Electronic Supplementary Material (ESI) for Physical Chemistry Chemical Physics.

Electronic Supplementary Information for: Fluorescence correlation spectroscopy for multiple-site equilibrium binding: a case of doxorubicin-DNA interaction

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S1. Diffusion coefficient of DOX

The diffusion coefficient of DOX in Tris-HCl, D_{DOX}, was determined by FCS based on the calibration measurements of rhodamine 110 (Rh110), whose diffusion coefficient D_{Rh110} in aqueous solution at 25 ℃ was known ($D_{\mathrm{Rh110}} = 4.70 \times 10^{-6}$ $\mu\mathrm{m}^2\mathrm{s}^{-1}$).[1] In brief, we recorded the diffusion of Rh110 and DOX in Tris-HCl solutions using FCS (Fig. S1). Their diffusion time through the FV was determined by fitting the experimental data with single-component model for ACF:

$$G_1(\tau) = G(0)\left(1 + \frac{p}{1-p}e^{\tau/\tau_{\mathrm{t}}}\right)\left(1 + \frac{\tau}{\tau_{\mathrm{p}}}\right)^{-1}\left(1 + \frac{\tau}{\kappa^2\tau_{\mathrm{p}}}\right)^{-1/2}, \tag{S1}$$

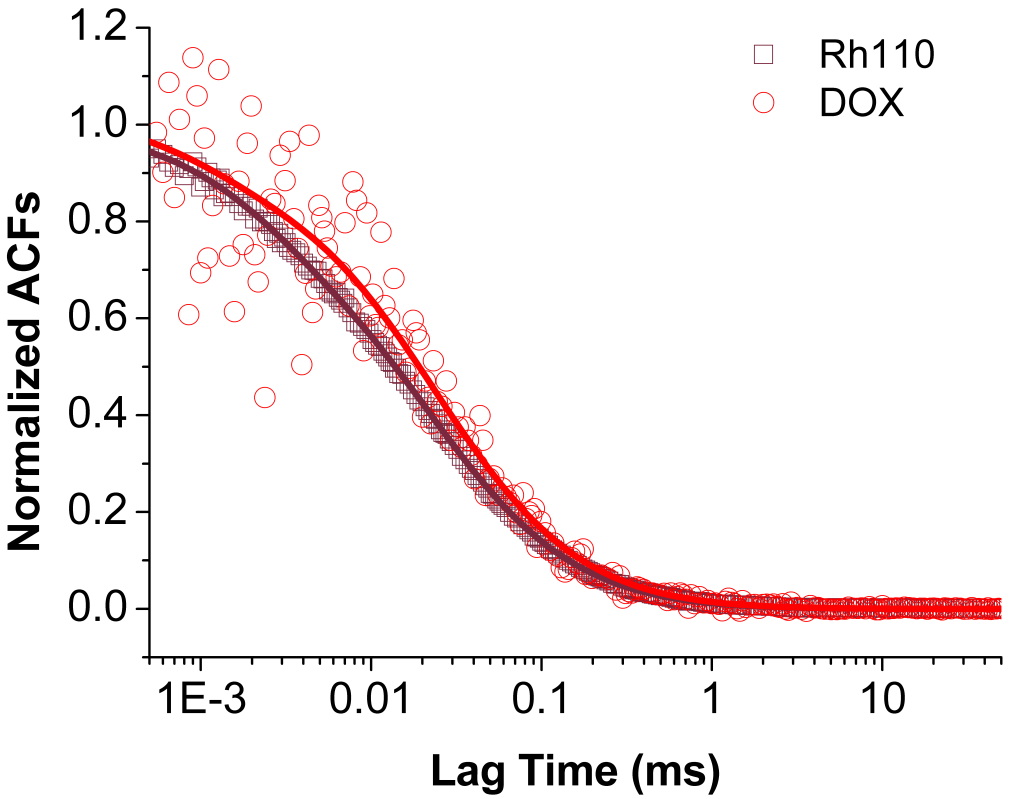

Figure S1: Experimental autocorrelation data for Rh110 and DOX (open symbols) diffusing in Tris-HCl solutions, and the fits (solid lines) using Eq. S1. The more pronounced scatter of data points for DOX is due to much lower quantum efficiency of this probe compared to Rh110.

