Electronic Supplementary Information

Fluorescence Correlation Spectroscopy Study of the Complexation of DNA Hybrids, IgG Antibody and a Chimeric Protein of IgG-binding ZZ Domains fused with a Carbohydrate Binding Module

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Determination of association constant using average diffusion times

Considering a fast exchange in the host-guest binding reaction during the transit time of the molecule in the confocal volume, Al-Soufi et al.[1] proposed that only an averaged diffusion time is obtained. This yielded mean diffusion time depends on the molar fractions of free (χ_F) and bound fluorescent species (χ_B), and is given by:

$$\bar{\tau}_D = \frac{\omega_0^2}{4\bar{D}} \tag{S1}$$

in which the average diffusion coefficient, $\bar{D}$, is the weighted sum of individual diffusion coefficients of free (D_F) and bound (D_B) species: $\bar{D} = \chi_B D_B + \chi_F D_F$.

The bound molar fraction χ_B can then be obtained from the corresponding average diffusion coefficient:

$$\chi_B = \frac{(\bar{D} - D_F)}{(D_B - D_F)} \tag{S2}$$

The bound molar fractions χ_B from a concentration series can be used to determine the apparent association constant value, K_a, by fitting equation 10 of the main text, while assuming a 1:1 interaction model between guest and host species. Alternatively, the average diffusion coefficient $\bar{D}$ can be used to determine the apparent association constant value K_a by fitting the variation of average diffusion coefficient in a concentration series:

$$\bar{D} = D_F + (D_B - D_F)\chi_B \tag{S3}$$

in which χ_B is defined by equation 10 of the main text.

In the case of the mixtures of DNA probe with increasing concentrations of complementary biotin-labeled DNA sequence, as shown in the inset of Figure 2A from the main text, an average (intermediate) diffusion time is obtained for each target DNA concentration from the fits of the correlation curves. Figure S1A shows the values of bound molar fraction χ_B obtained through equation S2. As expected, the molar fraction of hybridized DNA probe increases with the increment of target DNA until the DNA probe is completely bound. These values were adjusted using equation 10 of the main text, and a K_d value of 20 nM was obtained. On its turn, Figure S1B shows the values of average diffusion coefficient $\bar{D}$ obtained through equation S3. Similarly to the analysis presented in the main text, the fitted average diffusion coefficient decreases with the increment of target DNA, from ~136 μm^2/s for the free DNA probe to about 99 μm^2/s in the hybridized form (D_B). An apparent K_d value of 30 nM was obtained. These results are equivalent to the binding constant obtained for this molecular interaction through the two-component model global fit (see Figure 2B of the main text).

