Characterizing the interaction between DNA and GelRed fluorescent stain

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Introduction

Rational drug design is a process in which a new compound is conceived and synthesized to attack a specific biological target and/or to perform specific functions. In the health sciences, for example, there is much interest in developing new drugs to treat human diseases such as cancer. In biochemistry and molecular biology, new compounds have been developed to stain DNA and proteins, enabling visualization by fluorescence microscopy and the possibility of following the routes of biological processes.

GelRed is a fluorescent nucleic acid stain designed with the purpose of replacing highly toxic ethidium bromide (EtBr) in gel electrophoresis and other experimental techniques which depend on the fluorescence of stained DNA. When bound to DNA, GelRed has the same absorption and emission spectra as EtBr and, according to its manufacturer (Biotium, Hayward, CA, USA), the compound can be used in electrophoresis with greater sensitivity than EtBr, with the advantage of being much less toxic and mutagenic (Biotium 2013; Huang et al. 2010). This last property, according to the manufacturer, is because the chemical structure of the dye was designed such that the dye is incapable of crossing cell membranes (Biotium 2013). The chemical structure of GelRed is proprietary and has not been officially reported by the manufacturer. Nevertheless, one can find unofficial information on the internet claiming that GelRed is synthesized by linking two EtBr molecules with a linear spacer (Wikipedia 2014), which suggests the dye may be a bis-intercalator.

Although the manufacturer states that GelRed binds to DNA by a combination of intercalation and electrostatic binding (Biotium 2013), most details of the interaction have not yet been reported in the literature. In this work...
we performed single-molecule stretching and dynamic light scattering experiments to gain insight into this interaction. We recently developed a method that enables easy extraction of physicochemical data for DNA–ligand interactions from pure mechanical properties of DNA, which are readily obtained from single-molecule stretching. This method enables clarification of specific binding mechanism(s) (Siman et al. 2012; Cesconetto et al. 2013; Silva et al. 2013). The purpose of this work was to perform robust characterization of the DNA–GelRed interaction, by determining changes of the basic mechanical properties of the DNA molecule as GelRed binds, physicochemical data for the interaction, and the nature of the binding mechanism.

Materials and methods

Stretching experiments

In these experiments the samples were λ-DNA molecules end-labeled with biotin in phosphate-buffered saline (PBS) solution with [NaCl] = 140 mM. One end of the DNA molecule was attached to a streptavidin-coated glass coverslip by use of the procedure reported by Amitani et al. (2010). The other end of the molecule was attached to a streptavidin-coated polystyrene bead of diameter 3 µm (Bangs Laboratories). As reported by Crisafuli et al. (2012) and Cesconetto et al. (2013), in this configuration one can easily trap the polystyrene bead with the optical tweezers and stretch the DNA molecule by moving the microscope stage by use of a piezoelectric actuator. The sample chamber consisted of an o-ring glued in the coverslip, such that one can exchange the buffer and consequently change the ligand concentration, without affecting the trapped DNA molecule, by use of micropipettes. The DNA base-pair concentration used in all stretching experiments was $C_{bp} = 2.4 \, \mu M$.

The optical tweezers consisted of a 1,064-nm ytterbium-doped fiber laser with a maximum output power of 5.8 W (IPG Photonics) mounted on a Nikon Ti-S inverted microscope with a 100 × N.A. 1.4 objective. The apparatus had previously been calibrated by use of two independent methods as described by Crisafuli et al. (2012). Once calibrated, the optical tweezers were used to trap the polystyrene bead attached to a DNA molecule, enabling DNA stretching to be performed with high resolution and consequently enabling force × extension curves of the DNA–ligand complexes to be obtained for each situation studied. These curves were fitted to the Marko–Siggia worm-like chain (WLC) model (Marko and Siggia 1995). One can, therefore, study how the persistence and contour lengths of the DNA molecule vary as a function of ligand concentration. Representative data for the force × extension curves with the corresponding fitting can be found in Online Resource 1. To avoid enthalpic contributions to the values of the mechanical data, all stretching experiments were performed in the entropic low-force region ($\leq 2 \, \text{pN}$) (Crisafuli et al. 2012; Cesconetto et al. 2013). All other experimental details can be found elsewhere (Crisafuli et al. 2012; Cesconetto et al. 2013; Silva et al. 2013; Reis et al. 2013).