where κ is a fitting parameter representing the axial elongation of the FV and τ_{p} is the fitted diffusion time of probes through the FV, i.e., $\tau_{\mathrm{p,Rh110}}$ =0.022 ms for Rh110 and $\tau_{\mathrm{p,DOX}}$=0.024 ms for DOX. Then the value of D_{DOX} was determined from the relation $D_{\mathrm{DOX}} = D_{\mathrm{Rh110}} \cdot \tau_{\mathrm{p,DOX}}/\tau_{\mathrm{p,Rh110}} = 4.2 \times 10^{-6}\ \mu\mathrm{m^2 s^{-1}}$. Parameters τ_{t} and p are the fitted triplet state lifetime and fraction, respectively. The fitted triplet state lifetimes of the two probes ($\tau_{\mathrm{t,Rh110}} = 2\ \mu$s and $\tau_{\mathrm{t,DOX}}$= 0.6 μs) were shorter by more than an order of magnitude than their diffusion times. This allows for confidence in interpretation of the ACF, since the photophysical contribution is clearly separated from the diffusional part of the curve.

S2. Diffusion coefficient of ctDNA

The diffusion coefficient of ctDNA in Tris-HCl solution was measured by the dynamic light scattering (DLS) at 25 ℃. A detailed description of the method and experimental setup we employed here can be found in our previous publication.[2] Briefly, in each DLS measurement, we recorded the scattering of 514 nm laser light on ctDNA molecules suspended in Tris-HCl at a range of scattering angles. We obtained the characteristic correlation decay time τ by fitting the experimental data with the autocorrelation function $G_2(q,t) = \beta \exp(-t/\tau)^2$, where β is the experimental coherence factor and q is the wave vector, determined by the experimental setting. Then the cooperative diffusion coefficient of ctDNA (D_{c}) was calculated via the relation $1/\tau = q^2 D_{\mathrm{c}}$. The self-diffusion coefficient at infinite dilution of ctDNA, D_{ctDNA}, was determined from the relation:

$$D_{\mathrm{c}} = D_{\mathrm{ctDNA}}(1 + k \cdot C_{\mathrm{ctDNA}}), \tag{S2}$$

where C_{ctDNA} is the ctDNA concentration and k is a constant.

The determined value of D_{ctDNA}, 1.95 $\times 10^{-12}$ $\mathrm{m^2 s^{-1}}$, is in good agreement with previous published value of 2.0 $\times 10^{-12}$ $\mathrm{m^2 s^{-1}}$.[3] In addition, the lack of observable dependence of D_{c} on ctDNA concentration indicated there were no pronounced interactions between the