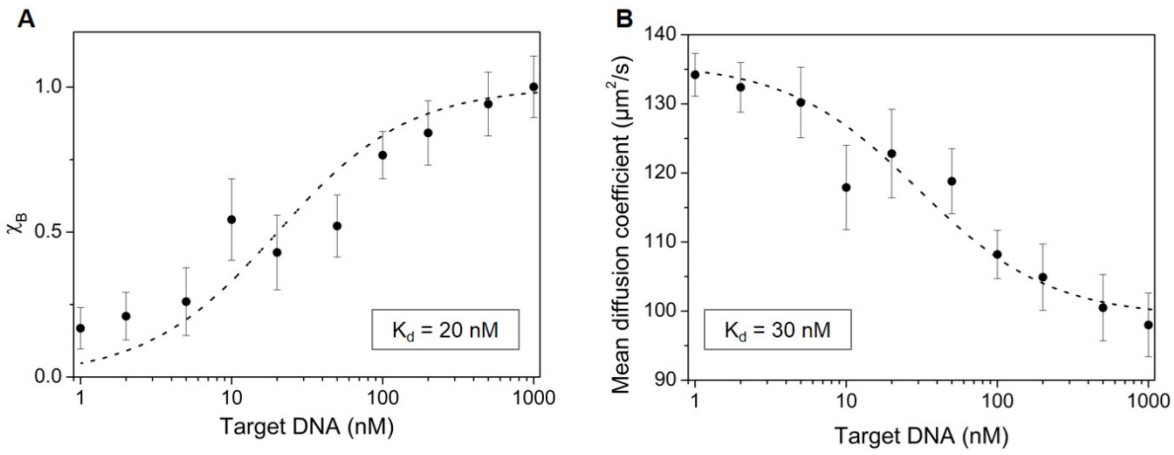

Figure S1. (A) Bound molar fraction (χ_B) of the Atto647N-labeled DNA probe (1 nM) at several complementary biotin-labeled target DNA concentrations (1-1000 nM) in PBS aqueous solution. (B) Average diffusion coefficient obtained for Atto647N-labeled DNA probe (1 nM) mixed with 0-1000 nM of complementary biotinylated target DNA. The dotted lines represents the best fit to a 1:1 binding model. Association constants were determined using average diffusing times assuming (A) experimental or (B) fitted values for diffusion coefficients of free, D_F, and bound, D_B, species.

Determination of association constant by two-component model global fit

A number of FCS experimental values were selected to exemplify the determination of association constant, K_a, by two-component model global fit (Figure S2). The fraction of bound species, χ_B, for each target molecule concentration was plotted over the best global fit used to determine the apparent K_a of the complex formed by the DNA probe and its complementary biotin-labeled DNA target (see Figure 2B of the main text).