Dynamic light scattering (DLS)

To confirm the results obtained from the stretching experiments by use of a second technique, we also performed DLS on the DNA–GelRed complexes. DLS performed at a fixed scattering angle enables evaluation of the hydrodynamic radius of the complexes, which is an estimate of the size of the complexes (Reis et al. 2013). The hydrodynamic radius was measured to enable investigation of the effect of the ligand on the effective size of the DNA molecule, which depends on its persistence and contour length. Thus, the results obtained from two very different experimental techniques can be compared (at least indirectly).

The DLS apparatus used was a ZetaSizer Nano-S (Malvern Instruments) with a low-volume cuvette (ZEN2112, Hellma Analytics). The backscattering angle used was 173° in all experiments. Particle size was determined using the non-negative least squares (NNLS) algorithm. Representative data of the intensity autocorrelation function of the scattered light can be found in Online Resource 1.

The DNA used in these experiments was a 3,000 bp molecule (Thermo Scientific) in the same buffer as used for the optical tweezers samples (it is difficult to use λ-DNA in DLS because of the long contour length). The DNA molecules are equilibrated with a specific concentration of GelRed directly in the cuvette used in the DLS apparatus. The DNA concentration used in all DLS experiments was the same as that used in the optical tweezers experiments (2.4 µM of base-pairs). This concentration is sufficiently low to avoid entanglements and relevant interactions between different DNA molecules (Hur and Shaqfeh 2001).

Results

Stretching experiments

In Fig. 1 (red circles) we show the behavior of the persistence length $A$ of DNA–GelRed complexes as a function of ligand total concentration in the sample $C_T$. Observe that it initially increases from the bare DNA value (∼46 nm) until it reaches a maximum value of ∼86 nm at $C_T = 4.0 \, \mu M$. For higher concentrations, it abruptly decays to ∼43 nm. For comparison purposes, we also show in Fig. 1 (blue squares) the persistence length of DNA–EtBr complexes.
Observe that the behavior of this mechanical property for the two drugs is quite similar, even quantitatively, which is the first evidence that the chemical structure and binding mode of the two compounds are somewhat similar.

In Fig. 2 (red circles) we show the behavior of the contour length $L$ of DNA–GelRed complexes as a function of total ligand concentration in the sample $C_T$. Observe that $L$ increases monotonically from the bare DNA value ($\sim 16.5 \, \mu m$) to a saturation value of $\sim 24 \, \mu m$. For comparison purposes, we also show in Fig. 2 (blue squares) the same data for the DNA–EtBr complexes. Observe that the qualitative behavior is, again, similar but here there is a major difference: the contour length of the DNA–GelRed complexes saturates at a very low concentration ($\sim 1 \, \mu M$) compared with results for the DNA–EtBr complexes, which saturate only at $\sim 10 \, \mu M$ with a saturation value of $\sim 23 \, \mu m$, as shown by Rocha et al. (2007).

To obtain these results, the experiments were performed as follows. First, we chose a specific bare DNA molecule and stretched it five times, determining the mean values of the persistence and contour lengths. We then changed the ligand concentration in the sample by use of a micropipette. After changing the concentration, we waited $\sim 20$ minutes for the ligand to equilibrate with DNA. This time scale is sufficient for both GelRed and EtBr to equilibrate with DNA under our experimental conditions, as was verified by analyzing the reversibility of the stretching curves and by performing some experiments after waiting longer, which resulted in no significant difference. We then performed another five stretching experiments, obtaining new values for the persistence and contour lengths for the chosen ligand concentration. This procedure was then repeated sequentially for each ligand concentration. We also repeated the entire procedure, scanning all the concentrations, for other DNA molecules using different samples. The error bars presented in Figs. 1 and 2 for each concentration are the standard errors obtained from this set of measurements.