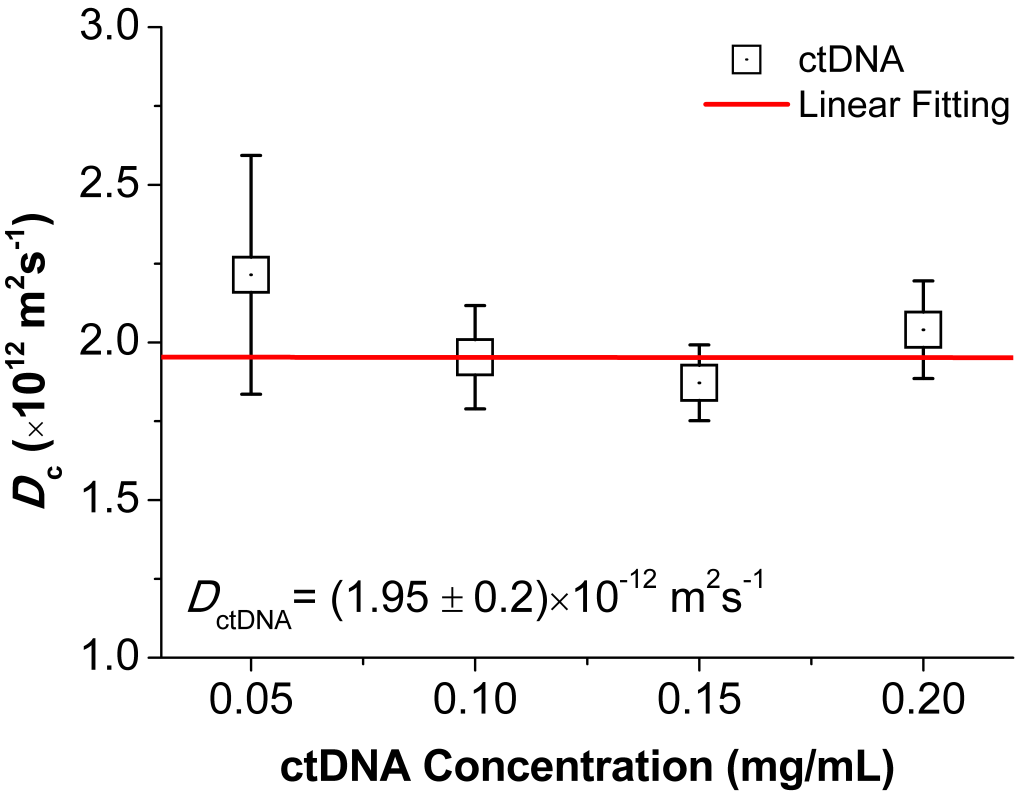

Figure S2: Linear fit of the cooperative diffusion coefficients of ctDNA in Tris-HCl via Eq. S2. Extrapolation of concentration to infinite dilution gives the self-diffusion coefficient of ctDNA.

ctDNA molecules within the studied concentration range (Fig. S2) that could complicate further analysis of the FCS data.

S3. Fluorescence spectra of DOX in ctDNA solutions

The fluorescence spectra of DOX (500 nM) in ctDNA solutions of various concentrations were performed using fluorescence spectrophotometer (Agilent, model: Cary Eclipse, excitation wavelength: 480 nm) at 25 ℃. We observed gradual decreases of fluorescence intensity emitted from DOX as the ctDNA concentration increase, demonstrating the quenching effect from the formation of DOX-DNA complexes (Fig. S3). This result agree with our countrate measurements of DOX in the ctDNA solutions.

