Electronic Supplementary Material (ESI) for Analyst.

Supporting Information for:

Single molecule brightness analysis for determination of anticancer drugs interactions with DNA

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1 Equilibrium constant determination with brightness analysis method

In brightness analysis method [1], the molecular brightness (MB) of a fluorescent molecule is defined as the number of photons ($N_{photons}$) emitted by a molecule in a time of period (t). It depends on the molecular properties of the fluorescent molecule (such as quantum yield), excitation conditions (laser power and wavelength) and measurement instruments (detector efficiency and emission filters). Since MB is sensitive to any local environment fluctuation (e.g. change of pH, solvent or formation of complex), under a given experimental condition, it can be expressed as:

$$MB = \frac{\frac{N_{\text{photons}}}{t}}{N_{\text{molecules}}} \tag{1}$$

As the number of emission photons in a period of time can be expressed as photon count rate (I):

$$I = \frac{N_{\text{photons}}}{t} \tag{2}$$

MB of a given molecule can be expressed as:

$$\text{MB} = \frac{I}{N_{\text{molecules}}} \tag{3}$$

The concentration (c) of fluorescent molecules is written as:

$$c = \frac{N_{\text{molecules}}}{N_{\text{A}} \cdot V_0} \tag{4}$$

where N_A represents Avogadro constant and V_0 is the confocal volume. With Equation (1), (2), (3) and (4), the count rate is equal to average number of fluorescent molecules in confocal volume:

$$c \cdot V_0 \cdot N_{\text{A}} \cdot \text{MB} = I \tag{5}$$

Equation (5) can be applied to any fluorescent molecule. And further it can be used to determine the equilibrium constant in a reaction. Here we illustrate DOX-DNA interaction as an example. When DOX molecules are reacting with DNA in a confocal with an volume of V_0:

$$DOX + DNA \overset{K_1}{\rightleftharpoons} \text{DOX-DNA}$$

the count rate of this reaction can be written as:

$$V_0 \cdot N_A \cdot \left(\alpha \cdot C_{DOX} + \beta \cdot C_{DNA} + \gamma_1 \cdot C_{DOX-DNA} \right) = I_1 \tag{6}$$

where α, β and γ_1 is the intrinsic molecular brightness of DOX, DNA and DOX-DNA molecules respectively. In this experimental setup, β is too small to be differentiated from the background noise. At equilibrium state, equation (6) is simplified as:

$$V_0 \cdot N_A \cdot \left(\alpha \cdot C^{eq}_{DOX} + \gamma_1 \cdot C^{eq}_{DOX-DNA} \right) = I_1 \tag{7}$$

The equilibrium constant (K_1) of this reaction is equal to:

$$K_1 = \frac{c^{eq}_{DOX\text{-}DNA}}{c^{eq}_{DOX} \cdot c^{eq}_{DNA}} = \frac{C^{eq}_{DOX\text{-}DNA}}{(c_{DOX} - c^{eq}_{DOX\text{-}DNA}) \cdot (c_{DNA} - c^{eq}_{DOX\text{-}DNA})} \tag{8}$$

where c_{DOX} and c_{DNA} are the initial concentrations of DOX and DNA in the reaction respectively. The Equation (8) can be solved as:

$$\boldsymbol{c^{eq}_{DOX\text{-}DNA}} = \frac{1}{2}\cdot\Big(c_{DOX}+c_{DNA}+\frac{1}{K_1}-\sqrt{\Big(-c_{DOX}-c_{DNA}-\frac{1}{K_1}\Big)^2-4\cdot c_{DOX}\cdot c_{DNA}}\Big) \quad (9)$$

Combining equation (9) and equation (7), there is:

$$V_0\cdot N_A\cdot\alpha\Big(\Big[c_{DOX}-\boldsymbol{c^{eq}_{DOX\text{-}DNA}}\Big]\cdot\Big[1+\frac{\gamma_1}{\alpha}\cdot K_1\cdot\Big(c_{DNA}-\boldsymbol{c^{eq}_{DOX\text{-}DNA}}\Big)\Big]=I_1 \quad (10)$$