The behavior of both persistence and contour lengths strongly suggests that the dominant mechanism of interaction between DNA and GelRed is intercalative binding, because the behavior is qualitatively identical to that shown in Figs. 1 and 2 for the well-known monointercalator EtBr. In fact, the general behavior of the persistence length $A$ shown in Fig. 1 has previously been verified by our group for the intercalators daunomycin, psoralen, diaminobenzidine, and even EtBr under nearly similar experimental conditions (Rocha et al. 2007, 2009; Rocha 2009; Reis et al. 2013), by using optical tweezers in the low-force region ($\leq 2 \, pN$). Some authors have also reported the same qualitative behavior of the persistence length for intercalators, finding that the value increases for low ligand concentrations and decreases for higher concentrations (Tessmer et al. 2003; Cassina et al. 2010; Kaji et al. 2001). Rocha (2009) and Rocha et al. (2009) have proposed that the
abrupt decrease of the persistence length shown in Fig. 1 for \( C_T > 4 \) µM is related to partial DNA denaturation, with the formation of denaturing bubbles, probably because of the pulling force.

It is also well established that intercalators always increase the DNA contour length when binding, by increasing the axial distance between two adjacent base-pairs in the intercalative site (Sischka et al. 2005; Fritzsche et al. 1982; Chaires et al. 1982). The other common types of interaction between DNA and ligands, for example groove binding, electrostatic interaction, or covalent binding, do not increase the DNA contour length. On the contrary, in some cases these types of interaction can cause compaction of the DNA with a decrease of the “apparent contour length” measured by force spectroscopy in the low-force region used in our experiments (Crisafulli et al. 2012; Silva et al. 2013). Therefore at least for \( C_T \leq 1 \) µM (the concentration range in which the contour length \( L \) increases strongly) intercalation is the dominant binding mode in the DNA–GelRed complexes.

The physicochemical properties of the DNA–GelRed interaction can be extracted from the contour length data. First, we used the data of Fig. 2 to determine the fractional increase of the contour length, \( \Theta = (L - L_0)/L_0 \), where \( L_0 \) is the bare DNA contour length, as a function of ligand concentration \( C_T \). These data were plotted in Fig. 3 (circles). For intercalators this fractional increase is proportional to the fraction of bound ligand \( r = C_b/C_{bp} \), where \( C_b \) is the concentration of bound ligand and \( C_{bp} \) is the DNA base-pair concentration. In other words, \( \Theta = \gamma r \), where \( \gamma \) is the ratio of the increase of the contour length because of a single intercalating molecule (\( \delta \)) to the mean distance between two consecutive base pairs in the bare DNA (\( \Delta \sim 0.34 \) nm) (Rocha et al. 2007; Rocha 2009; Daune 1999). For typical monointercalating molecules one has \( \delta \sim 0.34 \) nm and, consequently, \( \gamma \sim 1 \) (Sischka et al. 2005; Fritzsche et al. 1982).

The bound fraction \( r \) (and consequently the mechanical variable \( \Theta \)) can be linked to the physicochemical properties by use of a binding isotherm. The McGhee–von Hippel binding isotherm usually describes the physical chemistry of DNA interactions with intercalators very well, because it computes in detail the neighbor-exclusion effects which are usually associated with this type of interaction (McGhee and von Hippel 1974; Rocha 2010). The binding isotherm is:

\[
\frac{r}{C_f} = K_i(1-nr)\left[\frac{1-nr}{1-(n-1)r}\right]^{n-1},
\]

where \( n \) is the exclusion number (a measure of the effective number of base-pairs occupied by a single ligand molecule (McGhee and von Hippel 1974)), \( K_i \) is the intrinsic equilibrium association constant, and \( C_f = C_T - C_b \) is the free (not bound) concentration of ligand in solution.