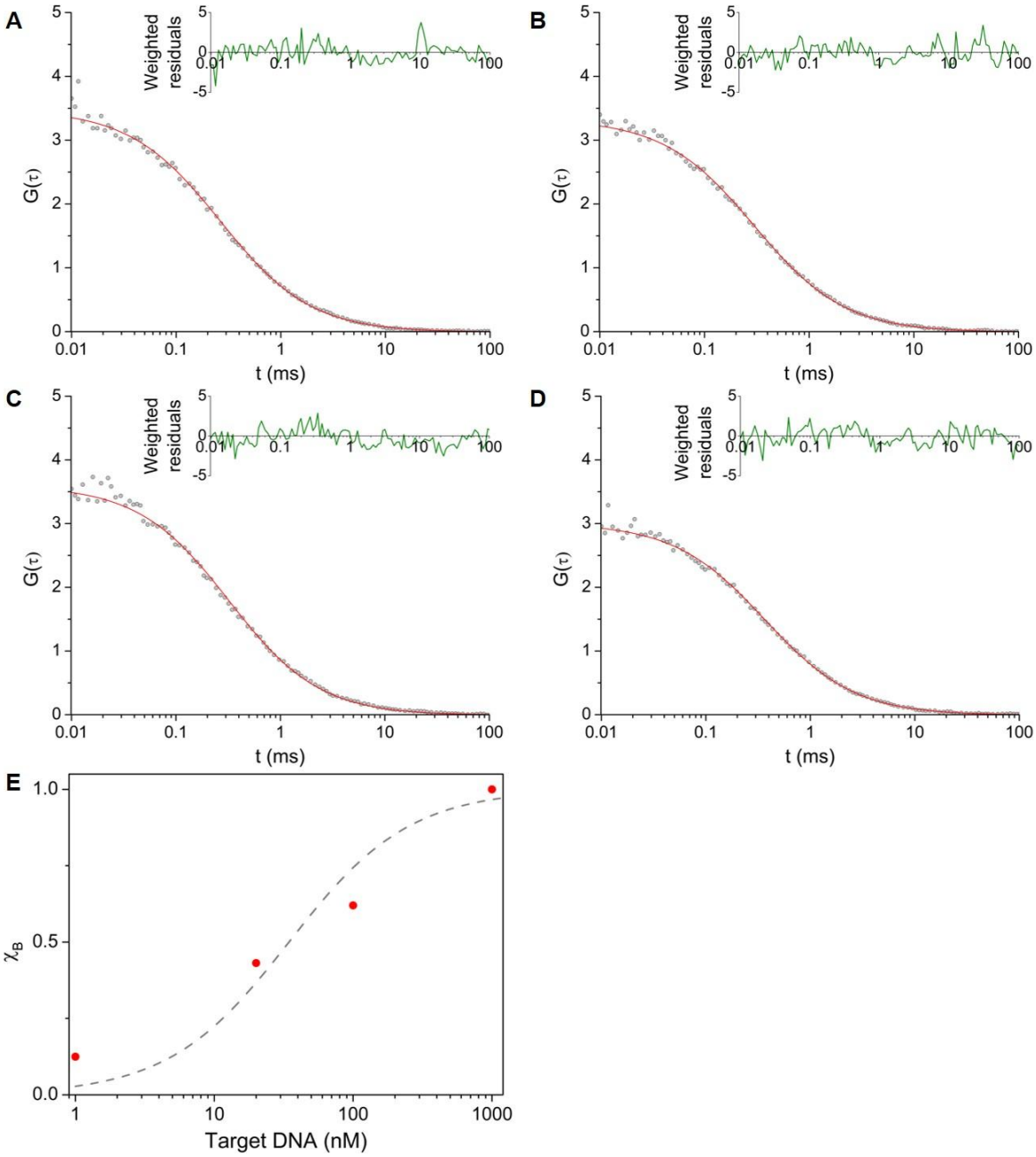

Figure S2. Determination of association constant, K_a, by two-component model global fit. A-D: Typical autocorrelation curves for Atto647N-DNA probe (1 nM) in aqueous solution in the presence of (A) 1 nM, (B) 20 nM, (C) 100 nM or (D) 1000 nM of complementary biotin-DNA target. The insets indicate the respective weighted residuals in function of time (ms), after autocorrelation curves were globally fitted by using fixed values for Q_f, Q_b, τ_f and τ_b (equation 7 of the main text). The average number of free and bound species in the confocal volume were fitted, minimizing the sum of squared residuals. (E) Bound molar fraction (χ_B) of the DNA probe at the selected target DNA concentrations were obtained from the fitted values (equation 8 of the main text), and plotted over the best global fit to a 1:1 biomolecular interaction model (equation 10 of the main text, dashed line).

In the case of the complexation of the biotinylated DNA hybrids with the anti-biotin IgG, the fluorescence intensity decreased about 65% upon binding to the antibody, as illustrated in Fig. S3A. Therefore, FCS data were globally fitted by a two-component model considering also the relative brightness, Q_b/Q_f, of ~35% for the DNA·DNA:antibody complex.

Considering this difference in the brightness of the fluorescent DNA·DNA hybrids free or bound to the antibody, it is expected that the correlation curves contain an additional slope denoting a "reaction term" with a relaxation rate of the reaction much faster than the typical diffusion times of guest and complex.[1] The correlation function $G_R(\tau)$ of the chemical equilibrium reaction is described by equation 11 of the main text. The amplitude of the reaction term A_R is given by equation S4, and it can be estimated from the relative brightness of the complex, Q_b/Q_f, and the equilibrium constant of the binding process, K_a:

$$A_R = \frac{K_a[Host](1 - Q_b/Q_f)^2}{(1 + Q_b/Q_f \times K_a[Host])^2} \tag{S4}$$

The relaxation time of the reaction term, τ_R, is defined through the following equation:

$$\tau_R = (k_+[Host] + k_-)^{-1} \tag{S5}$$

in which the association rate constant k_+ and the dissociation rate constant k_- relate to the equilibrium (association) constant by $K_a = k_+/k_-$. We have used equation S5 to estimate the relaxation time, τ_R, in the case of a binding reaction of guest to the host that is diffusion-limited. For this purpose, we have assumed that the bimolecular rate constant k_+ can be estimated from the Smoluchowski equation:

$$k_+ = 4\pi N_A R(D_{Guest} + D_{Host}) \quad \text{(S6)}$$

where N_A is Avogadro's number and R is the collision radius, which is generally assumed to be the sum of the molecular radii of each molecule.