In the case of multi-step reaction, the count rate is the result of individual molecular brightness inputs from each fluorescent component. In DOX-DNA interaction, the extra DOX can react with the formed DOX-DNA complexes, thus the reaction is written as:

$$DOX+DNA \overset{K_1}{\rightleftharpoons} \text{DOX-DNA} + DOX \overset{K_2}{\rightleftharpoons} \text{DOX-(DOX-DNA)}$$

At equilibrium state, the count rate is expressed as:

$$V_0\cdot\big(\,\alpha\cdot c^{eq}_{DOX}+\gamma_1\cdot c^{eq}_{DOX-DNA}+\gamma_2\cdot c^{eq}_{DOX-(DOX-DNA)}\,\big)=I_1 \quad (11)$$

where γ_2 is the intrinsic molecular brightness of DOX-(DOX-DNA) complexes. The equilibrium constant K_2 in step two is equal to:

$$K_2=\frac{c^{eq}_{DOX-(DOX\text{-}DNA)}}{c^{eq}_{DOX}\cdot c^{eq}_{DOX\text{-}DNA}} \quad (12)$$

After substitution and extraction (simmilar as in K_1), we obtain:

$$V_0\cdot N_A\cdot\alpha\Big[\Big(c_{DOX}-\boldsymbol{c^{eq}_{DOX\text{-}DNA}}\Big)\cdot\Big(1+\Big(\frac{\gamma_1}{\alpha}\Big)\cdot K_1\cdot\Big(c_{DNA}-\boldsymbol{c^{eq}_{DOX\text{-}DNA}}\Big)+\Big(\frac{\gamma_2}{\alpha}\Big)\cdot K_2\cdot\boldsymbol{c^{eq}_{DOX\text{-}DNA}}\Big)\Big]=I_1 \quad (13)$$

2 Aggregation of anthracyclines

The flat aromatic structure of anthracycline facilitates formation of dimers or higher aggregates. Since the self-aggregation process is competitive with anthracycline-DNA interaction, then the determination of K may be affected. In order to exclude the effect of self-aggregation at working concentration, we analyzed the fluorescence absorption and emission spectra of each anthracycline. The measurements were performed on UV-2700 spectrophotometer (Shimadzu) and Fluorolog-3 spectrofluorometer (HORIBA Scientific). Due to instrument detection limit, the lowest measuring concentration was set at 10000 nM, the results are shown in figure S1. We infer no aggregates formed from two aspects. Firstly, we analyzed the normalized absorption and emission spectral shape. For the DOX case, the shape did not depends on the drug concentrations because they were identical at 10000 nM, 50000 nM and 100000 nM. It was a good evidence of no aggregation. The emission band at 550 nm decreased with higher concentration because of the inner filter effect. The emission band at 550 nm was reabsorbed by the DOX solution itself due to the overlapping spectra.

Secondly, the peak of fluorescence intensity. According to reference, if it was monomer, one intensity peak should occur at 490 nm in absorption spectra and three distinct peaks should occur at around 560, 594 and 638 nm in emission spectra. [2] If dimers were formed, there would be a red shifted of the monomer spectra by 25 nm. From our results, no peak shift occurred, thus no aggregates formed in the range of 10000 nM to 100000 nM. Also according to the reported dimerization constant ($10^{4.8}$ M^{-1}), the number of dimers can be neglected in our working concentration (40 nM). Similar conclusions can be drawn from the spectra shape and characteristic monomer peak of DNR, EPR and IDR respectively. [3–5] The determination of K will not be affected by the self-aggregates of drugs at our working concentration.