Figure 3 shows the fit of this binding isotherm to the experimental data (solid line), obtained by use of the computational method described in detail by Cesconetto et al. (2013). Observe that the model describes the experimental data well. Fitting returns the values \( n = 3.7 \pm 0.4, K_i = (1.8 \pm 0.3) \times 10^7 \) M\(^{-1}\), and \( \gamma = 1.9 \pm 0.1 \). The same kind of analysis was performed by Rocha et al. (2007) for the DNA–EtBr complexes, and will not be repeated here. The results obtained from the fit shown in Fig. 3 strongly suggest that the GelRed dye is a DNA bis-intercalator. In fact, the exclusion number \( n \) indicates that each bound GelRed molecule effectively occupies 3.7 DNA base-pairs, a value substantially higher than the values obtained for most monointercalators, and approximately twice the value for EtBr (Rocha 2009; Chaires et al. 1982; Gaugain et al. 1978). The equilibrium constant is also higher than the result obtained for typical monointercalators (\( \sim 10^5 \) M\(^{-1}\)) (Rocha et al. 2007; Rocha 2009; Chaires et al. 1982; Gaugain et al. 1978), and within the range found for most bis-intercalators (\( 10^7 \) to \( 10^9 \) M\(^{-1}\) M) (Günter et al. 2010; Berge et al. 2002; Murade et al. 2009; Maaloum et al. 2013; Garbay-Jaureguierry et al. 1987). In particular, it is two orders of magnitude higher than the equilibrium constant for EtBr, a situation very similar to that for the bis-intercalator YOYO when compared with its precursor YO, a monointercalator, as is EtBr (Murade et al. 2009). Finally, the result \( \gamma = 1.9 \pm 0.1 \) is approximately twice the value obtained for typical monointercalators, suggesting that each bound GelRed molecule increases the DNA contour
length by $\sim 0.65$ nm, a result also typical of that for bis-intercalators (Günther et al. 2010; Maaloum et al. 2013). Observe that bis-intercalators should increase by a factor of approximately two the DNA contour length per bound molecule, because each ligand molecule contains two intercalating portions.

These results together are strong evidence that GelRed is, in fact, a bis-intercalator probably consisting of two EtBr molecules, as already claimed (Wikipedia 2014). In fact, if one supposes that GelRed is really a bis-intercalator formed by linking two EtBr molecules, it is straightforward to understand the statement of the dye manufacturer which claims that GelRed is more sensitive than EtBr in electrophoresis experiments (Biotium 2013). If one prepares two electrophoresis assays staining one with EtBr and the other with GelRed at the same molar concentration, the GelRed assay will have approximately twice as many DNA-bound sites (at least for concentrations far from saturation), which implies in more fluorescence signal and consequently more contrast. In addition, the fact that the absorption and emission spectra of the two compounds are the same (Biotium 2013) is easily understood on the basis of the discussion above.

Finally, one can ask about the possibility of another binding mode for the DNA–GelRed interaction, in addition to intercalation. We believe electrostatic interactions may also be relevant in this system. Because EtBr is a monocationic ligand, GelRed is expected to have two positive charges, one in each intercalating portion. In principle it is expected that long-range electrostatic interactions may have two relevant physical effects. The first is stabilization of the double-helix structure because of screening of the strong electrostatic repulsion between the negatively charged phosphate groups (Fu et al. 2009), which is reflected by the abrupt transition of the persistence length of the DNA–GelRed complexes which occurs at high concentration compared with the DNA–EtBr complexes. The second effect is because the two positive charges of the GelRed molecule are distant by a fixed value, defined by the linear spacer used to link the two EtBr molecules; this results in positional correlation of the positive charges along the double helix. This positional correlation is probably reflected in the fact that the persistence length of the DNA–GelRed complexes is lower than the corresponding value for the DNA–EtBr complexes for the same ligand concentration.