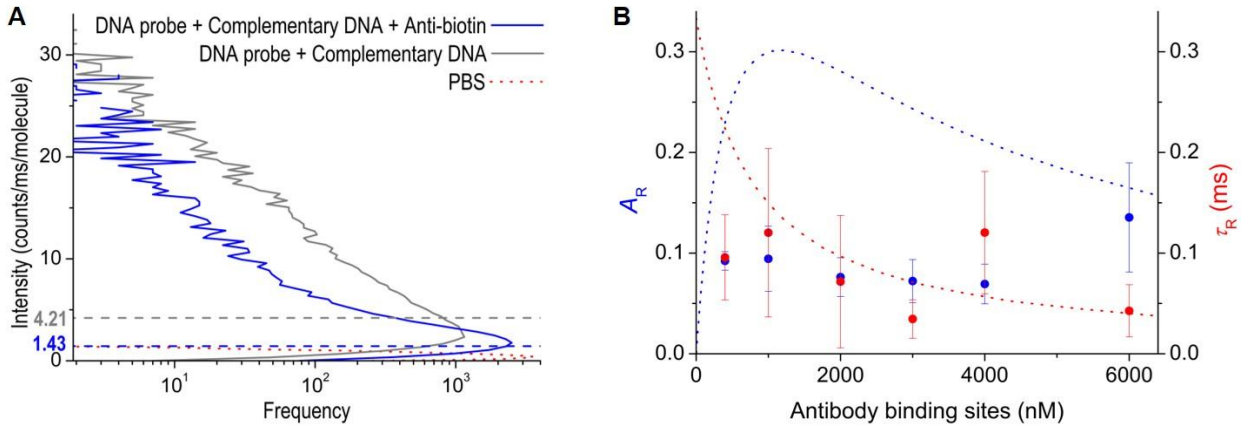

Figure S3. Complexation of biotinylated DNA·DNA hybrids with anti-biotin monoclonal IgG antibody. (A) Typical counts frequency of mixtures of 1 nM Atto647N-DNA and 200 nM biotin-DNA alone (gray) or in the presence of anti-biotin IgG at maximum concentration (3000 nM, blue). Their average fluorescence intensity is represented by the respective colored dashed line. The red dotted line indicates the background noise level measured in buffer solution. (B) Amplitude of the reaction term (A_R, blue) and the relaxation time (τ_R, red) as a function of the antibody binding sites concentration. The respective colored dotted lines represent the simulated variation of A_R and τ_R with increasing antibody binding sites concentration, following equations S4 and S5.

We have found that the fitted reaction times, τ_R, are comparable to the estimated values from equations S5 and S6, that is, assuming that the binding reaction is a diffusion-limited process (Fig. S3B). However, the relaxation time τ_R is expected to decrease with increasing host (antibody) concentration, but experimental data do not exhibit any tendency. The amplitude of the reaction term A_R obtained from experimental FCS data also does not follow the expected variation with increasing amounts of antibody. These results could be explained by the high uncertainty of the FCS data below 0.01 ms together with the time resolution of the equipment that could not allow the acquisition of data at short time range for an accurate description of the reaction term in the correlation curve (see Fig. S4). Nevertheless, the difference in the fitted

curves considering or not the correlation function $G_R(\tau)$ is noticeable and renders a lower residuals dispersion. Therefore, the reaction term was considered in the analysis of the binding of the DNA·DNA hybrids to the antibody, as well as in further analysis of the complexation with the ZZ-CBM fusion.