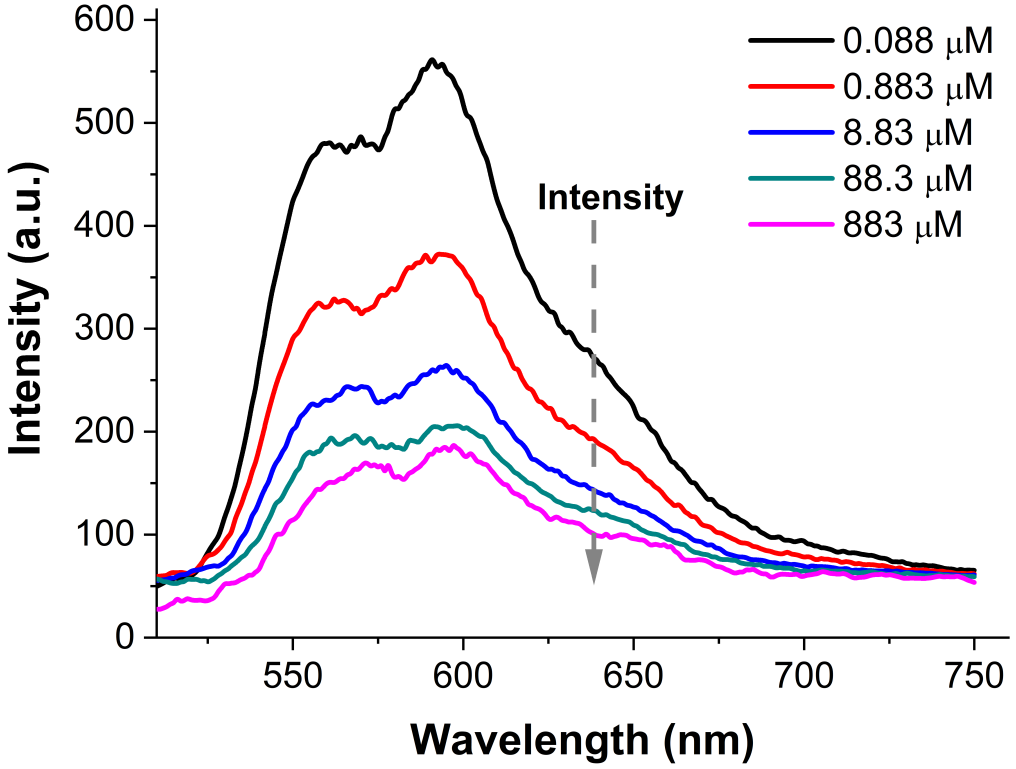

Figure S3: Fluorescence emission spectra of DOX (500 nM) in ctDNA solutions (Tris-HCl buffer, pH 7.4, I = 10 mM) of various concentrations (in terms of BP concentrations) at 25 ℃.

S4. Derivation of FCS-based formula for determination of K in multiple equilibria reaction

The FCS-based formula is derived based on the established equation for the chemical equilibrium condition in the case of macromolecules with identical independent binding sites for ligands.[4] We assume that binding of multiple DOX molecules (acting as ligands) to the binding sites of DNA (acting as macromolecule) occurs independently, since DNA molecules used in our experiments are much larger than DOX. The total concentration of bound DOX at equilibrium state, $[\mathrm{DOX}]_\mathrm{b}$, is equal to the sum of concentrations of all partially saturated

forms of complexes ($[\mathrm{DNA}-\mathrm{DOX}_i]$) according to the mass conservation principle:

$$[\mathrm{DOX}]_\mathrm{b} = \sum_{i=1}^{n} i[\mathrm{DNA}-\mathrm{DOX}_i], \tag{S3}$$

where n is the total number of binding sites on the DNA, equal to the quotient from the length of a DNA chain (i.e., BP number) to the size of binding site for single DOX molecule in the chain (i.e., exclusion parameter).

In the case of identical binding sites, the general binding equation describes the relation between the equilibrium concentration of free DOX $[\mathrm{DOX}]_\mathrm{f}$ and bound DOX $[\mathrm{DOX}]_\mathrm{b}$ is given:[4]

$$\frac{[\mathrm{DOX}]_\mathrm{b}}{[\mathrm{DNA}]_0} = \frac{\sum_{i=1}^{n} i\binom{n}{i}([\mathrm{DOX}]_\mathrm{f}K)^i}{1+\sum_{i=1}^{n}\binom{n}{i}([\mathrm{DOX}]_\mathrm{f}K)^i}, \tag{S4}$$

where K is the equilibrium constant for binding a DOX molecule to a DNA chain, and $[\mathrm{DNA}]_0$ is the initial concentration of DNA. Applying the binomial rule to Eq. S4, one gets a simplified expression:

$$\frac{[\mathrm{DOX}]_\mathrm{b}}{[\mathrm{DNA}]_0} = \frac{nK[\mathrm{DOX}]_\mathrm{f}}{1+K[\mathrm{DOX}]_\mathrm{f}}. \tag{S5}$$

In FCS experiments, both free and bound DOX molecules contribute to the signal fed to detectors. Due to the vast difference in the diffusion coefficients of these two populations, the respective components can be clearly discriminated in the autocorrelation curve. However, the magnitude of contributions of the two components to the total ACF, A_f and A_b, is not only proportional to their concentrations, but also to the squared brightness, which are expressed as:

$$\begin{aligned} A_\mathrm{f} &= B_\mathrm{f}^2 N_\mathrm{f}; \\ A_\mathrm{b} &= \sum_{i=1}^{n} i^2 B_\mathrm{b}^2 N_{\mathrm{b,i}}, \end{aligned} \tag{S6}$$

where N_f, $N_{\mathrm{b,i}}$ denote the number of free DOX molecules and complexes with amount of i

DOX molecules bound to the DNA in the FV of FCS, respectively (directly proportional to concentrations of each species), while B_f and B_b stand for the brightness of free and bound DOX (given as number of photons recorded in a time unit per single molecule from a given population in the FV). Although the absolute values of molecular brightness depend on the experiment conditions (e.g., laser power or filters used), their ratio $B_\mathrm{b}/B_\mathrm{f}$ is an intrinsic quality of the DOX-DNA complex. From Eq. S6 we get:

$$\frac{A_\mathrm{b}}{A_\mathrm{f}} \cdot (\frac{B_\mathrm{f}}{B_\mathrm{b}})^2 = \frac{\sum_{i=1}^{n} i^2[\mathrm{DNA}-\mathrm{DOX}_i]}{[\mathrm{DOX}]_\mathrm{f}}. \tag{S7}$$