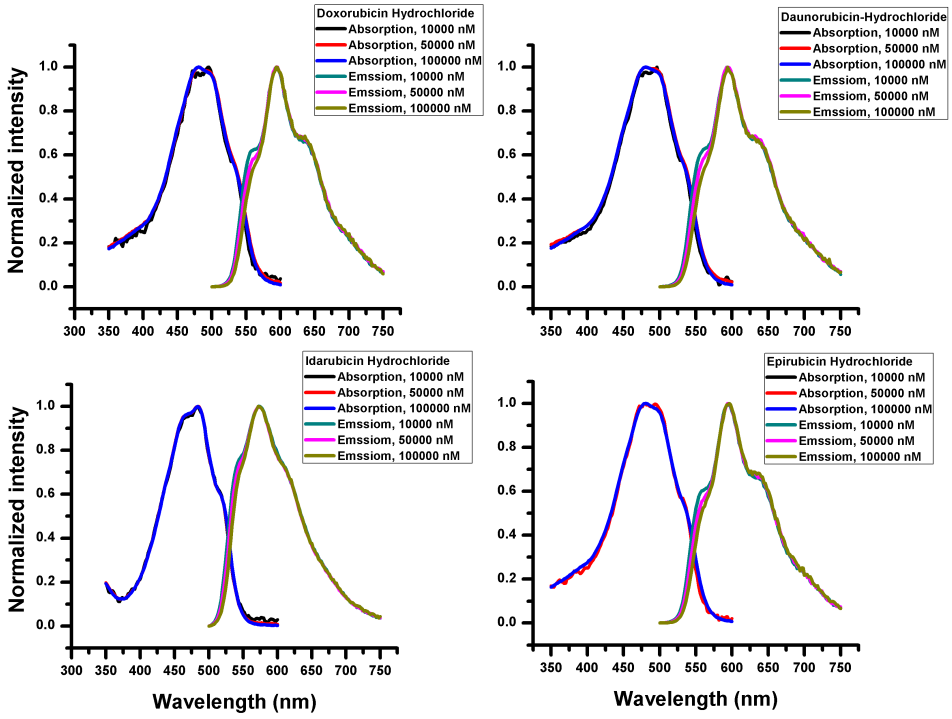

Fig S1: Absorption and emission spectra of anthracyclines in PB-EDTA buffer (pH=7.4). A, DOX; B, DNR; C, EPR; D, IDR.

3 Parameters in a typical measurement

In this section, we illustrate the critical parameters acquired in a typical measurement. All of the drugs were measured under same experimental conditions (i.e. emission filters, excitation laser power), thus obtained MB parameters for each component are comparable. Take the DOX-DNA interaction as an example: V_0 is the size of the confocal volume, it was known by using the standard calibration dye–rhodamine 110 with FCS method. Then, we measure the DOX sample. Drug molecules diffuse in and out of the confocal, and we recorded the photon count rate (I_{DOX}) over time. N_{DOX} was fit from the DOX autocorrelation curve with the known parameter V_0. The α was calculated by dividing I_{DOX} with N_{DOX}. Similarly, we recorded the photon count rate of DOX-DNA complexes ($I_{DOX-DNA}$) and DOX-(DOX-DNA) complexes ($I_{DOX-(DOX-DNA)}$) respectively, by dividing $I_{DOX-DNA}$ and $I_{DOX-(DOX-DNA)}$ with N_{DOX} separately, we got γ_1 and γ_2. We acquired these parameters for DNR, EPR, and IDR the same way as DOX.

Table S1: Parameters obtained in a typical measurement for different anthracyclines.

Drug	V_0 **(fL)**	$\mathbf{N}_{molecule}$	α **(counts/s/molecule)**	γ_1 **(counts/s/molecule)**	γ_2 **(counts/s/molecule)**
DOX	0.275	5.5	1020.4	93.3	447.5
DNR	0.276	7.7	586.0	85.4	328.8
EPR	0.297	8.1	679.2	44.8	336.3
IDR	0.241	4.5	993.9	137.2	434.4

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