC}$ values were found by ITC for the titration of bis-ANS with IgG, which points at a higher apparent $K_{d-ITC}$ being sampled as consequence of the
clearly larger concentration range being covered in this titration. In fact, for both ITC (Fig. 6) and steady-state fluorescence spectroscopy (Fig. 3), it could be observed that different apparent $K_d$ values were sampled depending on the concentration range covered during the titration and the direction of the titration (bis-ANS with IgG or IgG with bis-ANS). ITC and steady-state fluorescence measurements can be regarded as titrations of one binding component (A) to a second one at a virtually fixed amount (B). With both methods, no signs of heterogeneity were obtained from a single titration of A with B. However, titrations of A at increasing concentrations of B yielded dissociation constants that increased accordingly, as well as when changing the direction of the titration. This is indicative of heterogeneity in binding sites, with low-affinity binding sites becoming measurable only at higher concentrations of both binding partners.

Two factors have to be considered when discussing this observed behavior: (i) differences in bis-ANS affinity to the binding sites present in the sample, for example, high- and low-affinity binding sites and (ii) differences in sensitivity of the particular method to detect binding. Especially, the low-affinity binding sites, which result in higher corresponding apparent dissociation constants, require titrations performed over a wider concentration range in order to be sampled. However, those low-affinity binding sites may result in less intense fluorescence signals, as they are expected to be less hydrophobic, and also in lower binding enthalpies as probed by ITC. As a result, binding of bis-ANS to lower affinity sites can be overlooked, as it contributes only up to a minor degree to the total fluorescence intensity or binding enthalpies as compared with the strong signal of the more hydrophobic binding sites.

From the ITC titrations of bis-ANS with IgG, binding stoichiometries of about 5 and 9 have been calculated. However, as the sample is polydisperse and bis-ANS is expected to mainly interact with aggregates, more binding sites might be present on those species. Overall, the number of binding sites is in agreement with observations in the literature for dye binding to proteins. For ANS and bis-ANS, five and six binding sites, respectively, per bovine serum albumin molecule have been determined by steady-state fluorescence spectroscopy. For bis-ANS binding to tubulin, four low-affinity and 12 high-affinity binding sites have been measured by ITC.

Time-resolved fluorescence spectroscopy appeared to be particularly sensitive to high-affinity binding sites of bis-ANS due to the resulting long lifetimes. Even if the long lifetimes only account for a small percentage of the total fluorescence intensity, for example, within the first points of the titration, they can be clearly resolved in the decay curves (Fig. 4). The relative contribution of the lifetime of unbound dye ($\tau_{un}$, 0.14 ns) to the decay can be monitored from the fractional contribution, $f_1$. Although this parameter cannot provide quantitative information on the amount of free bis-ANS molecules, it shows that the contribution of the lifetime of free dye to the total fluorescence decay declined very rapidly at very low IgG concentrations. For data fitting, $\tau_{ave}$ and $f_1$ were used and the resulting apparent $K_{d,TR}$ values were in the nanomolar range. A comparison with fractionated heat-stressed IgG aggregates (Fig. 5) showed that the interaction of bis-ANS with those aggregates is mainly contributing to the signals measured in time-resolved fluorescence and the resulting low $K_{d,TR}$ values. This high sensitivity of time-resolved fluorescence could be an advantage when an early detection of small fractions of aggregates is required such as in formulation screening. However, the suitability of time-resolved fluorescence for formulation screening needs to be proven first due to the more complex measurement principle and data analysis as compared with steady-state fluorescence.

The described approach of measuring $K_d$ values by different methods, as well as the use of fluorescent dyes, in general, could be useful to gain more insight into aggregation mechanisms. In the example of IgG, the properties of the formed aggregates are known to depend on the selected stress temperature, as Fab domains are known to unfold readily at lower temperatures than the Fc domains. In fact, when IgG was stressed under milder conditions, for example, for 10 min at 70°C or 75°C, or for shorter periods at 80°C, weaker interactions (i.e., higher $K_d$ values) were observed as compared with the 10 min 80°C stressed sample (unpublished data). By studying binding affinities of bis-ANS to different types of aggregates, aggregation mechanisms could be further elucidated.

**CONCLUSIONS**

Steady-state fluorescence spectroscopy and ITC of aggregated IgG and bis-ANS determined binding constants in the micromolar range, whereas those from time-resolved fluorescence ranged in the lower nanomolar range. Steady-state fluorescence and ITC provide an “average” apparent bis-ANS binding constant for the various populations of heat-stressed IgG sampled in our studies. Time-resolved fluorescence is biased toward bis-ANS/heat-stressed IgG complexes with both high affinity and high quantum yield. This can be explained by the measurement principle, as even small fractions of bis-ANS molecules in such complexes can be detected by their prolonged lifetimes. This high sensitivity of time-resolved fluorescence can be an advantage when a sensitive detection of small fractions of aggregates is required, for example, during formulation screening or stability.
testing of therapeutic proteins. To gain a more general idea on the interaction between bis-ANS and IgG, steady-state fluorescence spectroscopy and ITC are preferred. By using the techniques in parallel and by testing various concentration ranges, a wide range of binding sites can be covered.

ACKNOWLEDGMENTS

This research is supported by the Dutch Technology Foundation STW, applied science division of NWO and the technology program of the Ministry of Economic Affairs.

REFERENCES