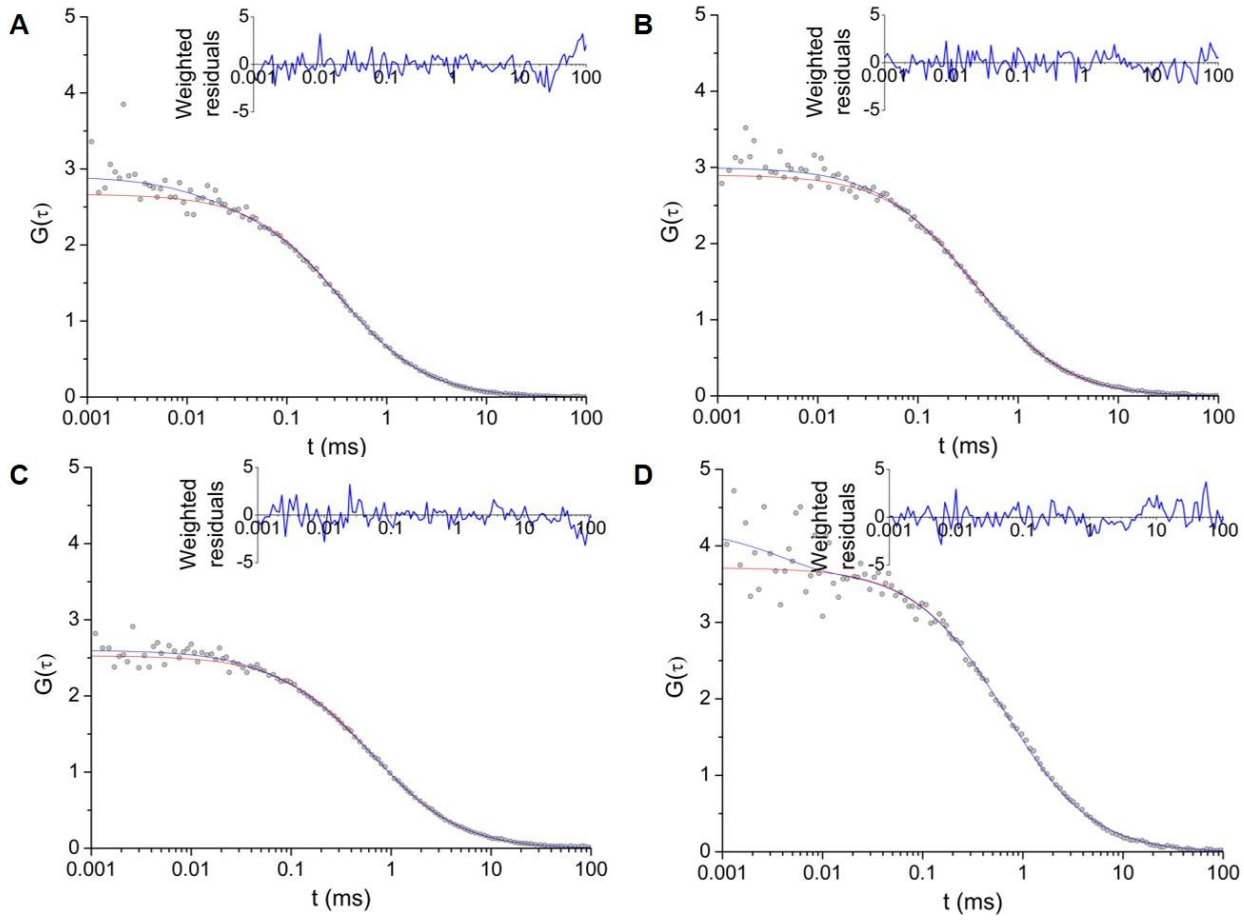

Figure S4. Determination of association constant, K_a, by two-component model global fit considering a change of brightness upon complex formation. A-D: Typical autocorrelation curves for mixtures of 1 nM Atto647N-DNA and 200 nM biotin-DNA in the presence of (A) 200 nM, (B) 500 nM, (C) 1500 nM or (D) 3000 nM of anti-biotin IgG. FCS data were globally fitted considering Q_b=0.35 and either only the diffusion model (red line, equation 7 of the main text) or with an equilibrium reaction term (blue line, equation 11 of the main text). The insets indicate the respective weighted residuals in function of time (ms), after autocorrelation curves were globally fitted considering a reaction term.