DLS experiments

In Fig. 4 (circles) we show the behavior of the hydrodynamic radius $R_H$ of DNA–GelRed complexes, obtained from DLS experiments, as a function of GelRed concentration. Observe that $R_H$ increases monotonically with GelRed concentration, from $\sim 87$ nm measured for bare DNA to $\sim 207$ nm obtained for $C_T = 6 \mu$M. Each experimental point is the mean value calculated from a set of $\sim 100$ measurements each 15 s long, and the error bars are the standard deviations. In the same figure, we show estimates of the radius of gyration $R_g$ for the DNA–GelRed complexes (squares). $R_g$ was calculated as a function of the mechanical properties obtained from the tweezers experiments as $R_g = \sqrt{A L \left(1 - \frac{2A}{L} + \cdots\right)}$ (Daune 1999). We used the values of the persistence length $A$ shown in Fig. 1 and assumed that the contour length $L$ of the 3000-bp DNA will increase with ligand concentration in the same ratio as shown in Fig. 3 for the $\lambda$-DNA. As remarked elsewhere (Reis et al. 2013), because the 3,000 bp DNA is sufficiently long (it contains $\sim 20$ persistence lengths), it is reasonable to expect base-pair sequence and other molecular details not to interfere with large-scale mechanical properties, for example persistence and contour lengths.

The radius of gyration $R_g$ increases with both $A$ and $L$. Thus, as shown in Fig. 4, $R_g$ decreases for the two largest concentrations used, because of the abrupt decrease of the persistence length, shown in Fig. 1. A similar decrease was not verified for the hydrodynamic radius $R_H$, indicating that the abrupt transition of the persistence length does
not occur for the samples used for the DLS experiments. The same result was previously verified by our group for the intercalator diaminobenzidine (Reis et al. 2013). As discussed in this previous work, this result was expected because the abrupt transition of the persistence length is probably related to the pulling force exerted in the stretching experiments, which may locally denature the previously deformed double-helix structure of the DNA–intercalator complex (Rocha 2009; Rocha et al. 2009). In this way, one can say that the behavior of \( K_H \) obtained in DLS experiments agrees qualitatively with the results from the optical tweezers experiments. Our DLS results also agree with the results obtained by most authors who have measured the persistence length of DNA–intercalator complexes with non-stretching techniques (fluorescence microscopy, electron microscopy, viscosimetry, among others; Yoshikawa et al. 1992; Quake et al. 1997). These authors found that intercalators, in addition to increasing the DNA contour length, usually also increase DNA persistence length under these conditions, thus increasing the effective size of the DNA–ligand complexes. Nevertheless, it is important to mention that some of the work performed by use of typical DNA-stretching techniques (optical or magnetic tweezers) have found a monotonic decrease of DNA persistence length as a function of intercalator concentration (Murade et al. 2009; Sischka et al. 2005; Lipfert et al. 2010). In our opinion such results are because of the range of forces used to perform the measurements, because, as the force used to stretch the DNA is increased, the probability of forming denaturation bubbles in the highly distorted double helix of the DNA–intercalator complexes increases accordingly, thus leading to a reduction in persistence length. Other factors which can certainly affect the results of such measurements are the salt concentration used in the buffers, the model used to fit force–extension data (which may include DNA stretch modulus if forces are \( \geq \)10 pN), and the ratio of ligand concentration to DNA base pair concentration (Rocha et al. 2007).

**Conclusion**

By using two very different experimental techniques (single-molecule stretching and dynamic light scattering) we have characterized the interaction of the DNA molecule with the fluorescent stain GelRed, by determining changes of the mechanical properties of DNA–GelRed complexes as a function of ligand concentration and extracting the physical chemistry of the interaction from these data. It was found that GelRed binds strongly to DNA (\( K_i \sim 10^7 \text{ M}^{-1} \)). We also found that each bound GelRed molecule effectively occupies \( \sim 3.7 \) DNA base-pairs and increases the contour length by \( \sim 0.65 \) nm. These values, which are compatible with results expected for bis-intercalating molecules, enabled us to determine the main binding mechanism of the GelRed dye and to understand the higher sensitivity of this compound than ethidium bromide in electrophoresis experiments.

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