To solve Eq. S7, we need to calculate the ratio:

$$\frac{\sum_{i=1}^{n} i^2[\mathrm{DNA}-\mathrm{DOX}_i]}{[\mathrm{DNA}]_0} = \frac{\sum_{i=1}^{n} i^2 \binom{n}{i} ([\mathrm{DOX}]_\mathrm{f}K)^i}{1+\sum_{i=1}^{n} \binom{n}{i} ([\mathrm{DOX}]_\mathrm{f}K)^i}. \tag{S8}$$

Applying the binomial rule again, we get:

$$\frac{\sum_{i=1}^{n} i^2[\mathrm{DNA}-\mathrm{DOX}_i]}{[\mathrm{DNA}]_0} = \frac{n[\mathrm{DOX}]_\mathrm{f}K(1+n[\mathrm{DOX}]_\mathrm{f})}{(1+[\mathrm{DOX}]_\mathrm{f})^2}, \tag{S9}$$

Combing Eq. S7 and Eq. S9, we obtain an expression connecting the experimentally accessible $A_\mathrm{b}/A_\mathrm{f}$ ratio with K as:

$$\frac{A_\mathrm{b}}{A_\mathrm{f}} \cdot (\frac{B_\mathrm{f}}{B_\mathrm{b}})^2 = \frac{nK[\mathrm{DNA}]_0(1+nK[\mathrm{DOX}]_\mathrm{f})}{(1+K[\mathrm{DOX}]_\mathrm{f})^2}. \tag{S10}$$

Since in the performed FCS experiments and also in the clinical usage of DOX, the amount of binding sites are much more excessive, i.e., $n[\mathrm{DNA}]_0 \gg [\mathrm{DOX}]_0$ (initial DOX concentration)

$> [\mathrm{DOX}]_\mathrm{b}$, we rewrite Eq. S5 as follows:

$$\frac{[\mathrm{DOX}]_\mathrm{b}}{n[\mathrm{DNA}]_0} = \frac{K[\mathrm{DOX}]_\mathrm{f}}{1 + K[\mathrm{DOX}]_\mathrm{f}} \ll 1. \tag{S11}$$

Applying the mass conservation principle for DOX:

$$\begin{aligned} [\mathrm{DOX}]_0 &= [\mathrm{DOX}]_\mathrm{f} + [\mathrm{DOX}]_\mathrm{b} \\ &= [\mathrm{DOX}]_\mathrm{f} + \frac{nK[\mathrm{DOX}]_\mathrm{f}}{1 + K[\mathrm{DOX}]_\mathrm{f}}[\mathrm{DNA}]_0 \\ &\approx [\mathrm{DOX}]_\mathrm{f} + nK[\mathrm{DOX}]_\mathrm{f}[\mathrm{DNA}]_0, \end{aligned} \tag{S12}$$

and substituting $[\mathrm{DOX}]_\mathrm{f}$ from Eq. S12 into Eq. S10, we derive the final formula for determination of K via FCS:

$$\frac{A_\mathrm{b}}{A_\mathrm{f}}\left(\frac{B_\mathrm{f}}{B_\mathrm{b}}\right)^2 = \frac{nK[\mathrm{DNA}]_0\left(1 + nK[\mathrm{DNA}]_0 + nK[\mathrm{DOX}]_0\right)}{1 + nK[\mathrm{DNA}]_0}. \tag{S13}$$

References

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(2) Zhang, X.; Poniewierski, A.; Jelińska, A.; Zagożdżon, A.; Wisniewska, A.; Hou, S.; Hołyst, R. *Soft matter* **2016**, *12*, 8186–8194.

(3) Roy, K.; Antony, T.; Saxena, A.; Bohidar, H. *The Journal of Physical Chemistry B* **1999**, *103*, 5117–5121.

(4) Bisswanger, H. *Enzyme kinetics: principles and methods*; John Wiley & Sons, 2017.