Cooperativity in the binding process between DNA·DNA and anti-biotin IgG

One important factor in the analysis of data in supramolecular systems is the cooperativity in a binding process, which can be assessed from Hill equation:

$$\chi_B = \frac{[Host]^{n_H}}{{K_{0.5}}^{n_H} + [Host]^{n_H}} \quad \text{(S7)}$$

where $K_{0.5}$ is the host molecule concentration at which half of the guest molecules are bound, and n_H is the Hill coefficient, which describes the cooperativity of host binding.[2] In the binding process of biotinylated DNA hybrids to IgG antibody, n_H will reflect the extent of cooperativity among the two IgG binding sites to capture biotin. An n_H of 1 describes a system where the binding sites are truly identical and independent of each other (non-cooperative binding). Positively ($n_H > 1$) or negatively cooperative binding ($n_H < 1$) refer to situations where the binding of biotinylated DNA·DNA to an IgG binding site enhances or weakens the binding of additional DNA hybrids to the remaining binding site, respectively.

Figure S5 shows the bound molar fraction (χ_B) of the DNA hybrids as a function of the antibody binding sites concentration with two fitted binding curves, which were obtained using equation S7 with either a fixed or floating value for n_H. The data is better fit with an n_H value of 1.6, suggesting positive cooperative binding between the biotinylated DNA hybrids and the anti-biotin antibody. Antibodies can bind bivalently to haptens and, therefore, their overall affinity depends upon both the first and second dissociation constants related to the eventual sequential binding to haptens.[3,4] However, previous studies reported non-cooperative behaviour between biotin and anti-biotin, either in bulk solution or in surface-based binding.[4–6] The $K_{0.5}$ values

obtained with $n_H = 1.0$ and $n_H = 1.6$ are of the same order of magnitude – 408 ± 77 nM and 533 ± 19 nM, respectively; but the degree of uncertainty is higher when a two-site, non-cooperative binding model is considered. Therefore, despite the absence of experimental support, positive cooperative binding should not be disregarded.

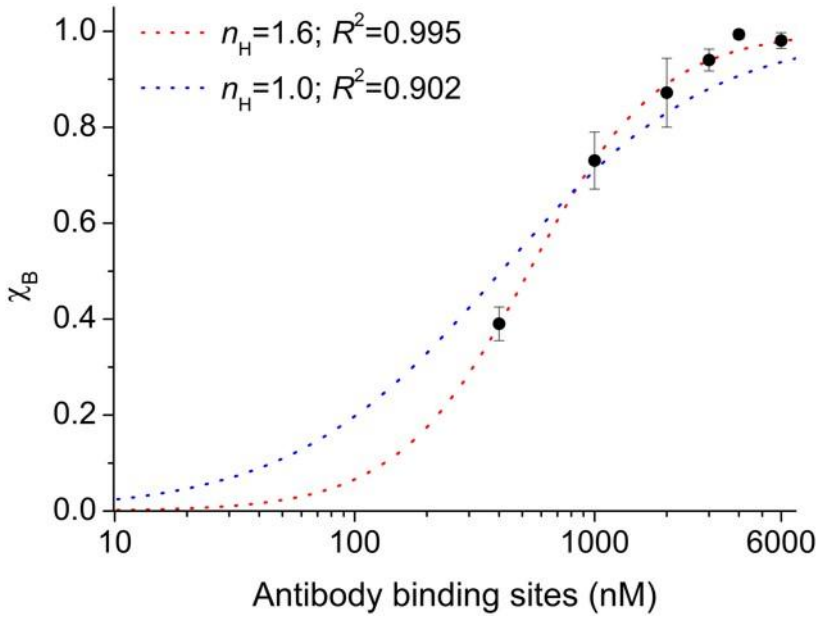

Figure S5. Assessment of the cooperativity in the complexation of biotinylated DNA·DNA hybrids with anti-biotin monoclonal IgG antibody. Closed circles represent the experimental data related to the bound molar fraction (χB) of the DNA·DNA hybrids (1 nM Atto647N-DNA and 200 nM biotin-DNA) at various antibody binding sites concentrations (0-6000 nM) in PBS. The blue and red dotted lines represent the best fit curve obtained with a fixed (n_H=1.0) or floating Hill coefficient, respectively. The resultant $K_{0.5}$ values with n_H=1.0 and n_H=1.6 are 408 ± 77 nM and 533 ± 19 nM, respectively.

References

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